Early Metabolite Levels Predict Long-Term Matrix Accumulation for Chondrocytes in Elastin-like Polypeptide Biopolymer Scaffolds

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The development of cartilage tissue engineering scaffolds could greatly benefit from methods to evaluate the interactions of cells with scaffolds that are rapid, are nondestructive, and can be carried out at early culture times. Motivated by this rationale, the objective of the current study was to evaluate whether the concentration of metabolites in scaffold–cell cultures at early culture times could predict matrix synthesis in the same samples at longer culture times. Metabolite and matrix synthesis were measured for 16 different formulations of cell-laden elastin-like polypeptide hydrogels. Metabolites were measured at days 4 and 7 of culture, while matrix accumulation was evaluated at day 28. Four of the 16 formulations resulted in molar ratios of lactate:glucose near 2, indicating anaerobic metabolism of glucose, which resulted in collagen:glycosaminoglycan accumulation ratios near those of native tissue. Lactate and pyruvate concentrations were found to significantly correlate with both sulfated glycosaminoglycan and hydroxyproline accumulation, with better fits for the latter. Lactate was found to be the strongest predictor of both matrix components, suggesting that measuring this metabolite at very early culture times may be useful for evaluating the status of tissue engineering constructs in a rapid and nondestructive manner.

Introduction

MANY METHODS FOR EVALUATING the success of *in vitro* cartilage tissue engineering strategies are destructive and require relatively long culture times. Examples include the commonly measured matrix constituents, sulfated glycosaminoglycans (sGAG), collagen, or cell number, parameters that are typically measured after culture that extends to 4 weeks and beyond. Studies seeking to simultaneously screen multiple types of biomaterial scaffolds or bioreactor culture conditions for an ability to promote long-term cartilage matrix accumulation *in vitro* would greatly benefit from nondestructive assays that can be performed at early or periodic culture times and that are predictive of the long-term functional outcome of the tissue engineering scaffold.

Cell metabolites measured from culture media lend themselves to this type of screening method, especially the substrates and products associated with glucose metabolism. The primary mechanism of glycolysis in articular cartilage is anaerobic metabolism,^{1–3} producing a molar ratio of lactate produced:glucose consumed of 2, and yielding ATP through substrate-level phosphorylation rather than through oxidative phosphorylation of the aerobic metabolism pathway.^{4,5}

Indeed, a principal end product of aerobic metabolism, pyruvate, is produced in very low amounts by chondrocytes under most conditions.⁴ Glucose metabolism is necessary for the synthesis of important cartilage matrix components, such as sGAG and collagen. Not only is sGAG synthesis dependent on glycolytic intermediates,³ but also inhibitors of glycolysis in cartilage have been shown to correspondingly inhibit sulfate incorporation and protein synthesis.^{4,6} It is thus apparent that glucose uptake and lactate production rates in cartilage may serve as useful surrogate indicators of both the metabolic state of cells and matrix synthesis.^{1,4,6,7}

In cartilage tissue engineering studies, media metabolites have been used as an indicator of glucose metabolism for cartilage and chondrocytes,^{4,7} as well as indicators of cellular response to perturbations in external oxygen supply,^{4,8} mechanical load,¹ and pH.⁹ Media metabolites have also been quantified for chondrocyte-laden constructs and found to relate to cell viability,¹⁰ oxygen tension, and available glucose,¹¹ as well as measures of gas exchange in bioreactor cultures.¹² The predictive value of early metabolite concentrations for longer-term matrix production in tissue engineering constructs is suggested by these earlier studies of both cartilage and chondrocytes, although it has not been

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demonstrated for cartilage tissue engineering constructs. Demonstrated utility of the nondestructive measurement of metabolites for assessing matrix production could facilitate rapid screening of biomaterial constructs for cartilage tissue engineering applications.

The objective of the current study was to evaluate the ability of short-term culture metabolite concentrations to predict extracellular matrix accumulation in long-term cultures. Primary porcine chondrocytes were encapsulated in varying formulations of chemically crosslinked, elastin-like polypeptide (ELP) hydrogel scaffolds. ELPs are artificial repetitive polypeptides, derived from a pentapeptide sequence in native elastin (Val-Pro-Gly-X-Gly, where X, termed the guest residue, may be any amino acid except proline).^{13,14} ELPs are attractive as 3D scaffolds, as they will undergo a reversible and thermally triggered phase transition that allows for convenient and efficient cell encapsulation.¹⁵

Further, recombinant expression of ELPs in *Escherichia coli* or other hosts ensures that they can be produced with precise control of molecular weight (MW) and the incorporation of precisely positioned chemical or enzymatic crosslinking sites.^{16–18} ELPs have been crosslinked using amine-reactive crosslinkers,^{16,17,19,20} γ -irradiation,^{16,21} photoinitiated crosslinking,²² enzymatic crosslinking,²³ as well as physical crosslinking and self-assembly.^{24–27} The monodispersity of ELPs and their facile and precise crosslinking has allowed hydrogels to be synthesized with a well-controlled array of physical properties. For example, the mechanical properties of ELP hydrogels have been shown to be dependent on the amino acid sequence,¹⁹ the crosslinking system used,¹⁶ as well as the starting concentration of polymer and crosslinker.^{16,17,19,28}

Prior work has shown that primary chondrocytes and progenitor cells are capable of synthesizing and accumulating cartilage extracellular matrix when encapsulated in ELP.^{15,23,29} In this study, ELP formulations were designed to contain lysine (K)^{17,19,28} to enable chemical crosslinking via a biologically benign, amine-reactive, trifunctional crosslinker, β -[tris(hydroxymethyl) phosphino] propionic acid (betaine)

(THPP).¹⁹ ELPs with a wide range of lysine residues can be crosslinked with this system within a clinically relevant time frame (<5 min),¹⁹ resulting in scaffolds with varying crosslink densities, mechanical strength, and elasticity.^{19,28} Chondrocytes entrapped within these crosslinked ELPs were evaluated for an ability to produce the matrix components proteoglycan and collagen, and assayed for production and consumption of relevant metabolites over a range of ELP formulations to test for relationships between these measures of long- and short-term culture. The resulting data were also used to test for differences in the chondrogenic performance of cells in varying ELP biopolymer scaffolds, toward the goal of screening a wide range of ELP scaffold compositions.

Materials and Methods

ELP synthesis

Genes for all ELPs investigated herein were available from previous studies.^{17,19,28} The nomenclature for ELPs provides the stoichiometric ratio of valines (V) to lysines (K) in the guest residue position (X in VPGXG) as well as the total number of pentapeptides in the polymer. For example, ELP [KV₆]-56 denotes an ELP with a K:V ratio in successive pentapeptides of 1:6 and a total of 56 pentapeptides. Different K-containing ELP genes were chosen in this study to provide a wide variation in the crosslink density of the ELP hydrogels (determined by the substitution of V with K), MWs, as well as ELP architectures, as shown in Table 1. This was achieved by inclusion of five different ELP series: (1) the KV₆ series, with a K:V substitution ratio of 1:6. These ELPs thus have a K-period of 7, meaning a lysine (crosslinking site) appears in every 7th pentapeptide; (2) the KV₁₆ series, with a K:V substitution ratio of 1:16 (K-period = 17); (3) the KV₂F series, an ELP having a K or F every 4th pentapeptide (K-period = 4); (4) the KV₇F series, an ELP having a K or F every 9th pentapeptide (K-period = 9); and (5) a triblock ELP composed of [KV₇F]-72-[VG₇A₈]-64-[KV₇F]-72, with two identical outer blocks of [KV₇F] that provide crosslinking sites that are separated by the [VG₇A₈] block that does not

TABLE 1. ELP FORMULATIONS

Formulation	ELP amino acid sequence-pentapeptide repeats	MW (kDa)	K-Period	Concentration (mg/mL)
1	[KV ₆]-56	23.9	7	250
2	[KV ₆]-112	47.1	7	150
3	[KV ₆]-112	47.1	7	200
4	[KV ₆]-224	93.4	7	100
5	[KV ₆]-224	93.4	7	150
6	[KV ₆]-224	93.4	7	200
7	[KV ₁₆]-102	42.7	17	200
8	[KV ₁₆]-204	84.8	17	150
9	[KV ₁₆]-204	84.8	17	200
10	[KV ₂ F]-128	55.7	4	100
11	[KV ₂ F]-128	55.7	4	150
12	[KV ₂ F]-128	55.7	4	200
13	[KV ₇ F]-144	61.1	9	100
14	[KV ₇ F]-144	61.1	9	150
15	[KV ₇ F]-72-[VG ₇ A ₈]-64-[KV ₇ F]-72	85.2	12	100
16	[KV ₇ F]-72-[VG ₇ A ₈]-64-[KV ₇ F]-72	85.2	12	200

ELP formulations of eight different molecular weights, five periods of lysine repeats (K-period) and four different concentrations were studied.

contain a lysine residue. The average K substitution across this entire polypeptide was calculated to be ~ 12 . The triblock ELP copolymer was included in this study to increase the diversity of the architectures of the ELP and to thereby probe the possible impact of architecture on its performance as a tissue engineering scaffold. Both ELPs in series 3 and 4 were more hydrophobic by inclusion of the phenylalanine (F), as was the ELP triblock that apposed two hydrophobic blocks with a more hydrophilic block.

ELPs were expressed from plasmid-borne genes in *E. coli* as previously described,^{17,19,28} and purified from *E. coli* lysate by inverse transition cycling, a nonchromatographic purification method that exploits the inverse phase transition behavior of ELPs and their fusion proteins.^{30,31} Typical yields of these ELP polymers were in the range of 200–400 mg ELP/L of growth media. All ELPs incorporated a C-terminal tryptophan residue for determination of the concentration of purified protein by UV-Vis spectrophotometry (Shimadzu Scientific Instruments UV mini 1240, Columbia, MD; ELP molar extinction coefficient at 280 nm of $5690 \text{ M}^{-1} \text{ cm}^{-1}$). Purified ELP proteins were concentrated to 100, 150, 200, or 250 mg/mL (Table 1) in HEPES-buffered saline and stored at -80°C until further use.

Crosslinker preparation

A biocompatible, trifunctional, amine-reactive crosslinker, THPP (Pierce Biotechnology, Rockford, IL) was dissolved in 200 μL of 25 mM HEPES-buffered saline to a final concentration of 250 mg/mL. Aliquots of this solution were stored at -80°C until further use.

Chondrocyte isolation and encapsulation in ELP

Primary chondrocytes were isolated from skeletally immature porcine femoral condyles via overnight digestion in type II collagenase (Worthington Biochemical, Lakewood, NJ) at 37°C .³² Cells ($100 \times 10^6/\text{mL}$) were mixed with each ELP solution, and THPP was added at a 1:1 molar ratio of reactive ELP amines to THPP (hydroxyl)methylphosphines.¹⁹ The byproducts of this reaction are only water and chemically stable aminomethyl-phosphines ($>\text{P-CH}_2\text{-N}<$). The solution was drawn into a 1 mL syringe using a 20-gauge needle and injected into custom molds³³ to create a slab of the ELP–cell mixture (Fig. 1). Molds were incubated at 37°C in a humidified, 5% CO_2 environment to promote crosslinking. Slabs were then cored using a biopsy punch to obtain 4-mm-diameter $\times$ 2-mm-thick samples ($n = 6$ per formulation). Each sample was overlaid with 1.5 mL culture media (Ham's F-12 culture medium supplemented with 10% FBS [Hyclone, Thermo Fisher Scientific, Waltham, MA], 50 $\mu\text{g}/\text{mL}$ L-ascorbic acid 2 phosphate [Sigma, St. Louis, MO], 5 mL $100 \times$ pen/strep [Sigma], and 25 mM HEPES buffer [Gibco–Invitrogen, Carlsbad, CA]) and cultured at 37°C and 5% CO_2 with orbital shaking at 25 rpm. Media was not changed for the first 7 days, and 50% volume changes were made every 3–4 days following for 28 days. This was done so that a cumulative difference in metabolites could be measured on days 4 and 7 (see Measurement of Media Metabolites section). An excess of media was supplied to constructs so that they would not be in limiting conditions during this first 7 days of culture. Acellular constructs were prepared following the same procedure, without the mixing of cells, and served as negative controls.

FIG. 1. Encapsulation of chondrocytes in ELP–THPP solutions, showing the (1) cell–ELP mixture being (2) injected into the mold to create a slab, which after (3) crosslinking, was (4) cored into 4-mm-diameter samples.

Determination of cell content

On days 1 ($n = 6$ per formulation) and 28 ($n = 6$ per formulation) of culture, culture media was aspirated from sample vials, and samples were lyophilized. Samples of ELP containing no cells were also lyophilized and served as negative controls. All samples and controls were digested in a 300 $\mu\text{g}/\text{mL}$ papain (Sigma) solution prepared in PBS containing 5 mM EDTA (Mallinckrodt, Hazelwood, MO) and 5 mM cysteine-HCl anhydrous (Sigma). Samples were digested in 0.5 mL of papain solution overnight at 65°C and then stored at -80°C until further use. On the day of analysis, samples were thawed, vortexed, and reacted with a fluorescent Quant-iT DNA reactive dye from the Quant-iT™ Pico Green® dsDNA Assay Kit (Molecular Probes–Invitrogen, Carlsbad, CA), and fluorescent intensity was measured on a Tecan plate reader (GENios; Phenix Research Systems, Candler, NC) fit with excitation (485 nm) and emission (535 nm) filters. Because some ELP sequences have been shown in prior studies to autofluoresce at this wavelength, all samples were normalized to the fluorescence intensity emitted by the matching blank ELP sequence (no cells). The amount of DNA was determined using a standard curve of DNA provided in the kit. The number of cells contained within each sample was also determined using a value of 7.7 pg DNA per cell.³⁴

Measurement of media metabolites

Media aliquots from ELP–cell samples, as well as from media samples containing no ELP or cells ($n = 6$ per formulation), were obtained on days 4 and 7 and stored at -80°C until further use. On the day of analysis, media aliquots were thawed, mixed using a vortex mixer, and filtered through a 10 kDa MW cut-off centrifugal filter device (Nanosep; Pall Life Sciences, East Hills, NY) to remove particles larger than 10 kDa. Fifteen microliter samples from the filtered aliquots were then transferred to plastic vials, briefly spun, and analyzed in batch mode for glucose, lactate, and pyruvate

concentration on a CMA 600 microdialysis analyzer (CMA Microdialysis, North Chelmsford, MA), which uses reagents supplied by CMA that contain either glucose, lactate, or pyruvate oxidase, and a catalyst. When mixed with the sample, a red-violet colored quinoneimine product is produced in the presence of the given metabolite that is detected spectrophotometrically and used to calculate metabolite concentration from a calibration curve. All measured sample concentrations were corrected for the metabolite concentration in media without cells or ELP. The ratio of lactate produced to glucose consumed was also calculated for each sample for comparison to ratios of ~ 2 that are commonly reported for cells in healthy articular cartilage.⁵

Determination of accumulated sGAG

On the day of analysis, aliquots of papain-digested samples and controls were thawed and mixed on a vortex mixer before being analyzed for sGAG content via the dimethylmethylene blue dye-binding assay.³⁵ Samples and controls were incubated with the dye in a 96-well assay plate, absorbances were read at 540 nm on a Tecan plate reader (GENios; Phenix Research Systems), and sGAG content was calculated from a standard curve prepared from commercial chondroitin-4-sulfate (Sigma). The mean absorbance for each cell-free ELP formulation was subtracted from the matched cellular counterpart for final determination of the accumulated sGAG concentration.

Determination of hydroxyproline content

Aliquots of papain-digested samples and controls along with standard solutions of hydroxyl-L-proline (Sigma-Aldrich, St. Louis, MO) were hydrolyzed in 6 M HCl for 16 h at 110°C.³⁶ Acid was then removed from the hydrolyzed samples, controls, and standards (Thermo-Savant SpeedVac Plus SC210A; Thermo Fisher Scientific, Waltham, MA) before rehydration in citrate-acetate buffer (pH 6.5). Solutions were spun through activated charcoal (0.45 μ m nylon Costar Spin-X HPLC microcentrifuge filters; Corning Life Sciences, Lowell, MA) and aliquoted into separate wells of a 96-well plate. Samples, standards, and controls were incubated with 0.062 M chloramine-T (Mallinckrodt) for 15 min and subsequently with 0.94 M p-dimethylaminobenzaldehyde for 30 min at 37°C. Absorbances were read at 540 nm (Tecan 96-well plate reader), and hydroxyproline (OHP) content was determined from a standard curve of known hydroxyl-L-proline content. Mean absorbances for each cell-free ELP formulation were subtracted from corresponding ELP samples to obtain an accumulated OHP concentration for each formulation. Total collagen is reported based on an OHP:collagen ratio of 1:7.46.^{36,37} Lastly, the ratio of total collagen to sGAG accumulated was calculated for each matched sample.

Histological visualization

To visualize accumulated matrix components and cell distribution, on day 28 of culture, samples ($n=2$ or 3 per formulation) were flash frozen in liquid nitrogen, cut on their circular plane (where possible) to 8 μ m on a cryomicrotome, adhered to glass slides, and fixed in 10% neutral-buffered formalin for 10 min. One section from each sample was

stained with safranin-o to visualize negatively charged proteoglycans, and was counterstained with hematoxylin. Other sections (one per sample per each formulation for each stain) were processed for immunohistochemical labeling of types I and II collagen using the HistoStain Plus Broad Spectrum staining kit (Invitrogen, Carlsbad, CA). To label type I collagen, samples were incubated in a peroxidase-quenching solution (9:1 methanol:10% peroxide) to quench endogenous peroxidase activity (20 min), followed by washing in PBS and incubation with blocking serum at room temperature (30 min, 10% normal goat serum; Invitrogen). Primary antibody (C2456; Sigma) was then added to the slides at a dilution of 1:300 in 10% normal goat serum and incubated at room temperature for 1 h. Following washing in PBS, samples were incubated with a secondary antibody at room temperature diluted by half with 10% normal goat serum (30 min), followed by washing and incubation with an enzyme conjugate (30 min, room temperature). Again slides were washed and incubated with the chromagen, 3-amino-9-ethylcarbazole, which results in a deep red color, to visualize type I collagen (10 min, room temperature). Samples were counterstained with hematoxylin to visualize individual cells. Negative controls were processed in parallel following the same protocol with the omission of the primary antibody.

Samples stained for type II collagen followed the same protocol with an additional digestion step. Following peroxidase quenching, samples were digested for 10 min at 37°C with Digest-All 3 (Invitrogen), a pepsin-containing enzyme solution that helps expose the type II collagen epitope recognized by the II-II6B3 antibody (Developmental Studies Hybridoma Bank, Iowa City, IA). Negative controls were processed in parallel with the omission of the type II collagen primary antibody.

Statistical analysis

ANOVA was used to determine the effects of the lysine (K) concentration in each ELP (as a surrogate of ELP crosslink density, and termed K-period), and total ELP concentration on cell number (days 1 and 28), sGAG, and OHP accumulation (day 28), as well as metabolite concentrations measured at days 4 and 7 using an α value of 0.05 to determine statistical significance. Tukey's *post hoc* test was used to determine differences among groups at a significance level of 0.05.

To assess the predictive capability of metabolites for matrix accumulation, regressions of sGAG accumulation or OHP accumulation were performed on each measured metabolite. Before regressions were performed, samples whose consumption or production of a metabolite was within the 95% confidence interval of the media value were excluded from regressions. ANOVA was used to determine statistical significance of the regressions ($p < 0.05$). Statistics were performed using JMP software (SAS Institute, Cary, NC). Confidence intervals were calculated using Matlab software (The Math Works, Natick, MA).

Results

Cell number

At day 1, there was a significant effect of ELP K-period on the number of cells encapsulated per sample (ANOVA,

FIG. 2. Mean cell number per sample for each formulation at (A) 1 day and (B) 28 days of culture. Asterisks (*) indicate that groups are significantly different for day 1 only; “a” denotes that groups are significantly different from K-period 17; “b” denotes that groups are significantly different from K-period 4; “c” denotes that groups are significantly different from K-period 9 (Tukey’s *post hoc*, $\alpha = 0.05$).

$p < 0.0001$) with formulations with a K-period of 17 encapsulating significantly more cells than ELPs of any other K-period except those with a K-period of 4 (Fig. 2A; Tukey’s *post hoc*, $\alpha = 0.05$). In addition, ELPs with a K-period of 4 encapsulated significantly more cells than ELPs with a K-period of 7 or 9 (Tukey’s *post hoc*, $\alpha = 0.05$). ELP concentration also had a significant effect of the number of cells encapsulated per sample at day 1 (ANOVA, $p < 0.0001$), with samples prepared with an ELP concentration of 100 mg/mL encapsulating a significantly lower number of cells than formulations of 150 or 200 mg/mL (Tukey’s *post hoc*, $\alpha = 0.05$). By day 28, K-period still had a significant effect on the number of cells per sample (Fig. 2B; ANOVA, $p < 0.0001$), with formulations with a K-period of 17 retaining significantly more cells than all other K-periods, and formulations

with a K-period of 9 retaining significantly more cells than formulations with a K-period of 7 (Tukey’s *post hoc*, $\alpha = 0.05$). ELP concentration did not significantly affect the number of cells per sample at day 28 (ANOVA, $p = 0.61$).

Cell metabolites in culture media

Glucose, lactate, and pyruvate concentrations in conditioned media were easily detected with as little as 15 μ L of sample. Both the K-period of the ELP (ANOVA, $p < 0.01$) and total ELP concentration (ANOVA, $p < 0.0001$) were found to have a significant effect on the concentrations of all three metabolites in culture media at both time points (Table 2, data not shown for day 7 metabolite values). In general, cells in formulations prepared from ELPs having an intermediate

TABLE 2. RESULTS FROM QUANTITATIVE ANALYSIS OF MATRIX COMPONENTS AT DAY 28 AND MEDIA METABOLITES AT DAY 4

ELP formulation number	Accumulated ECM (μ g/sample)			Metabolites (mM/sample)			
	sGAG	OHP	Ratio Col/sGAG	Lactate	Glucose	Ratio Lac/Glu	Pyruvate
1	7.39 $\pm$ 3.29	0.34 $\pm$ 0.51	0.24 $\pm$ 0.32	0.66 $\pm$ 0.06	0.15 $\pm$ 0.47	-1.54 $\pm$ 4.61	-0.29 $\pm$ 0.05
2	7.32 $\pm$ 1.69	0.00 $\pm$ 0.00	0.00 $\pm$ 0.00	0.73 $\pm$ 0.22	0.19 $\pm$ 0.59	-0.82 $\pm$ 0.94	-0.13 $\pm$ 0.03
3	4.95 $\pm$ 1.58	0.00 $\pm$ 0.00	0.00 $\pm$ 0.00	0.24 $\pm$ 0.01	0.20 $\pm$ 0.25	-0.5 $\pm$ 1.19	-0.08 $\pm$ 0.04
4	26.13 $\pm$ 15.55	8.41 $\pm$ 2.74	1.55 $\pm$ 0.80	3.00 $\pm$ 0.82	-1.52 $\pm$ 0.40	2.01 $\pm$ 0.31	-0.22 $\pm$ 0.03
5	5.91 $\pm$ 1.61	0.00 $\pm$ 0.00	0.00 $\pm$ 0.00	0.71 $\pm$ 0.35	-0.49 $\pm$ 0.40	0.11 $\pm$ 3.01	-0.19 $\pm$ 0.04
6	4.60 $\pm$ 0.94	0.00 $\pm$ 0.00	0.00 $\pm$ 0.00	0.16 $\pm$ 0.18	-0.53 $\pm$ 0.38	0.09 $\pm$ 0.55	-0.19 $\pm$ 0.07
7	12.37 $\pm$ 9.50	1.19 $\pm$ 0.97	0.76 $\pm$ 0.55	1.31 $\pm$ 1.44	-0.33 $\pm$ 0.48	0.38 $\pm$ 2.15	-0.21 $\pm$ 0.18
8	25.26 $\pm$ 22.44	4.91 $\pm$ 5.17	1.32 $\pm$ 0.75	1.70 $\pm$ 0.74	-1.29 $\pm$ 0.75	1.88 $\pm$ 1.35	-0.17 $\pm$ 0.02
9	7.19 $\pm$ 1.99	0.10 $\pm$ 0.21	0.09 $\pm$ 0.18	0.12 $\pm$ 0.08	-0.17 $\pm$ 0.55	-0.22 $\pm$ 0.05	-0.12 $\pm$ 0.03
10	6.1 $\pm$ 0.69	0.18 $\pm$ 0.39	0.25 $\pm$ 0.56	0.15 $\pm$ 0.14	-0.12 $\pm$ 0.68	0.12 $\pm$ 0.25	-0.10 $\pm$ 0.06
11	5.32 $\pm$ 0.93	0.00 $\pm$ 0.00	0.00 $\pm$ 0.00	0.15 $\pm$ 0.12	-0.52 $\pm$ 0.85	1.08 $\pm$ 1.28	-0.09 $\pm$ 0.01
12	5.56 $\pm$ 2.00	0.46 $\pm$ 0.18	0.58 $\pm$ 1.04	0.04 $\pm$ 0.02	-0.24 $\pm$ 0.34	-0.09 $\pm$ 0.26	-0.11 $\pm$ 0.04
13	77.28 $\pm$ 34.82	22.56 $\pm$ 7.23	2.34 $\pm$ 0.58	3.23 $\pm$ 0.82	-2.36 $\pm$ 0.79	1.41 $\pm$ 0.28	-0.32 $\pm$ 0.07
14	13.88 $\pm$ 4.17	2.07 $\pm$ 2.05	0.97 $\pm$ 0.83	1.37 $\pm$ 1.46	-1.03 $\pm$ 0.92	0.63 $\pm$ 1.27	-0.30 $\pm$ 0.08
15	27.53 $\pm$ 14.50	7.41 $\pm$ 12.40	1.74 $\pm$ 2.92	1.75 $\pm$ 2.39	-2.45 $\pm$ 1.27	0.54 $\pm$ 0.42	-0.33 $\pm$ 0.15
16	12.00 $\pm$ 9.66	1.18 $\pm$ 2.71	0.31 $\pm$ 0.64	0.28 $\pm$ 0.07	1.03 $\pm$ 0.29	-0.28 $\pm$ 0.08	0.02 $\pm$ 0.04

Shaded rows indicate formulations resulting in lactate:glucose ratios near 2 and collagen:sGAG ratios near 1.3 by day 7. A negative sign of the value for glucose indicates glucose uptake. A positive sign indicates measured concentrations near media values (i.e., no uptake). Likewise, the lactate:glucose ratio assumes glucose uptake but does not retain the negative sign; thus, formulations exhibiting glucose uptake have a positive lactate:glucose ratio.

K-period (9 or 12) or prepared from the lowest ELP concentration (100 mg/mL) consumed more glucose and pyruvate and produced more lactate than cells encapsulated in ELPs of all other K-periods and concentrations. In addition, at day 4, cells in lower concentration ELP formulations with a K-period of 7 or 17 showed a lactate to glucose ratio near 2, suggesting that cells in these formulations were operating largely via anaerobic glucose metabolism (formulations 4 and 8, Table 2). By day 7, ELP formulations with K-period of 9 or 12 prepared at low concentrations also exhibited a ratio of 2 (data not shown). It is noteworthy that all ELP formulations associated with lactate to glucose ratios of ~ 2 were prepared at low to medium concentrations (100–150 mg/mL) and/or intermediate to high K-periods (K-period > 7 , Table 1).

Accumulated sGAG and OHP in crosslinked ELP

sGAG and OHP accumulation were analyzed for all samples following 28 days of culture (Table 2). Results indicate that both ELP concentration and K-period had a significant effect on sGAG and OHP accumulation (ANOVA, $p < 0.0001$). Formulations prepared at 100 mg/mL or those with a K-period of 9 produced significantly more sGAG and OHP than formulations of other concentrations or K-periods (Tukey's *post hoc*, $\alpha = 0.05$).

The ratio of total collagen to sGAG was calculated for each formulation (Table 2). This ratio is estimated to be $\sim 1.3^{38}$ for native tissue. Interestingly, formulations that demonstrated ratios of collagen to sGAG similar to that of native tissue are the same formulations that demonstrated a lactate to glucose ratio near 2 (formulations 4, 8, and 15, Table 2).

Histology

Histological appearances of samples generally corroborated findings from quantitative analysis (Fig. 3). Relatively even cell distribution was noted in most samples, whether or not the presence of matrix was detected. Samples that led to the detection of abundant sGAG or OHP by quantitative means were generally stained bright red (safranin-o) or deep red (type II collagen) throughout their cross section (Fig. 3A, C), as opposed to samples that led to the detection of very little sGAG or OHP by quantitative means, which appeared to accumulate little to no sGAG, type I, or type II collagen upon histological staining (Fig. 3B, D). It is important to note that very little type I collagen was detected in any histological sample, suggesting that quantitative measurement of OHP may be mostly attributed to type II collagen accumulation.

Correlations of metabolites with matrix accumulation

Regression analyses for lactate with sGAG and OHP at days 4 (Fig. 4) and 7 (Fig. 5) revealed significant positive correlations at both days for both matrix constituents ($p < 0.0001$). Results indicate that sGAG accumulation was negatively correlated with pyruvate depletion at both days 4 ($y = -110.02 - 6.21x$, $R^2 = 0.2$, $p < 0.001$) and 7 ($y = -102.84 - 7.03x$, $R^2 = 0.5$, $p < 0.0001$) of culture. Similar trends were observed for the regressions of OHP accumulation on lactate and pyruvate, with evidence of improved regression of the data for both OHP and sGAG at 7 days of culture compared to day 4.

Discussion

The objective of this study was to determine whether metabolite concentrations in media at early culture times were predictive of longer-term matrix synthesis in cartilage tissue engineering constructs, toward the goal of developing a nondestructive method to screen the chondrogenic capacity of varying scaffolds. To evaluate this possibility, chondrocytes encapsulated in 16 different formulations of cross-linked ELPs were cultured *in vitro* for 28 days. Media concentrations of glucose, lactate, and pyruvate were obtained over a wide range of ELP molecular parameters at days 4 and 7 of culture, and the matrix composition of sGAG and OHP was evaluated at day 28. Lactate was found to be the strongest predictor of both sGAG and OHP accumulation by regression analyses, with pyruvate shown to be a predictor of primarily OHP accumulation. At 7 days after the start of culture of cell-ELP constructs, regressions were generally stronger for OHP than sGAG for all metabolites. Taken together, these results suggest that monitoring both or even one of the measured metabolites at 7 days of culture may be sufficient to predict longer-term matrix accumulation for these cell-ELP constructs.

A secondary goal of this study was to rapidly screen varying formulations of ELP scaffolds for those that resulted in more successful tissue-engineered constructs, using measures of metabolites in culture media as an early screening tool. Results indicated that 4 of 16 ELP formulations provided an environment wherein encapsulated cells produced markers of anaerobic glucose metabolism by days 4 or 7, resulting in lactate to glucose ratios nearly equivalent to 2. It is of further significance that many of these same samples accumulated collagen to sGAG ratios nearly equivalent to 1.3, the value for native cartilage. The four formulations providing environments conducive for cartilage matrix synthesis were formulations with intermediate K-periods (9 and 12) or low ELP concentration (100 mg/mL), while formulations with extremely small K-periods (high crosslink density) or high ELP concentrations led to poor cell viability or low matrix synthesis, with few exceptions. In general, these results suggest that ELPs with the prescribed crosslink densities and of low to medium concentrations could be suitable as cartilage scaffolds, independent of the composition of the ELP or its architecture (i.e., single segment ELP or triblock ELP).

An important feature of the analysis of metabolites used here was the elimination of all data for constructs where metabolite levels were effectively zero (i.e., within 95% confidence limits of control values). This suggests that an initial cut-off determination based on metabolite values could also assist in the rapid screening of successful tissue-engineered cartilage constructs. As used here, this protocol suggested that 75% of the ELP scaffold cultures could be eliminated after testing media at days 4 or 7. By eliminating further analyses of these samples in the future, this cut-off determination has the potential to significantly decrease resources needed to process large datasets for a wide array of biomaterial scaffolds. While application of this precise protocol and its predictive ability is limited here to chondrocytes in ELP scaffolds, this approach will likely be useful for other biomaterial scaffolds and culture conditions where synthesis of sGAG and synthesis of OHP are important outcomes.

FIG. 3. Representative histological images stained with safranin-o (A, B) and for type II collagen (C, D). Panels (A) and (C) represent formulation 15, while panels (B) and (D) represent formulation 10 (see Table 1). Scale bars = 100 μm .

Glucose consumption and lactate production, as well as matrix production, have previously been shown to be influenced by the availability of glucose in the culture medium, with reported findings that a media-to-cell ratio of at least 6.4 mL media/ 10^6 chondrocytes is necessary for optimal anaerobic metabolism of glucose.¹⁰ Glucose consumption and lactate production have been shown to depend on oxygen concentration within the culture or construct.^{7,11,12} Values for glucose availability or oxygen concentration within the constructs were not directly measured in the current study, although incorporation of these measures into the regression model predictions may provide additional insight or strength to the use of metabolites for evaluating cartilage tissue engineering constructs. The prior findings for a dependence of chondrocyte glucose metabolism and matrix production on both glucose supply and oxygen tension do contribute a potential interpretation of this study's finding that only ELP formulations of low to medium crosslink density, or low ELP concentration, contribute to suitable levels of cartilage matrix production. It is probably that the lack of metabolic and synthetic activity in ELP formulations with high crosslink densities and higher ELP concentrations is at least partly related to diffusional limitations of oxygen or glucose to cells in these formulations.

Interestingly, glucose consumption was not found to significantly correlate with either matrix component at either time point. Glucose measurements were observed to have higher variability than the other metabolites used in this study, even for media-only samples. While this may be due to error associated with the measurement method, it suggests that caution be used when using glucose concentrations as predictors of long-term matrix synthesis for this system.

FIG. 4. Regressions for concentrations of lactate measured on day 4 of culture against (A) sGAG and (B) OHP accumulation after 28 days of culture.

FIG. 5. Regressions for concentrations of lactate measured on day 7 of culture against (A) sGAG and (B) OHP accumulation after 28 days of culture.

Pyruvate concentrations in culture media containing ELP-encapsulated chondrocytes were observed to decrease from day 0 to 4 or 7 of culture, and correlate with increased matrix accumulation. Pyruvate has been shown to function as an oxidant (electron acceptor) for chondrocytes when supplied externally at high (20 mM) concentrations under anoxic conditions, and stimulates sulfate incorporation and lactate production.³⁹ ELP formulations, whose external pyruvate concentration decreased markedly, resulted in improved matrix accumulation as well as molar ratios of lactate production:glucose uptake near, but not greater than, 2, suggesting utilization of pyruvate in a manner not related to aerobic metabolism that resulted in increased matrix accumulation.

In conclusion, metabolites were easily measured as early as 4 days after the start of culture of cartilage tissue engineering constructs and found to correlate with longer-term matrix accumulation. Media lactate concentration was found to be a strong predictor of matrix accumulation, with the ratio of lactate to glucose serving as a good predictor of collagen to sGAG ratio at later times. Further, key features were identified of crosslinked ELP scaffolds that have a high potential to support chondrogenic matrix production and accumulation, pointing toward the importance of the ELP parameters, crosslink density and protein concentration, rather than MW and amino acid sequence, for engineering a desirable tissue engineering construct. These findings suggest that measuring metabolites at early culture times may

provide a relatively inexpensive and rapid method for screening new biomaterials, and possibly cell sources or culture conditions, for their ability to support cartilage matrix synthesis in tissue engineering applications.

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Disclosure Statement

No competing financial interests exist.

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