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Maximizing cartilage formation and integration via a trajectory-based tissue engineering approach

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Abstract

Given the limitations of current surgical approaches to treat articular cartilage injuries, tissue engineering (TE) approaches have been aggressively pursued. Despite reproduction of key mechanical attributes of native tissue, the ability of TE cartilage constructs to integrate with native tissue must also be optimized for clinical success. In this paper, we propose a “trajectory-based” tissue engineering (TB-TE) approach, based on a hypothesis that time-dependent increases in construct maturation in-vitro prior to implantation (i.e. positive rates) may provide a reliable predictor of in-vivo success. As an example TE system, we utilized hyaluronic acid hydrogels laden with mesenchymal stem cells. We first modeled the maturation of these constructs in-vitro to capture time-dependent changes. We then performed a sensitivity analysis of the model to optimize the timing and amount of data collection. Finally, we showed that integration to cartilage in-vitro is not correlated to the maturation state of TE constructs, but rather their maturation rate, providing a proof-of-concept for the use of TB-TE to enhance treatment outcomes following cartilage injury. This new approach challenges the traditional TE paradigm of matching only native state parameters of maturity and emphasizes the importance of also establishing an in-vitro trajectory in constructs in order to improve the chance of in-vivo success.

Keywords

Tissue Engineering; Hyaluronic Acid; Hydrogels; Cartilage; Integration; Maturation

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Disclosures

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1. Introduction

Given the limitations of current surgical approaches to treat articular cartilage injuries [1–5], tissue engineering (TE) approaches have been pursued extensively over the past two decades. Using a variety of scaffolding materials, cell types, and culture conditions, engineered tissues with biochemical (e.g., glycosaminoglycan (GAG) content) and biomechanical properties (e.g. compressive modulus) on the order of the native tissue have been achieved with extended in-vitro culture durations [6–13] (Fig. 1A). Despite this progress, the ability of these TE cartilage constructs to integrate with native tissue must also be optimized for successful clinical therapies to be realized. Indeed, functional integration may be just as important (if not more) than functional properties of the construct itself [14]. Failure to integrate results in marked stress concentrations at the implant boundaries, predisposing both the construct (and the surrounding native tissue) to further degenerative processes [15, 16].

The cartilage tissue engineering community has not yet come to a consensus on the best means by which to integrate an engineered cartilage construct with the native tissue [6, 17–20]. The prevailing notion is that as TE cartilage constructs mature, their ability to integrate into the native tissue is diminished (Fig. 1B). Indeed, one clinical cartilage repair strategy, osteochondral allograft transplantation (or OATs), involves the transfer of a cylinder of cartilage and bone from a non-load-bearing region to a cartilage defect site [4]. This immediately restores load transfer capacity [21], but is plagued by poor integration at the cartilage margins [22, 23]. Such findings suggest that there may exist a “trade-off” between functional maturation (to provide load transmission) and integration (to evenly distribute stress across the repaired cartilage surface) (Fig. 1B). Indeed, in one of the earliest papers to examine in-vitro integration of engineered constructs to native cartilage, Obradovic et al. reported that immature constructs (5 days of culture) integrated with native cartilage to a much greater extent than mature constructs (5 weeks of culture) [6]. However, other studies revealed a more complicated situation [20, 24, 25]. For example, using similar TE constructs, Hunter et al. saw little to no difference in the integration potential between immature and mature constructs pre-cultured for the same period [17]. In our laboratory, constructs made from hyaluronic acid (HA) hydrogels seeded with mesenchymal stem cells (MSCs) that had been pre-cultured for 4 weeks integrated better than constructs that were formed immediately within a cartilage defect [18]. Similarly, Miot et al. recently examined the role of maturation state at the time of implantation in the goat model [19]. Autologous chondrocytes were harvested and cultured in-vitro within hydroxyapatite/hyaluronic acid sponges for two days, two weeks, or six weeks prior to implantation into an osteochondral defects. Interestingly, the constructs cultured for two weeks showed superior results in terms of histological scoring at 8 months compared to those cultured for shorter or longer period of time. These studies depict a more complex relationship between construct maturity and integration potential (Fig. 1C).

In light of these conflicting data, we posited that “static” measures of construct maturity (e.g. compressive modulus) alone may not be the best indicator of in-vivo success. An ideal TE construct will be required to mature, remodel, and integrate with the host over time. Since the growth state that best promotes such activities need not be (and likely is not) the most mature state, less mature but rapidly developing constructs may need to be selected for implantation, thereby eliminating the “trade-off” between maturity and integration potential (blue dot in Fig. 1B).

To formalize this concept, we propose the general notion of “trajectory-based” tissue engineering (TB-TE). This is based on a hypothesis that time-dependent increases in construct maturation in-vitro prior to implantation (i.e. positive rates) may provide a better

predictor of in-vivo success than static measures of construct maturation. Under this hypothesis, the shape of construct maturation (i.e., its trajectory) becomes critically important for determining the ideal time for implantation. Such an approach requires almost real-time assessment of construct maturation to identify the correct shape of the growth trajectory. However, given this limited sampling frequency in traditional tissue engineering endeavors, several general shapes are possible, which leaves the ideal time for implantation an open question (Fig. 1D).

To overcome this limitation and to validate the TB-TE concept, our first objective was to rigorously assess and model how engineered cartilage constructs mature over time in-vitro using a well-defined culture platform. After determining the general shape of maturation, and its perturbation by changing input parameters (such as cell seeding density and mixing environment), the second objective was to model this response, as well as to perform a sensitivity analysis to determine how often and how much data should be collected to accurately describe the shape of the maturation. Finally, to test this concept in a scenario relevant to cartilage repair, the third objective was to correlate biochemical and biomechanical properties of the engineered cartilage constructs and their time-dependent variation in integration potential as a function of the derived TB-TE parameters using an in-vitro integration assay. Our findings provide quantitative selection criteria, based on a TB-TE approach, to improve cartilage repair.

2. Materials and Methods

2.1 Experimental Design and Analyses- Study 1

2.1.1 MeHA macromer synthesis—Methacrylated HA (MeHA) was synthesized by reacting methacrylic anhydride (Sigma, St Louis, MO) and 74 kDa HA (Lifecore, Chaska, MN) as previously described [7, 18, 26]. Using ¹H NMR characterization, the final MeHA product was determined to be 20–25% methacrylated. HA macromer was then lyophilized and stored at –20 °C. One day prior to construct formation, the MeHA macromer was sterilized by exposure to a biocidal UV lamp for 15 min and dissolved in sterile PBS at a concentration of 1% (mass/volume) with 0.05% Irgacure-2959 photoinitiator (2-methyl-1-[4-(hydroxyethoxy)phenyl]-2-methyl-1-propanone; Ciba-Geigy, Tarrytown, NY).

2.1.2 Mesenchymal Stem Cell Isolation and Construct Formation—Juvenile bovine hind limbs (3–6 months old) were obtained (Research 87, Boylston, MA). For each study replicate, two donors were used. MSCs from the femoral bone marrow were isolated as previously described [7, 18, 27] and expanded through passage 2 in basal medium consisting of DMEM with 10% fetal bovine serum and 1% penicillin–streptomycin–fungizone (PSF) (Invitrogen, Carlsbad, CA).

For the first experiment, cells were encapsulated within methacrylated HA (1% w/v). Following polymerization via UV light, cylindrical constructs (4 mm diameter) were formed and cultured in chemically-defined media (1 mL per construct) containing TGF-β3 (10 ng/mL) for up to 9 weeks in free-swelling conditions [7, 18, 27]. To assess repeatability, this experiment was replicated three separate times with different donor sources at a seeding density of 60 million cells/mL. To examine the impact of cell density, another replicate study included groups with constructs containing MSCs at a density of 20 or 60 million/mL. To examine the impact of environment, one replicate study included a group that underwent orbital shaking (1 Hz) during in-vitro culture.

2.1.3 Biomechanical Analysis—The mechanical properties of free-swelling constructs (n=4–5/group/time point) were assessed via uniaxial unconfined compression as previously described [7, 18, 27]. Constructs were first equilibrated under creep (0.02N tare load) for

300 seconds. Then, a stress relaxation test was performed by applying 10% strain at a strain rate of 0.05%/second followed by a 1000 second relaxation phase. Equilibrium modulus was calculated from equilibrium load and sample cross-sectional geometry. After the stress relaxation test, a 1% sinusoidal strain was applied at 1 Hz. The dynamic modulus was calculated from the slope of the resulting stress-strain response. Samples were then frozen at -20°C for biochemical assessment.

2.1.4 Biochemical Analysis—Following mechanical testing, constructs were digested via papain at 60°C for 24 hours to solubilize matrix components. Glycosaminoglycan (GAG) content was quantified using the 1,9-dimethylmethylene blue dye-binding assay [7, 28]. Collagen content quantified using the orthohydroxyproline assay [7, 29] using an OHP: collagen ratio correction factor of 7.14 [30].

2.1.5 Mathematical Modeling of Growth Trajectories—Biomechanical (equilibrium and dynamic moduli) and biochemical (GAG and collagen content) data from each study replicate were plotted versus time. To determine the best fit for the shape of maturation, two possible shapes were compared (linear and sigmoidal). Data were fit individually with either a line or a sigmoidal curve ($y=C1*e^{(C2*e^{(C3*x)})}$) using a custom MATLAB program and nonlinear regression features (version R2012a, Mathworks, Inc., Natick, MA). Correlation coefficients were calculated and compared between fits using the mean values at each time point to eliminate the inherent variability caused within the regression due to sampling multiple samples at the same data point.

Additionally, using the sigmoidal fit, an example analysis was run using the replicate study comparing cell density. The data from both seeding densities were fit using the sigmoidal function as above. Using the determined parameters ($C1$, $C2$, and $C3$), the 1st derivative of the function was calculated. These data were compared between groups at time points throughout the culture period.

2.2 Experimental Design and Analyses- Study 2

When designing studies in which the shape of maturation is important, an additional consideration is the time interval between data collection and the number of samples collected at each time point. To expend the minimum amount of resources (and sacrifice the fewest possible samples), it would be advantageous to know precisely the minimum amount of data necessary to accurately model the growth trajectory. Thus, for the second experiment, the MATLAB code was modified to perform a sensitivity analysis of the sigmoidal fit using a block Bootstrapping technique [31, 32]. Bootstrapping is a statistical technique used to evaluate variability of a measurement or process. In general, this process involves selecting a sample data set randomly from a larger set of population data and performing the analysis using the sample data. By performing this operation many times, the variability of the measurement or process can be determined.

Specifically, the experimental factors of interest were 1) the interval between data collection time points and 2) the number of samples tested at each time point. Using given parameters describing a sigmoidal curve as above ($C1=500$, $C2=-15$, $C3=-0.6$), a large synthetic data set was created. At each potential time point (1 day intervals), a set of “population data” of 10,000 data points was created by assuming the actual data to be normally distributed around the true mean at that time point, with a variance of 0.18. This is a block bootstrapping approach since the population data at each time point was different and sampled independently.

Data points were then randomly selected from the population data at each time point to represent an experimental data set. The model was fit to this data set and specific parameters

(peak growth rate and time of the peak growth rate) were obtained. This process was repeated 10,000 times to obtain 99% confidence intervals for these parameters, which were then compared to the true values. Sensitivity was deemed acceptable if the 99% confidence interval for a given parameter fell within $\pm 10\%$ of the actual value. To assess the impact of the number of constructs tested at each time point, separate trials were run while varying the number of samples (3, 4, 5, 6, 8, or 10 samples). To assess the impact of time interval for data collection, this variable was altered (1, 3, 7, 14, or 21 days).

2.3 Experimental Design and Analyses- Study 3

2.3.1 MSC and Cartilage Isolation and Construct Formation—Juvenile bovine hind limbs were obtained, and MSCs were isolated and cultured as in Study 1. Additionally, for integration testing, cartilage was cored from the trochlear grooves of juvenile bovine femurs (3–6 months of age) using 8 mm diameter biopsy punches (Miltex, York, PA) and cultured in basal medium for 1–2 weeks.

Constructs were created and cultured as in Study 1, with similar biomechanical and biochemical assessments up to 17 weeks of culture. At 1, 2, 3, 4, 5, 6, 8, or 11 weeks of culture, some constructs were used to assess their ability to integrate to cartilage, as previously described [6, 17, 18]. On the day of the experiment, a custom mold was used to trim the cartilage explants to ~ 3 mm in thickness. A core (4 mm diameter) was removed from the center of the cartilage discs, creating a cartilage “defect”. Constructs were press-fit into the cartilage rings and cultured in chemically-defined media (3 mL per construct) containing TGF- $\beta 3$ for 6 weeks. Cartilage cores were also placed back into the cartilage rings to serve as a control. At 3 and 6 weeks, integration testing ($n=5-6$) and histological assessment ($n=2$) was performed. This experiment was repeated three times with similar findings. The results of a single replicate are shown.

2.3.2 Integration Testing—The integration strength of the constructs to native cartilage was assessed using an established push-out test [6, 17, 18]. Prior to testing, constructs were trimmed using a freezing stage microtome to remove the outer capsule that forms during culture. A cylindrical flat end indenter (3.8 mm diameter) was affixed to an Instron 5848 mechanical testing device (Instron, Norwood, MA) and centered over the sample, which was placed within a custom fixture attached to the base. The crosshead was lowered at 0.2 mm/sec to push the TE construct or cartilage core out of the cartilage annulus until failure, while recording the load. Integration strength was calculated as the load at failure normalized to the interface area (height $\times$ circumference).

2.3.3 Histological Analysis—For integration studies, additional constructs ($n=2$) were fixed in 4% paraformaldehyde (FD NeuroTechnologies, Inc, Ellicott City, MD), dehydrated, and paraffin processed. Sections (8 μ m) were taken from the center of the constructs and stained for proteoglycans (Alcian Blue, Rowley Biochemical, Inc, Danvers, MA).

2.4 Statistical Analyses

All statistical analysis was performed using SYSTAT (version 13, Systat Software, Inc., Chicago, IL). For Study 1, biomechanical and biochemical data were compared using a two-way ANOVA with group and time as independent variables, followed by Bonferroni post-hoc tests. Experimental significance for the 1st derivative from the model was defined as a difference greater than 25% of the peak value. For Study 3, Pearson correlation coefficients were calculated to compare the modulus of constructs or the 1st derivative to the integration strength. For all studies, statistical significance was set at $p < 0.05$.

3. Results

3.1 Study 1- Shape of Maturation

In order to model the maturation trajectory of the TE constructs, data were collected to ascertain the general shape of maturation. While the data for equilibrium modulus is described in detail, data for dynamic modulus, GAG content, and collagen content followed similar trends. For all study replicates, the equilibrium modulus of the MSC-seeded HA constructs followed a sigmoidal growth trajectory over time, with an initial lag phase for the first 2 to 3 weeks, followed by a linear region with increased slope, before slowing down by 7 to 9 weeks (Fig. 2). This general shape was repeatable, as the same experiment repeated three times yielded similar results, although the durations of the lag, linear, and plateau regions varied (Fig. 2A). Altering other experimental variables also resulted in a sigmoidal maturation shape that varied in scale based on input parameters. When the seeding density was reduced to 20 million cells/mL, the shape remained the same, but with lower magnitudes (Fig. 2B). Alternatively, when the culture conditions were changed from a static, free-swelling condition to dynamic shaking, the shape was again sigmoidal, but with slightly higher magnitudes (Fig. 2C). To quantify these findings, data for all studies were fit using either linear or sigmoidal functions (Table 1). The linear fit produced R^2 values ranging from 0.54 to 0.99. With the sigmoidal fit, these R^2 values improved to 0.88 to 0.99. Together, these data show the robustness of the shape of maturation to changes in experimental variables.

To further illustrate the information that can be obtained by modeling maturation, data for the equilibrium modulus and its first derivative were plotted and compared between the 20M and 60M cell density groups (Fig. 3). In terms of equilibrium modulus, statistical analyses revealed a significant effect due to group and time ($p < 0.05$) and a significant interaction between these variables ($p < 0.05$). Within each group, the magnitude of the equilibrium modulus increased over time with higher values for the 7 and 9 week time points compared to the 3 week time point ($p < 0.05$). Additionally, for the 60M group, the 7 and 9 week time points were greater than the 5 week time point ($p < 0.05$), and the 5 week time point was also greater than the 3 week time point ($p < 0.05$). The first derivative featured a parabolic shape over time (Fig. 3B), with the greatest values found at 5 weeks for both groups (experimentally significant versus all other time points). Additionally, for the 60M group, the first derivative at 7 weeks was significantly higher than the 3 and 9 week time points.

Between the 20M and 60M groups, similar values were found at 3 and 5 weeks ($p > 0.05$), but the equilibrium modulus for the 60M group increased by 2- and 3-fold relative to the 20M group at 7 and 9 weeks, respectively ($p < 0.05$) (Fig. 3C). In terms of the first derivative (Fig. 3D), since statistical analyses could not be performed, experimental significance was defined as a difference greater than 25% of the peak value for the 60M group. The 60M group was 3 to 4 times higher than the 20M group at 5 and 7 weeks, respectively (experimentally significant), while both groups had similar values at 3 and 9 weeks. These data highlight the ability to determine earlier differences between experimental groups by close examination of the time-dependent changes in their maturation.

3.2 Study 2- Modeling Maturation

To evaluate the sensitivity of the model to variables associated with data collection, a sensitivity analysis was performed using a synthetic data set (Fig. 4A). For each set of input variables, random data sets were created and compared to the true data (Fig. 4A&B). Simulations revealed substantial differences in the accuracy and variability of the model as the time interval between data collection and the number of samples at each time point was varied (Fig. 4C&D). Not surprisingly, as the number of data samples or the frequency of

data collection increased, the sensitivity of the model improved. For the peak growth rate, the 99% confidence interval was acceptable (within $\pm 10\%$ of the true mean) only for data collected with a 1 day sampling interval and 8 or more samples collected per time point. For the time of the peak growth rate, the 99% confidence interval was acceptable for 5 or more samples collected at intervals of 7 days or less. Since the primary TB-TE hypothesis depends on the time of the peak growth rate, 5 samples were collected weekly for Study 3.

3.3 Study 3- Correlation of Growth Trajectory Parameters with Functional Integration

To correlate maturation trajectory parameters with integration, TE constructs were cultured for up to 17 weeks and modeled as in previous sections (Fig. 5A&B). At 1, 2, 3, 4, 5, 6, 8, or 11 weeks of culture, constructs were placed within native cartilage rings and cultured for an additional 3 or 6 weeks to assess integration. Interestingly, no significant correlation was found between equilibrium modulus or dynamic modulus of the constructs at implantation and the resulting integration strength at 3 weeks ($R^2=0.01$ and 0.01 , respectively, $p>0.05$, Fig. 5C, Table 2). Similar findings were obtained for the GAG and collagen content ($R^2=0.01$ and 0.01 , respectively, $p>0.05$). However, a clear correlation was achieved between the first derivative of all biochemical and biomechanical measures and integration strength ($R^2=0.59-0.86$, $p<0.05$, Fig. 5D, Table 2). Similar trends were observed at 6 weeks, with R^2 values ranging from 0.10 to 0.27 for the biochemical and biomechanical measures ($p>0.05$) and R^2 values ranging from 0.67 to 0.83 for their 1st derivatives ($p<0.05$). The only exception was for the 1st derivative of the collagen content at 6 weeks, which was not significantly correlated to the integration strength ($R^2=0.24$, $p>0.05$).

Histological assessments matched the trends in mechanical data with the greatest integration apparent with constructs pre-cultured for 4–6 weeks, and noticeable gapping at earlier or later time points (Fig. 6). Interestingly, substantial variation was found for the cartilage-to-cartilage controls both from a mechanical and histological perspective, with some specimens showing good integration while others showed little or incomplete integration.

4. Discussion

In this study, we developed a mathematical approach to model the maturation of MSC-laden HA hydrogels during in-vitro culture and determined the ability of time-dependent parameters (extracted from biochemical and biomechanical growth trajectories) to predict construct integration with native cartilage. In the initial phase of the study, we modeled the maturation of constructs under multiple conditions and determined that the constructs had a repeatable maturation trajectory that was sigmoidal in shape. Then, we tested the sensitivity of the model (based on the experimental data) to determine the number of samples and sampling frequency required to obtain an accurate prediction of the growth trajectory. Lastly, we correlated the construct maturation and time-dependent changes in growth parameters with the ability of these constructs to integrate with native cartilage. In support of our hypothesis, integration of constructs to cartilage was linearly correlated with the change in maturation as a function of time (its rate), but not the maturation state itself. This findings support the use of a trajectory based tissue engineering (TB-TE) approach in directing translation of engineered constructs for cartilage repair studies.

In order to define and validate the concept of TB-TE, it is essential to know the time course of how TE constructs mature, namely, the shape of maturation. Most TE studies only examine construct properties at 3–4 week intervals, preventing accurate assessment of growth trajectory, and its variation with time. In this study, multiple replicate studies confirmed a sigmoidal maturation curve, which was generally consistent between studies and independent of experimental variables, such as biochemical or biomechanical measurement type, cell density, or culture conditions. This shape is consistent with previous

histological and biochemical findings using this TE system [7, 8, 18]. In initial weeks, cells produce abundant pericellular matrix; however, open spaces exist between cells that limit the impact of formed matrix on bulk mechanical properties [33, 34]. Over time, the matrix becomes more homogeneously distributed throughout the constructs, resulting in substantial increases in mechanical properties. As the constructs approach native tissue levels, cells within reach a homeostatic state and reduce matrix production, resulting in a plateau phase. Depending on culture conditions and cell type, this plateau can either be stable over extended culture durations, or regress in conditions where cell health is compromised or cell phenotype is unstable (for instance, in MSCs that begin to undergo spontaneous hypertrophy [35, 36]). Maturation rates and shapes may also be scaffold dependent, and additional studies with other common biomaterials, such as felts or lyophilized scaffolds, are warranted.

In our initial studies, we also assessed the sensitivity of the model to sample number and sampling frequency. As expected, both had an impact on the accuracy of the model, where increased numbers and increased frequency decreased error in predicting trajectory. It should be noted that these analyses apply to separate samples collected at each time point. One possible solution is a system, such as the recently developed mechanoactive transduction and evaluation bioreactor (MATE) system [37], that allows for repeated measures of mechanical properties of the same set of specimens as they develop in culture. This system would provide for almost continuous analysis of constructs, eliminating the substantial variation in the model and allow for determination of maturation curves from individual specimens as opposed to using different samples at each time point as in this study. Alternatively, high throughput mechanical test systems [38] would allow for many samples to be tested, substantially reducing the time needed for data collection. Our sensitivity analysis suggests that both approaches could easily yield the required information to adequately predict the point at which maturation rate is maximal.

As a final step in this process, and as an initial proof of the TB-TE concept, we correlated the trajectory of maturation with the ability of the constructs to integrate with native cartilage. Strikingly, we did not find any correlation between measures of ‘state’ (construct biochemical or biomechanical content) and integration capacity, similar to previous reports [17]. However, the trajectory of these state parameters were important, where the time-dependent changes (i.e., ‘rate’ parameters) were strongly correlated with the integration strength. This finding supports the importance of maturation “rate” rather than “state” in choosing the most auspicious time point at which to implant TE constructs. By tracking the time-dependent changes in these TE systems, this mechanism may also rectify conflicting data in the literature, where some studies suggest implanting immature scaffolds may be better, while others suggest using mature scaffolds [6, 17–20, 24, 25].

This study has an interesting parallel to current clinical strategies to treat focal cartilage defects. Microfracture of the underlying bone is commonly performed to encourage marrow into the defect, creating a soft immature clot [39–41]. Although the clot integrates well to the surrounding cartilage, it remodels into a fibrocartilaginous tissue and does not reach native levels of maturity. On the other hand, allogeneic or autologous osteochondral grafts comprised of mature native tissue are also used, but the ability of these grafts to integrate laterally is limited [42, 43]. Indeed, in our study, we noted variable levels of integration capacity in native tissue (Fig. 7) that could be due to donor-to-donor variability among other experimental variables. In either case, the time-dependent changes in maturation state are likely minimal in these native tissue specimens. It is of further note that improved native tissue to native tissue integration has been reported when the boundaries are pre-digested with matrix degrading enzymes [6, 25, 44]. It may well be that the same TB-TE principle applies here as well, where a zone of integration is reconfigured towards a maturation state

that is compatible with tissue integration. Other recent repair techniques, including matrix-assisted chondrocyte implantation (MACI), attempt to find a middle ground between maturity and integration potential by expanding chondrocytes, placing them onto a collagen scaffold, and culturing these constructs prior to implantation back into the patient [45, 46]. However, the maturation rates of these approaches have not yet been explored.

One potential advantage of the use of TE constructs is the ability to better control their maturation trajectory in order to optimize the time of implantation. Indeed, we showed in this study that a linear region exists that spans several weeks during which the slope is high and relatively constant with similar levels of integration capacity. Thus, there is a multi-week period where the rate is near maximum, providing a fairly large timespan for selection prior to implantation. Additionally, systems that allow more “real time” feedback of construct maturation (as proposed above) will allow the determination of the beginning of this optimal time frame for implantation using only the data up to that point, as opposed to the entire data set, which is a limitation of the current work.

With regard to clinical translation, an ideal construct would be one with a high maturation rate as well as a sufficient mechanical state so as to enable immediate function. However, for procedures such as microfracture and autologous chondrocyte implantation, patients are often fully or partially non-weight bearing for at least several weeks after surgery [39–41, 45, 46]. Such a period would allow a less mature construct additional time to mature before full mechanical load bearing was required, while still taking advantage of a growth trajectory that fosters the best integration with surrounding tissues.

Having established the TB-TE framework and validated with an in-vitro integration assay, future work will determine whether this framework holds in-vivo as well. Specifically, we are currently assessing whether in-vitro maturation rates at the time of implantation correlate to in-vivo outcomes using a large animal model of cartilage repair. If successful, additional work will aim to maximize maturation rates by altering intrinsic variables (e.g. cell types and number, scaffold type and properties) or extrinsic variables (e.g. chemical factors, mechanical stimulation).

5. Conclusions

In conclusion, this study provides a proof-of-concept for the use of trajectory-based TE to enhance treatment outcomes following cartilage injury. Using hyaluronic acid hydrogels seeded with MSCs as a case example, and an in-vitro model of cartilage injury and treatment, we demonstrate the importance of the time-dependent changes in construct maturation on the ability of a construct to integrate with the surrounding cartilage. This new approach challenges the traditional TE paradigm of seeking to match native ‘state’ parameters of maturity at the time of implantation. Instead, we emphasize the need to establish an in-vitro trajectory in constructs such that native levels can be restored in-vivo both in terms of continued maturation and integration with surrounding tissue.

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Figure 1.

Schematic illustration of the question of construct state versus trajectory. Current practice in cartilage TE allows for the formation of constructs with some properties matching native tissue (A). While there is a general negative correlation between construct maturity and its ability to integrate with native tissue (B), individual studies are less clear regarding this trend and are limited by few data points (C). One important factor in correlating construct maturity and integration potential might be its “trajectory” or time-dependent properties, however, the shape of maturation for these constructs has yet to be elucidated, which could influence the ideal time for implantation (D).

Figure 2.

The shape of maturation is generally sigmoidal, independent of culture conditions. Data for equilibrium modulus is shown as a representative parameter to measure maturation. A) Maturation of constructs under the same conditions from three different trials. B) Effect of cell density within constructs (20 or 60 million cells per mL) on maturation shape. C) Effect of culture conditions (free-swelling (FS) or dynamic shaking (DS)) on maturation shape.

Figure 3.

Example fitting of maturation trajectory, comparing MSC-laden HA constructs with different cell seeding densities (20 or 60 million cells/mL, 20M and 60M, respectively). Equilibrium modulus as a function of time for 20M and 60M groups and respective model fits (A) (solid lines). First derivative of equilibrium modulus with respect to time (B). Average values for modulus (C) and first derivative (D) during periods of rapidly changing growth trajectory. (C: * $p < 0.05$ vs. 60M group, # $p < 0.05$ between time points; D: #experimentally significant vs. 60M group, bars=experimentally significant between time points)

Figure 4.

Sensitivity analysis for the assessment of maturation (A) and maturation rate (B) showing the input data (blue line), a random data set normally centered around the input data (green circles), and the fit of the random data set (green line). Impact of varying the time between sampling and the number of samples per time point on estimation of the peak growth rate (C) and the time of the peak growth rate (D). Data are represented by the mean value and 99% confidence interval. The true mean is represented by a black line and 10% error in the mean represented by the dashed lines.

Figure 5.

Correlation of maturation state or trajectory to integration capacity. Equilibrium modulus (A) and its first derivative (B). Dashed lines represent duration of pre-culture prior to initiating integration assay. Correlations between integration strength of constructs to native cartilage and equilibrium modulus (C) or the first derivative (D) of constructs at time of implantation.

Figure 6.

Histological staining for proteoglycans at the tissue-construct or tissue-tissue interface following integration periods of 3 and 6 weeks (C-C= cartilage-cartilage controls, C-TEC= cartilage-tissue engineered construct). TECs were cultured for varying pre-culture durations prior to implantation (scale bar = 200μm).

Correlation coefficients (R^2) for linear and sigmoidal fits of maturation defined by equilibrium modulus. Studies on repeatability and the effects of cell density and culture conditions are shown (20M and 60M indicate seeding density of 20 million and 60 million cells per mL, respectively; FS= free swelling conditions; DS= dynamic shaking conditions).

Table 1

		Equilibrium Modulus		Dynamic Modulus		GAG Content		Collagen Content	
		Lin.	Sigm.	Lin.	Sigm.	Lin.	Sigm.	Lin.	Sigm.
Repeatability	Study 1	0.84	0.97	0.92	0.98	0.97	0.99	0.90	0.95
	Study 2	0.88	0.97	0.89	0.94	0.99	0.99	0.90	0.94
	Study 3	0.68	0.94	0.75	0.97	0.54	0.96	0.91	0.92
Cell Density	20M	0.82	0.92	0.79	0.86	0.83	0.99	0.90	0.96
	60M	0.85	0.98	0.89	0.97	0.96	0.98	0.92	0.96
Culture	FS	0.88	0.97	0.89	0.94	0.99	0.99	0.90	0.94
Conditions	DS	0.91	0.97	0.92	0.96	0.95	0.93	0.86	0.88

Table 2

Correlation coefficients (R^2) between integration strength of constructs to native cartilage and the maturation state and rate of biochemical and biomechanical properties of constructs at the time of implantation (* $p < 0.05$).

Values	Normalized Integration Strength			
	3w		6w	
	p-value	R ²	p-value	R ²
Equilibrium Modulus	0.817	0.01	0.432	0.11
Dynamic Modulus	0.861	0.01	0.409	0.12
GAG Content	0.837	0.01	0.457	0.10
Collagen Content	0.794	0.01	0.186	0.27
1st Derivative				
Equilibrium Modulus	0.004*	0.78	0.013*	0.67
Dynamic Modulus	0.002*	0.82	0.004*	0.78
GAG Content	0.010*	0.70	0.002*	0.83
Collagen Content	0.027*	0.59	0.222	0.24