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TISSUE ENGINEERING: Part A
Volume 22, Numbers 1 and 2, 2016

DOI: 10.1089/ten.tea.2015.0203

termis
Tissue Engineering
& Regenerative Medicine
International Society

ORIGINAL ARTICLE

Superior Tissue Evolution in Slow-Degrading Scaffolds for Valvular Tissue Engineering

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Synthetic polymers are widely used to fabricate porous scaffolds for the regeneration of cardiovascular tissues. To ensure mechanical integrity, a balance between the rate of scaffold absorption and tissue formation is of high importance. A higher rate of tissue formation is expected in fast-degrading materials than in slow-degrading materials. This could be a result of synthetic cells, which aim to compensate for the fast loss of mechanical integrity of the scaffold by deposition of collagen fibers. Here, we studied the effect of fast-degrading polyglycolic acid scaffolds coated with poly-4-hydroxybutyrate (PGA-P4HB) and slow-degrading poly-ε-caprolactone (PCL) scaffolds on amount of tissue, composition, and mechanical characteristics in time, and compared these engineered values with values for native human heart valves. Electrospun PGA-P4HB and PCL scaffolds were either kept unseeded in culture or were seeded with human vascular-derived cells. Tissue formation, extracellular matrix (ECM) composition, remaining scaffold weight, tissue-to-scaffold weight ratio, and mechanical properties were analyzed every week up to 6 weeks. Mass of unseeded PCL scaffolds remained stable during culture, whereas PGA-P4HB scaffolds degraded rapidly. When seeded with cells, both scaffold types demonstrated increasing amounts of tissue with time, which was more pronounced for PGA-P4HB-based tissues during the first 2 weeks; however, PCL-based tissues resulted in the highest amount of tissue after 6 weeks. This study is the first to provide insight into the tissue-to-scaffold weight ratio, therewith allowing for a fair comparison between engineered tissues cultured on scaffolds as well as between native heart valve tissues. Although the absolute amount of ECM components differed between the engineered tissues, the ratio between ECM components was similar after 6 weeks. PCL-based tissues maintained their shape, whereas the PGA-P4HB-based tissues deformed during culture. After 6 weeks, PCL-based engineered tissues showed amounts of cells and ECM that were comparable to the number of human native heart valve leaflets, whereas values were lower in the PGA-P4HB-based tissues. Although increasing in time, the number of collagen crosslinks were below native values in all engineered tissues. In conclusion, this study indicates that slow-degrading scaffold materials are favored over fast-degrading materials to create organized ECM-rich tissues *in vitro*, which keep their three-dimensional structure before implantation.

Introduction

BIODEGRADABLE SYNTHETIC POLYMERS are used extensively in the field of cardiovascular tissue engineering (TE) to fabricate three-dimensional (3D) porous scaffolds, aiming for the regeneration of different types of tissues, such as heart valves and blood vessels.[1,2] In the classical *in vitro* TE approach, tissue replacements are developed by seeding cells into synthetic biodegradable porous scaffolds, which can be implanted as a living autologous replacement after *in vitro* culture. Alternatively, the engineered tissue can be decellularized after culture to create allogenic off-the-shelf replacements that are rapidly repopulated to function as a living replacement that is able to adapt and remodel.[3–6]

As we focus on cardiovascular applications such as in heart valves, we use human primary vascular-derived cells to grow tissue in fast-degrading polyglycolic acid scaffolds coated with poly-4-hydroxybutyrate (PGA-P4HB)-based scaffolds or slow-degrading poly-ε-caprolactone (PCL)-based scaffolds. Both scaffold types have previously shown

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excellent results in terms of biocompatibility, processing ability, and cell infiltration.[3,5,7–10] Different research groups have studied the absorption of these scaffolds, mainly *in vivo*. It appeared that the site of implantation, presence of enzymes, molecular weight of the material, and scaffold porosity all affect absorption rates *in vivo*.[11] Reported complete absorption times of PGA vary from 1.5 months[12] to 4–6 months.[13] For electrospun PCL scaffolds, absorption takes much longer, and this type of scaffold is reported to be completely absorbed *in vivo* after at least 2 years.[14] Obviously, scaffolds with slow- and fast-absorption rates will contribute to the mechanical integrity of the tissue differently with time, both *in vivo* and *in vitro*.

We hypothesize that cells in fast-absorbing scaffold materials will comprehend increased rates of tissue production to compensate the loss of mechanical integrity by formation of collagen fibers, compared to cells in slow-absorbing scaffold materials, where mechanical integrity is maintained for a longer period of time. How tissue composition changes during *in vitro* culture and how this affects mechanical integrity due to different *in vitro* degradation properties of the scaffold materials was never fully assessed. In an attempt to balance scaffold degradation, tissue stability, and mechanical integrity for *in vitro* TE, we determined the weight ratio between scaffold and tissue every week, during a 6-week culture period, using both a slow- and a fast-degrading scaffold. In addition, we analyzed absolute and relative amounts of extracellular matrix (ECM) and mechanical properties of the constructs with time. Mechanical loading is essential in valvular tissue formation and was applied to the constructs in a static manner by constraining the growing tissues. This allows for tension to develop in the tissues by the contractile nature of the cells,[15,16] and this tension provides increased functionality of engineered tissues.[17]

Previously, attempts were undertaken to compare tissue composition of engineered constructs to that of native cardiovascular tissues.[12,18–20] Results from conventional tissue composition assays express tissue composition relative to the dry weight of the sample. Often the weight of the remaining scaffold is integrated into the weight that is used to compare tissue composition of engineered tissues with that of native tissues.[13,21–23] This results in an overall overestimation of the actual formed tissue weight and, therewith, an underestimation of the amount of ECM components per milligram formed tissue. To make a fair comparison between engineered and native tissues, the obtained data should be corrected for the remaining scaffold weight. During aging, heart valves are known to change in terms of ECM composition. We compared our engineered tissue data with native valvular human data of several age groups as determined by van Geemen *et al*[24] to assess similarity of the engineered tissues to native tissues.

Materials and Methods

Cell culture

Human vascular-derived cells were harvested from segments of a vena saphena magna from a patient who underwent bypass surgery and were obtained according to the Dutch guidelines for secondary used materials. Cells were obtained using the outgrowth method and cultured using standard culture methods in a humidified atmosphere containing 5% CO_2 at 37°C as described previously.[8] These cells were previously characterized as α-smooth-muscle-actin positive and smoothelin and S100A4 negative,[7] indicating activated smooth muscle cells, which are also often referred to as myofibroblasts. Isolation and expansion medium consisted of advanced Dulbecco's modified Eagle's medium (Invitrogen), supplemented with 1% GlutaMax (Gibco), 1% Penicillin/Streptomycin (P/S; Lonza), and 10% fetal bovine serum (FBS) (Greiner Bio-one).

Scaffold preparation and sterilization

Rectangular strips (25 × 5 mm) were cut out of PGA meshes (PGA; specific gravity, 70 mg/cm^3; Cellon) and conventionally electrospun PCL meshes, both with a thickness of 1 mm and comparable fiber diameter. PGA scaffolds were additionally coated with P4HB (received through a collaboration with Professor Hoerstrup of the University Hospital Zurich) to provide structural integrity to the mesh. The outer 3–4 mm of both PGA and PCL scaffold strips was attached to stainless steel rings (RVS Paleis) using 15% polyurethane–tetrahydrofuran (PU; DSM) glue, leaving an 18 × 5 mm area for cell seeding. The solvent was allowed to evaporate overnight in a vacuum oven. The rings with the scaffold strips were placed in 6-well plates and sterilization was achieved by immersion in an antibiotic/antifungi solution, consisting of 10% P/S (Lonza) and 50 μg/mL Fungin in sterile phosphate-buffered saline (PBS) (Sigma) for 30 min on a shaker at 37°C. Subsequently, the antibiotics/antifungi solution was removed and 70% ethanol was added for 15 min. The ethanol step was repeated and, thereafter, the strips were washed twice in PBS. To facilitate cell attachment, the scaffolds were incubated overnight with TE medium, consisting of expansion medium supplemented with 0.25 mg/mL L-ascorbic acid 2-phosphate (Sigma).

Experimental design

Scaffold strips of both materials ($n = 58$) were either kept unseeded in culture ($n = 4$ per week) or were seeded with cells ($n = 4$–5 per week). Passage 7 cells were used and seeded onto both PGA-P4HB and PCL scaffolds with a seeding density of 2.0×10^6 per cm^3 using fibrin as a cell carrier.[25] In short, cells were suspended in TE medium containing thrombin (10 U/mL; Sigma). This cell suspension was mixed with an equal volume of TE medium containing fibrinogen (10 mg/mL; Sigma) and dripped onto one side of the scaffolds. After seeding, the constructs were placed in an incubator at 37°C for 30 min to allow polymerization of the fibrin gel. Thereafter, 6 mL of TE medium was added to each scaffold. The constructs were cultured for up to 6 weeks and the TE medium was changed twice a week. After 1–6 weeks, seeded strips ($n = 4$–5 per week) were sacrificed. One strip was used for histology and the remaining strips were used for mechanical testing followed by biochemical assays. At weeks 0–6, unseeded strips ($n = 4$ per week) were sacrificed. These strips were used for mechanical testing only.

Biochemical assays

For the quantification of tissue formation during culture, engineered constructs were lyophilized after mechanical testing ($n = 3$–4 per group) and used for biochemical

assays. The total amount of DNA was determined as an indicator of number of cells, the amount of hydroxyproline (hyp) as an indicator for collagen content, and the amount of sulfated glycosaminoglycans (sGAG) was measured. Measurements were averaged per group. Lyophilized constructs were weighed and digested in papain solution (100 mM phosphate buffer [pH = 6.5], 5 mM L-cysteine, 5 mM ethylene-di-amine-tetra-acetic acid [EDTA], and 125–140 μg papain per mL, all from Sigma) at 50°C for 16 h. To compare DNA, sGAG, and collagen within the engineered constructs in time and with values found in the native tissue, the weight of the engineered tissues without scaffold needs to be calculated. To obtain these values, the weight of the remaining scaffold of the unseeded strips of equal time points was subtracted from the weight of the seeded strips. The digested supernatant was collected and used for the DNA, sGAG, and collagen assays. The amount of DNA in the constructs was determined using the Hoechst dye method[26] and a standard curve was prepared from calf thymus DNA (Sigma). As described before, the sGAG content was determined with a modification of the protocol described by Farndale *et al.*[8,27] Collagen content was determined by an assay as described by Huszar *et al.*[28] and a standard curve was prepared from *trans*-4-hydroxyproline (Sigma). The number of mature collagen hydroxylysyl pyridinoline (HP) and lysyl pyridinoline (LP) crosslinks, as a measure of tissue maturity, was measured in the digests of the constructs using high-performance liquid chromatography as described previously.[29–31] The number of HP and LP crosslinks was expressed per triple helix (TH) and the ratio of HP and LP crosslinks was determined.

Mechanical testing

After 0 (only for the unseeded group), 1–6 weeks of culture, the mechanical properties of the engineered constructs ($n = 3$–4 per group) were assessed by uniaxial tensile tests in the longitudinal direction of the constructs, using a BioTester 5000 (CellScale). The samples were stretched to 5%, 10%, and 15% strain for five times to precondition the samples. Mechanical test data were averaged per group. Sample thickness and width were measured with an electronic caliper. Stress–strain curves were obtained and Young's modulus was determined as the slope of the curve at a strain of 15%, as a measure for tissue stiffness.

Histology

To analyze tissue formation qualitatively, constructs were processed for histology ($n = 1$ per group). Representative samples were fixed with 3.7% formaldehyde (Merck) and embedded in paraffin. Tissue sections of 10 μm were cut and studied by Masson trichrome (MT) staining (MTC kit; Sigma) for collagen deposition. The stainings were analyzed using light microscopy (Axio Observer; Zeiss).

Statistical analyses

Statistics was performed using GraphPad Prism (version 5.04) and differences were considered significant for $p < 0.05$. All data were presented as mean ± standard error of the mean. Regression analyses were performed to determine changes in tissue weight, scaffold weight, amount of ECM components, stiffness of the samples, and crosslinks over time. In case of a significant increase or decrease, the percentage increase or decrease was calculated using the predicted model equation. Also, the plateau and slope of the different curves were compared using regression analyses. One-way ANOVA, followed by a Tukey's multiple comparison *post-hoc* test, was used to compare TE composition with native tissues.

Results

Scaffold-to-tissue ratio changes over time

Dry weight of PCL scaffold material remained constant during culture time, whereas dry weight of PGA-P4HB scaffolds indicated mass loss starting after week 1, with a decrease of 93% compared to the initial values after 6 weeks of culture ($p < 0.05$, Fig. 1A). A contribution in weight due to tissue formation was observed in both scaffold types, as weight of tissues cultured in both PCL- and PGA-P4HB-based scaffolds increased during culture. When comparing the ratio between tissue weight and the remaining scaffold weight, a percentage decrease in scaffold contribution and a percentage increase in tissue weight were observed in both the PGA-P4HB-based (Fig. 1B) and PCL-based (Fig. 1C) scaffold groups ($p < 0.05$). After seeding, mainly scaffold weight contributed to the total weight of the constructs, as no tissue was formed yet. Although tissue was formed within the PGA-P4HB constructs, a decrease in total weight was observed during culture, which was due to the fast degradation, and thus mass loss, of PGA-P4HB scaffolds. In the PGA-P4HB-based constructs, a change was observed after roughly 2 weeks, as after this time point, mainly tissue weight contributed to the total weight of the constructs. PGA-P4HB scaffolds were completely resorbed after 6 weeks, with only tissue weight contributing to the total weight of the constructs. This change was not observed in the PCL-based constructs, as PCL scaffolds did not degrade as fast as PGA-P4HB scaffolds and primarily contributed to the total weight.

Tissue evolution in slow- and fast-degrading scaffold

Total amounts of DNA, sGAG, and collagen per construct increased during culture in both the PGA-P4HB (Fig. 2A) and the PCL (Fig. 2B) groups (all $p < 0.01$). When comparing the sGAG formation between the scaffold groups (Fig. 2C), production rates were similar; however, the total amount of the PGA-P4HB-based tissues formed was significantly lower than that of their PCL counterparts ($p < 0.01$). Furthermore, although collagen production was increased in the PGA-P4HB groups compared to that in the PCL groups during the first 2 weeks of culture, the total amount of the PGA-P4HB groups formed was lower than that of the PCL group after 6 weeks ($p < 0.01$, Fig. 2D). Total ECM values in the PCL-based constructs after 6 weeks of culture were higher than those in the PGA-P4HB-based constructs, which were 192 ± 3 and 166 ± 12 μg for the PCL and PGA-P4HB constructs, respectively. Although lower total amounts were observed for the PGA-P4HB-based tissues, cells in the PGA-P4HB-based tissues seemed to be more synthetic during the first 2 weeks of culture than cells in PCL-based scaffolds, with increased total amounts of ECM when corrected for the amount of DNA. Synthetic activity, in terms

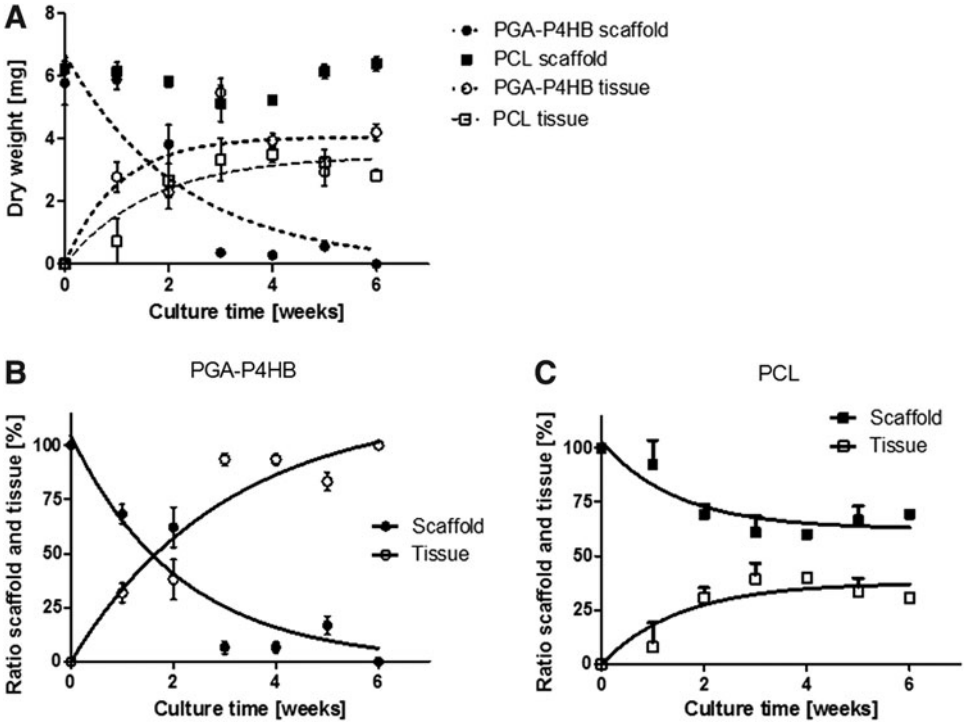

FIG. 1. Dry weights of scaffolds and tissues during culture **(A)**. Weights of the scaffolds and tissues are given as mean ± standard error of the mean (SEM). Poly-ε-caprolactone (PCL) scaffold remains stable during culture, whereas polyglycolic acid scaffolds coated with poly-4-hydroxybutyrate (PGA-P4HB) started to degrade even after 1 week. Newly formed tissue contributes to the total weight of the strips. Ratio of the fast-degrading PGA-P4HB **(B)** and the slow-degrading PCL **(C)** scaffold to tissue during culture, given in percentages. Weight of tissue and scaffold is given as mean percentage, as a section of the total weight of the samples. Total weight of the whole samples is set at 100%.

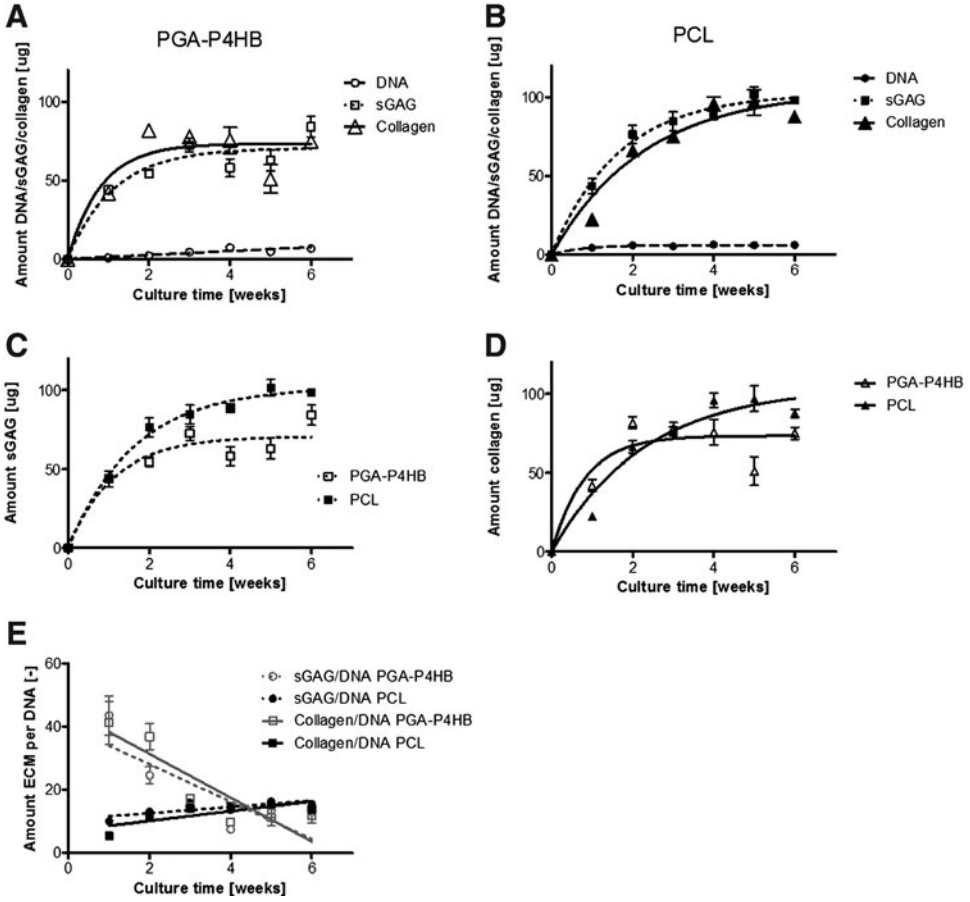

FIG. 2. Combined results of DNA, sulfated glycosaminoglycans (sGAG), and collagen per strip during culture on PGA-P4HB **(A)** and PCL **(B)** scaffolds. During culture, the total amount of extracellular matrix (ECM) increased, which was more pronounced for PCL-based tissues. PGA-P4HB-based constructs demonstrated lower plateau levels of the amount of sGAG **(C)** and collagen **(D)** than PCL-based constructs. sGAG and collagen production per DNA **(E)** of PGA-P4HB-based tissues was increased during the first weeks and became comparable to those of PCL-based tissues after 3 weeks. All results are given as mean ± SEM.

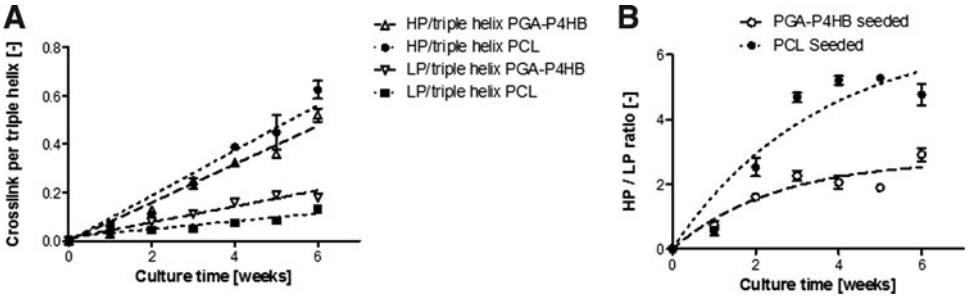

FIG. 3. Collagen crosslinks in both scaffold groups given as hydroxylysyl pyridinoline (HP)/triple helix (TH) and lysyl pyridinoline (LP)/TH **(A)**. Crosslinks within tissues grown on both type of scaffolds increased with culture time, whereas HP/TH increased with a higher rate than LP/TH. HP/LP ratio **(B)** was increased in PCL-based tissues compared to PGA-P4HB-based tissues.

of sGAG per DNA and collagen per DNA, decreased with time for cells in the PGA-P4HB scaffolds ($p<0.01$), whereas this increased with time for the cells in the PCL scaffolds ($p<0.01$) (Fig. 2E).

Both the HP and LP crosslinks per TH increased during culture time in tissues cultured on both the PCL and PGA-P4HB scaffolds ($p<0.01$, Fig. 3A), with increased production rates of HP per TH compared to LP per TH in both scaffold groups ($p<0.01$). When compared between the scaffold groups, faster production of LP per TH was observed in the PGA-P4HB-based tissues ($p<0.05$) than in the PCL-based tissues, whereas no difference in the production rates of HP per TH was found between the scaffold groups.

Engineered tissues versus native heart valves

After 6 weeks of culture, the amount of ECM per milligram tissue (Fig. 4A) and ECM ratio (Fig. 4B) of engineered tissues were compared with those of human aortic heart valves. DNA per milligram tissue was comparable between engineered tissue based on both scaffold types and native values of different age groups. sGAG per milligram tissue decreases during aging of humans, whereas the amount of collagen per milligram tissue increases ($p<0.05$). All engineered tissues resulted in sGAG values comparable to native adolescent and adult values, whereas PGA-P4HB-based tissues demonstrated lower sGAG values than native values in children ($p<0.05$). Collagen values of PCL-based tissues were not significantly different from native values, whereas PGA-P4HB-based tissues resulted in lower values ($p<0.05$ compared to children and $p<0.001$ compared to adolescents and adults). Although the amounts of ECM differed between the engineered tissue groups, their ECM ratios were comparable. These ratios were also similar to ratios found in children and adolescents. When compared to adult tissues, the percentage of collagen differed between adults and both engineered groups ($p<0.05$), whereas the percentage of sGAG was only significantly different from the PCL-based tissues ($p<0.05$). Although the amount of newly formed collagen in PCL-based engineered tissues after 6 weeks was similar to values measured in native tissues, the HP collagen crosslinks of both PCL and PGA-based tissues do not reach native values during culture (data not shown). After 6 weeks of culture, HP crosslinks of PCL- and PGA-P4HB-based tissues were 0.63 ± 0.04 and 0.52 ± 0.03 HP/TH, respectively, whereas values observed in children, adolescents, and adults were 2.0 ± 0.1, 2.0 ± 0.03, and 2.6 ± 0.1 HP/TH, respectively. LP/TH also increased during culture time and directed toward values measured in children (0.2 ± 0.06 LP/TH) at the end of culture. HP/LP ratio of engineered tissues was similar to native values for adolescents and adults; however, it differed significantly with the ratio found in children ($p<0.001$) (Fig. 4C). During aging, the ratio drops rapidly from 14.4 in children to 5.7 and 4.8 in adolescents and adults, respectively, as a result of a fast increase in LP/TH in adolescents.

Mechanical characteristics of formed tissues based on fast- or slow-degrading scaffold

Contribution of tissue formation to the mechanical properties was observed in both seeded PGA-P4HB and PCL samples, as samples became stiffer with culture time ($p<0.01$ for PCL and $p<0.05$ for PGA-P4HB, Fig. 5A), whereas Young's modulus remained constant in the unseeded PCL scaffold strips. This is also visualized by the stress–strain curves after 6 weeks of culture (Fig. 5B). The curves demonstrate the additional effect of tissue formation on the PCL strips compared to the unseeded PCL strips. Due to fast loss of mechanical integrity of the PGA-P4HB scaffold strips, mechanical tests on the unseeded PGA-P4HB samples over time could not be performed. This indicates that the observed mechanical properties in the seeded PGA-P4HB constructs are solely determined by the tissue. The stress–strain curves of both PCL- and PGA-P4HB-seeded strips demonstrate nonlinear curves, representing tissue behavior, which is more pronounced in the PGA-P4HB strips.

Histological visualization of engineered tissues in time

Histology of the constructs revealed cellular tissues with dense surface layers, which was more pronounced in PGA-P4HB-based tissues. MT stainings showed collagen fibers throughout the strips of all groups during culture. Collagen is less homogeneously distributed in the PGA-P4HB-based strips (Fig. 6A–F) than in the PCL-based strips (Fig. 6H–M). Furthermore, PCL-based tissues resulted in interstitial growth of tissue, whereas appositional growth was observed in the PGA-P4HB-based tissues, where a thick layer of tissue was formed around the scaffold. In addition, PGA-P4HB constructs showed compaction (decreased scaffold width) during culture, with significant differences compared to the original

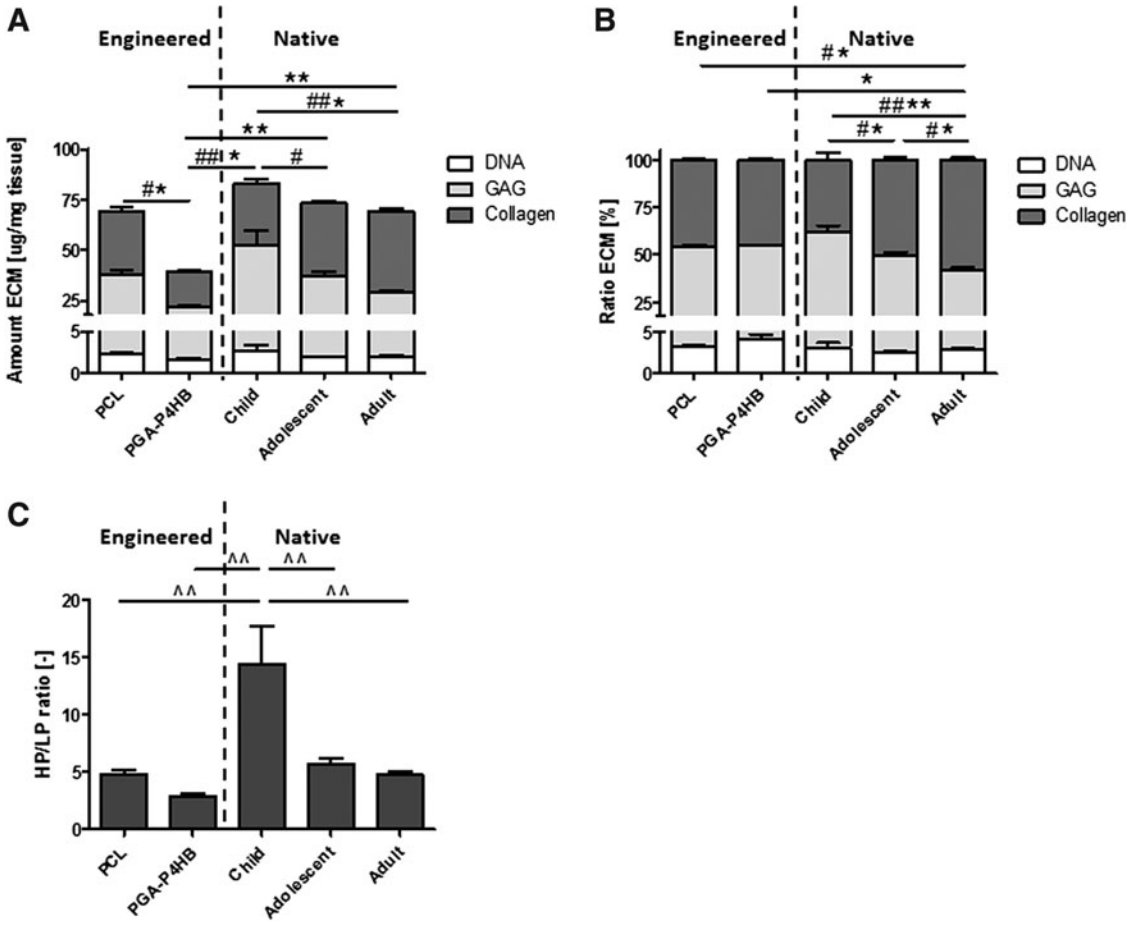

FIG. 4. Comparison between amount of ECM **(A)** and ECM ratio **(B)** per milligram formed engineered tissue with native data. Results are given as mean ± SEM. Tissues based on PCL scaffolds showed amounts of ECM comparable to native human aortic valve values, whereas amounts found in PGA-P4HB-based tissues were lower than in their native counterparts. ECM ratio was similar in all engineered tissues and comparable to ratios found in children and adolescents, whereas it differed when compared to the ratio observed in adults. The HP/LP ratio **(C)** of the engineered tissues was comparable to the ratio observed in aortic valves of adolescents and adults, whereas HP/LP ratio was lower than that of children. #, *, and ^ represent significant differences of sGAG, collagen, and HP/LP ratio, respectively. Single or double symbols indicate $p < 0.05$ and $p < 0.001$.

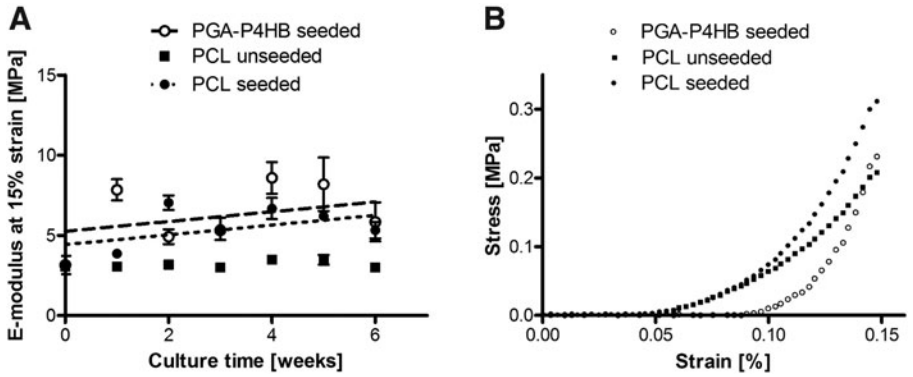

FIG. 5. Young's modulus of seeded and unseeded scaffold strips during culture, given as mean ± SEM **(A)**. Stress–strain curves of the seeded and unseeded scaffold strips after 6 weeks of culture at 15% strain **(B)**. Scaffold in PCL-based constructs still contributes to the mechanical properties, whereas for the PGA-P4HB-based constructs, mechanical properties are determined by tissue only. Newly formed tissue showed an additional effect on Young's modulus during culture, as demonstrated in both figures with an increased stiffness in the seeded samples than in the unseeded samples.

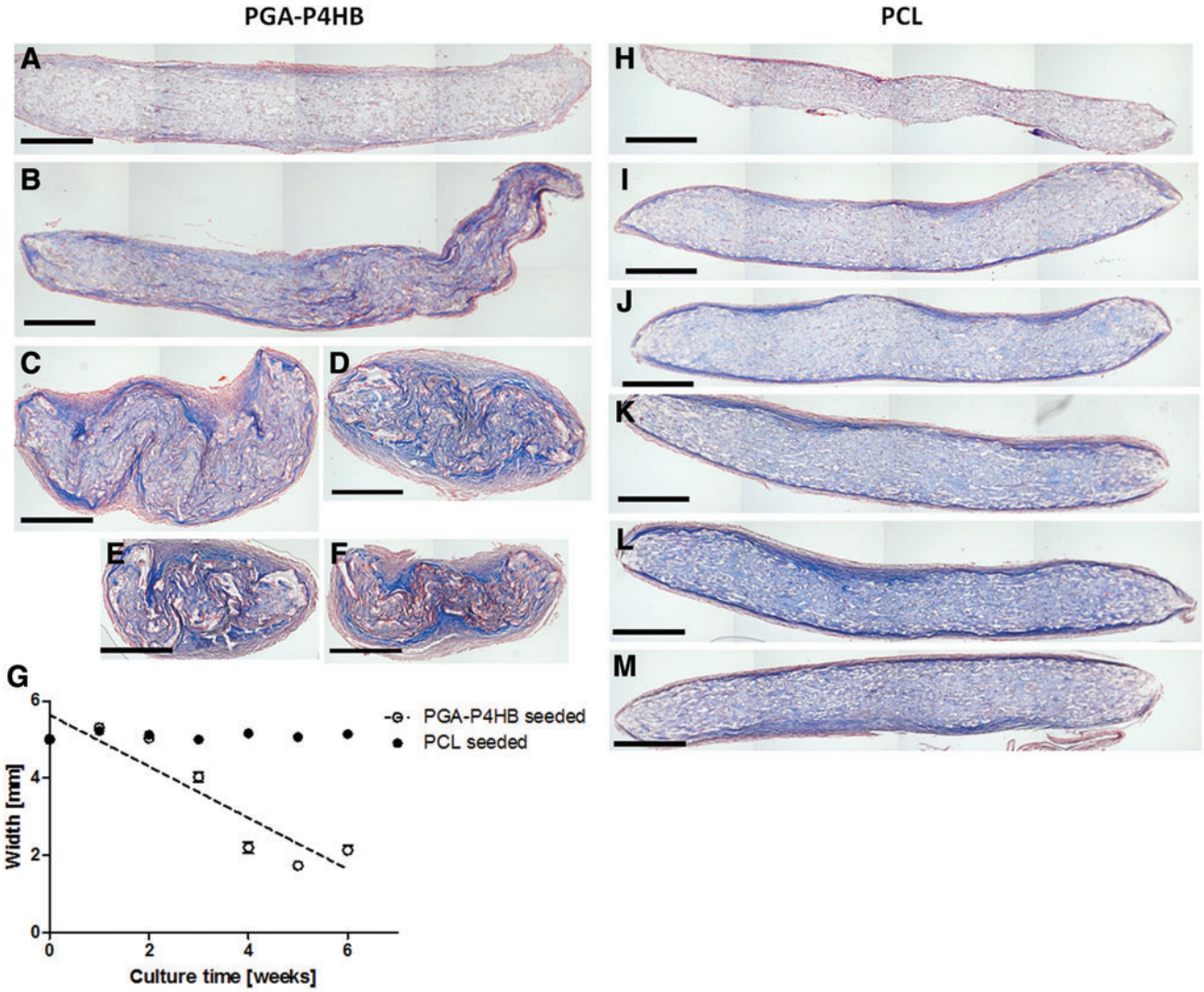

FIG. 6. Masson trichrome staining of PGA-P4HB (weeks 1–6 represented by **A–F**) and PCL (weeks 1–6 represented by **H–M**) sections. The *black* scale bars represent 600 μm. Collagen is shown in *blue* and *red* represents cytoplasm and muscle tissue. Vacuoles within the PCL sections are due to scaffolds remnants, which are dissolved during the dehydration step. PGA-P4HB sections do still show scaffold remnants (uncolored parts between tissue). Collagen is more homogeneously distributed in the PCL strips than in the PGA-P4HB strips. Thickness of the strips **(G)** remains stable for PCL strips, whereas PGA-P4HB strips showed compaction. Color images available online at www.liebertpub.com/tea

width ($p < 0.01$), whereas the width of PCL constructs remained stable during culture (Fig. 6G).

Discussion

A balance between the rate of scaffold absorption and tissue formation is crucial for maintaining mechanical integrity of the replaced tissues. We estimated the influence of slow- versus fast-degrading scaffolds on the amount and composition of engineered tissues and mechanical integrity during culture. In addition, we compared these values of the engineered tissues to values found in native human heart valve leaflets.

In vitro *evolution of tissue formation*

The unseeded PCL scaffold strips did not degrade *in vitro,* in terms of weight, whereas the unseeded PGA-P4HB scaffold started to loose mass already after 1 week. This resulted, together with the contribution in weight of the tissues, in different scaffold-to-tissue ratios during culture, with tissue weight being the main contributing factor in PGA-P4HB constructs, whereas for PCL constructs, both tissue and scaffold weight contributed to the total weight. Our results on scaffold degradation are comparable with findings by Klouda,[32] where a mass loss of 0.9% and 11% for PCL and PGA-P4HB scaffolds, respectively, was found after 15 days of static incubation. However, we observed a more severe mass loss of the PGA-P4HB scaffold, as it decreased by 33% after 14 days. This might be due to the fact that in the study of Klouda, samples were incubated with PBS, whereas our samples were incubated in a culture medium containing FBS. As certain enzymes present in serum are known for degrading scaffolds,[33–38] this might have led to accelerated degradation of the PGA-P4HB scaffold strips compared to that shown in the study of Klouda.

Although increased amounts of ECM components were shown in both scaffold groups with time, differences in

tissue composition, when cultured using fast- or slow-degrading scaffolds, were observed. A first observation was that cells in the PGA-P4HB-based tissues seemed to be highly synthetic as sGAG and collagen per DNA were higher than in PCL-based tissues. However, this was only observed during the first 2 weeks of culture, whereafter the cells became less synthetic. At the end of culture, higher total amounts of sGAG and collagen for PCL-based tissues were observed. We hypothesize that this difference in tissue evolution is due to the fast degradation of the PGA-P4HB scaffolds, resulting in highly synthetic cells during the first weeks to compensate for the loss of mechanical integrity of the scaffold by depositing newly formed collagen fibers. Compaction of the PGA-P4HB scaffolds resulted in a smaller surface area and less volume for the cells within these tissues to lay down their ECM, compared to PCL-based tissues. This smaller total volume available for cells to deposit matrix in the PGA-P4HB scaffolds may explain the lower ultimate overall level of ECM in the PGA-P4HB scaffolds than in the compaction-resistant PCL scaffolds. Although in this study the the formed tissue was analyzed over time, in future studies detailed analyses on the dynamic changes in cell behavior may be performed through multiple time point PCR analyses to elucidate the mechanisms behind the observed differences in tissue formation in slow- versus fast-degrading scaffolds.

It is well described that degradation of PGA scaffolds with or without P4HB can alter the pH of the environment due to their acid degradation products.[11,39–41] A low pH possibly affects the viability, proliferation, or tissue synthesis of the cells. Higgins *et al.* showed that the number of porcine smooth muscle cells decreased, and cells dedifferentiated due to PGA degradation products.[39] However, in their study, the medium was collected after 7 days only, whereas in our study, the medium was changed twice a week to prevent building up of degradation products and thus an acidic environment. We, therefore, assume that degradation products released into the culture medium did not have a profound effect on the viability, proliferation, and tissue synthesis in our experiments.

All tissues demonstrated a continuous increase in LP and HP crosslinks during culture. However, the ratio between these crosslink types differed between tissues. The HP/LP ratio was lower for PGA-P4HB-based tissues than for PCL-based tissues. Wassen *et al.*[42] described that a lower HP/LP ratio caused by a relative high amount of LP/TH, as observed in our PGA-P4HB samples, is seen in mineralized tissues only. This might assume that tissues cultured in PGA-P4HB scaffolds are more prone to mineralization than their PCL counterparts. They also hypothesize that mineralization of collagen fibrils is promoted by specific orientation of the molecules within these fibrils, which might be different between the PCL- and PGA-P4HB-based tissues due to a potentially higher degree of tissue remodeling of the PGA-P4HB-based tissues as a result of faster degradation of PGA-P4HB scaffolds.

The growing tissues were exposed to static conditioning to enhance tissue formation. Previous work in our group demonstrated that the addition of a dynamic conditioning component has minimal added value in terms of tissue formation and mainly enhances tissue organization,[43] which was beyond the scope of the current study. Furthermore, previous work has demonstrated the predictive nature of statically conditioned constructs to valvular engineered tissues.[7] Adding a dynamic component to the growing tissues is expected to enhance tissue organization in both slow- and fast-degrading scaffolds. Then, histology of multiple samples per time point may be informative to derive quantitative information on architectural changes in the ECM in fast- versus slow-degrading scaffolds.

Comparison between tissue formation

In the literature, different methods are described to compare ECM components and amounts of tissue, between engineered tissues or between their native counterparts. These include a noninvasive monitoring system to correlate biomarkers present in culture medium with those in the synthesized tissue[23] and a method in which ECM components are expressed as mg/cm^3 tissue.[20] However, these are suboptimal methods, as the first method does not include the total amount of tissue formed and, in the second method, the remaining scaffold can contribute to the dimensions and, therefore, possibly influence the outcome, especially when the scaffold is not degraded yet. To allow for accurate insight into tissue evolution during culture and a fair comparison between engineered and native tissues, only tissue weight without the contribution of remaining scaffold should be used. Our study is the first that provides these insights as we corrected for the presence of remaining scaffold. This correction is of importance when comparing properties of tissues that were cultured using scaffolds with different degradation rates and when comparing engineered tissues that were grown on slow-degrading scaffolds, which is still (partly) present with native tissues. A limitation of this method is that we do not account for the effect of cells and tissue on scaffold degradation. The presence of cells can

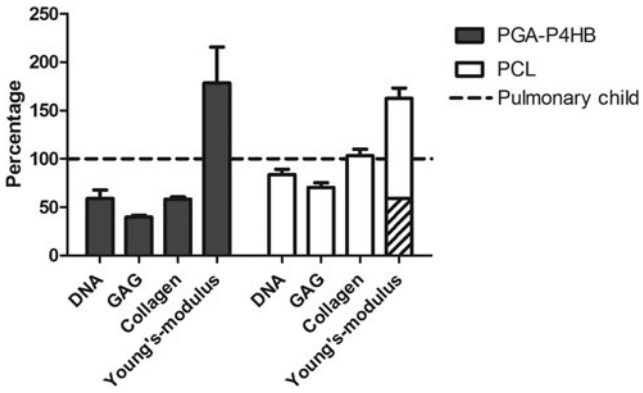

FIG. 7. Comparison of native values of a child's pulmonary valves with engineered tissues after 6 weeks of both PCL- and PGA-P4HB-based tissues. Values of pulmonary valves of children are set at 100% (*horizontal dashed line*). Values of PCL- and PGA-P4HB-based tissues are given as percentage compared to native values of children. PCL shows values that are similar or closer to native values in terms of ECM, whereas PGA-P4HB shows lower values. Engineered tissues are stiffer than their native counterparts. Although PGA-P4HB scaffold does not influence the mechanical properties of the tissue after 6 weeks, stiffness is similar to PCL-based tissues that are still partly influenced by the remaining scaffold (marked area in the bar).

result in accelerated degradation, as cells might release enzymes that stimulate this degradation. Furthermore, *in vivo* macrophages will migrate to the scaffold materials and start to degrade the materials, which is not the case in our *in vitro* setup. Despite this limitation, this new method comes nearest to the actual values, compared to all other studies performed until this day.

Pulmonary valves for children are the first target of tissue-engineered valves. Therefore, an overview of the generated amount of ECM and mechanical properties of PCL- and PGA-P4HB-based tissues after 6 weeks of culture compared to values of pulmonary valves of children is given in Figure 7. PCL-based tissues show ECM values that are most similar to native values found in children, whereas PGA-P4HB-based tissues showed a somewhat lower amount of ECM. Similar stiffness values were observed in both PCL- and PGA-P4HB-based tissues, whereas the values of the latter are determined only by the newly formed tissue and not by remaining scaffold, as observed for PCL. Stiffness of engineered samples is higher than that of native values; however, we do not expect difficulties in opening or closing of the leaflets after implantation, as PGA-P4HB valves with a similar stiffness were successfully implanted before.[12]

In conclusion, tissues based on slow-degrading materials, which maintained weight and mechanical integrity during culture, preserved their 3D shape. Tissues based on fast-degrading material, which quickly demonstrated mass loss and loss of mechanical integrity, resulted in compaction during culture and different tissue-to-scaffold ratios. Although cells in PGA-P4HB constructs produced tissue at a higher rate during the first weeks of culture than cells in PCL constructs, the amount of tissue after 6 weeks was higher in the latter. ECM ratios were comparable between the scaffold groups and also between engineered and native human values. This study demonstrates the importance of using slow-degrading scaffolds to create constructs with stable mechanical integrity, which maintain their configuration upon implantation. Further long-term research is needed to investigate properties of PCL-based tissues when this scaffold material is completely degraded.

Acknowledgments

This work was supported by a grant from the Dutch Government to the Netherlands Institute for Regenerative Medicine (NIRM, grant no. FES0908). This research also forms part of the Project P1.01 iValve of the research program of the BioMedical Materials institute, cofunded by the Dutch Ministry of Economic Affairs. The financial contribution of the Nederlandse Hartstichting is gratefully acknowledged. The authors gratefully thank Marc Simonet for electrospinning of the PCL scaffolds.

Disclosure Statement

M.B. is employed by Xeltis. M.C.is employed by Xeltis and has shares in Xeltis. F.B. and A.D.-M. have shares in Xeltis.

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Received: May 11, 2015
Accepted: September 28, 2015
Online Publication Date: November 23, 2015