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Relationships Between Degradability of Silk Scaffolds and Osteogenesis

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Abstract

Bone repairs represent a major focus in orthopedic medicine with biomaterials as a critical aspect of the regenerative process. However, only a limited set of biomaterials are utilized today and few studies relate biomaterial scaffold design to degradation rate and new bone formation. Matching biomaterial remodeling rate towards new bone formation is important in terms of the overall rate and quality of bone regeneration outcomes. We report on the osteogenesis and metabolism of human bone marrow derived mesenchymal stem cells (hMSCs) in 3D silk scaffolds. The scaffolds were prepared with two different degradation rates in order to study relationships between matrix degradation, cell metabolism and bone tissue formation in vitro. SEM, histology, chemical assays, real-time PCR and metabolic analyses were assessed to investigate these relationships. More extensively mineralized ECM formed in the scaffolds designed to degrade more rapidly, based on SEM, von Kossa and type I collagen staining and calcium content. Measures of osteogenic ECM were significantly higher in the more rapidly degrading scaffolds than in the more slowly degrading scaffolds over 56 days of study in vitro. Metabolic analysis, including glucose and lactate levels, confirmed the degradation rate differences with the two types of scaffolds, with the more rapidly degrading scaffolds supporting higher levels of glucose consumption and lactate synthesis by the hMSCs upon osteogenesis, in comparison to the more slowly degrading scaffolds. The results demonstrate that scaffold degradation rates directly impact the metabolism of hMSCs, and in turn the rate of osteogenesis. An understanding of the interplay between cellular metabolism and scaffold degradability should aid in the more rational design of scaffolds for bone regeneration needs both in vitro and in vivo.

Introduction

Successful tissue engineering strategies usually require three dimensional scaffolds with controllable structural and morphological features matched to the targeted clinical application. In addition, environmental factors are critical to cell differentiation toward specific cell and tissue outcomes in these scaffolds [1,2]. The scaffolds provide critical cues to the cells to direct function and fate, including interacting via integrins, leading to downstream signaling events [3]. Polymeric biomaterials studied for tissue engineering scaffolds related to bone

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regeneration present many challenges, including architectural control for pore size, pore size distribution and porosity, mechanical properties, rates of degradation, and chemistry related to cell adhesion [4-6]. Previously, we reported the importance of processing conditions in determining the morphology and structure of silk protein scaffolds, with direct relevance to bone tissue formation *in vitro* with human bone marrow derived mesenchymal stem cells (hMSCs) [7]. We have also shown that the structural features of these degradable silk scaffolds, mainly the crystalline beta sheet content, directly influenced the degradation rate *in vivo* [8]. A critical factor in the overall process of tissue regeneration is the relationship between scaffold degradation rate and cell functions leading toward the target tissue formation [9,10].

To date, most studies that address the role of scaffold features in cell differentiation have focused on the immediate consequences the morphology and chemistry related to the presentation of ligands for integrin signaling [11,12]. Less attention has been given to matching scaffold degradation rate to new tissue formation or to cell metabolic activity, despite the importance of these relationships to the rate and quality of tissue formed. For example, we have previously demonstrated that the rate of collagen remodeling directly impacts the rate of new collagen-extracellular matrix formation by human fibroblasts, based on metabolic flux analysis [9,10]. To determine key scaffold features related to bone healing, modeling has been used to describe the interactions between bone regeneration and scaffold properties after implantation [13]. The results of the model suggest that bone formation occurred gradually over time whereas scaffold resorption started quickly. This type of modeling used numerical simulation of microstructure and mechanical strength related to bone tissue engineering and provides a useful tool to identify optimal patient-specific designs, however, experimental validation is required [13]. Composite scaffolds that combine biodegradability and mechanical strength may offer advantages for applications towards bone engineering [14]. Mechanical properties of scaffolds (elasticity and stiffness) play an important role in bone regeneration [15,16]. Therefore, scaffolds should be biodegradable and possess a degradation rate that matches that of new bone formation in terms of mechanical load [17].

For bone repairs, the biomaterial scaffold should degrade over time, giving way to new bone regeneration *in vivo* to allow full restoration of native tissue structure and function [18]. If the degradation rate is too rapid, the scaffold porous structure may collapse, hindering mass transfer and leading to necrosis [19]. If the degradation is too slow, tissue regeneration may be hampered by fibrotic encapsulation and lack of host integration [20]. Therefore the kinetics of scaffold degradation are important in fostering optimal bone tissue regeneration. To address this issue, alginate gels were studied related to degradation rate and bone tissue formation, and more quickly degrading alginate gels permitted the more rapid development of a bone tissue [21]. *In vitro*, poly(ethylene glycol) hydrogel degradation was correlated to extracellular matrix formation, with increased polymer biodegradation rate resulting in higher cell proliferation/survival and extracellular matrix distribution with chondrocytes [22]. Matching degradation rates of poly-lactic-co-glycolic acid (PLGA) scaffolds to new bone formation facilitated new bone tissue formation and maturation over 24 weeks in both intra-periosteum and iliac bone defect models [23].

The hypothesis of the present study was that scaffolds with different degradation rates would support different cellular metabolic activities and thus influence the rate of new bone formation *in vitro*. To address this hypothesis, we utilized silk protein scaffolds with different degradation rates. A unique feature of this scaffold system is that the degradation rate can be adjusted at the materials processing stage, affording controlled studies on this variable in determining osteogenic outcomes. The silk scaffolds utilized in the study have shown promise in bone tissue engineering both *in vitro* and *in vivo* due to their impressive mechanical properties, biocompatibility and biodegradability [24-26]. In addition, silk fibroin scaffolds can be sterilized with ethanol or by autoclaving without loss of structural integrity [8]. To fabricate

the two different study groups used in the present work, we employed the same silk protein, but processed and sterilized the protein in two different ways. For fast degrading scaffolds, aqueous based processing and sterilization by ethanol was used, while the slow degrading scaffolds were prepared from hexafluoroisopropanol (HFIP) and autoclaving. In the preparation of the two study groups, the materials were also characterized for structure and pore size to assure these were not variables to confound data interpretation. (S. Figs. 1 and 2). To assess cell responses, hMSCs were analyzed with a variety of phenotypic and genotypic assays. Since our previous work on collagen matrix remodeling by fibroblasts demonstrated that the degradation rate of scaffolds directly impacted the metabolic activity of the cells [9, 10], we also characterized the metabolic profiles of the hMSCs undergoing osteogenesis. Glucose, lactate and amino acids (proline, lysine, glutamate, glutamine) were chosen as metabolic indicators of cell activity and matrix synthesis [27].

Materials and Methods

Preparation of silk fibroin porous 3D scaffolds

Cocoons of *Bombyx mori* were boiled for 20 min in an aqueous solution of 0.02 M Na₂CO₃, and then rinsed thoroughly with distilled water to extract the glue-like sericin proteins as we have previously reported [28]. The extracted silk fibroin was dissolved in 9.3 M LiBr solution at 60°C for 4 h, yielding a 20 w/v % solution. This solution was dialyzed in distilled water using Slide-a-Lyzer dialysis cassettes (MWCO 3,500, Thermo Fisher Scientific Inc., Rockford, IL) for 2 days. The final concentration of silk fibroin in aqueous solution was around 8 w/v%, determined by weighing the remaining solid after drying. To prepare the aqueous-derived more rapidly degrading scaffolds, 4 g of granular NaCl (particle size; 710~850 µm) was added to 2 ml of silk fibroin solution. The containers were covered and left at room temperature for 24 hrs, and then immersed in water to extract the salt for 2 days.

To prepare the organic solvent-based 3D porous scaffolds using HFIP, first the silk fibroin aqueous solution was lyophilized and then dissolved in HFIP at a concentration of 8 w/v % [7,29]. Granular NaCl particles (particle size; 710 µm to 850 µm) were added and the solvent allowed to evaporate overnight. Subsequently, the silk/porogen matrix was then treated in methanol for 30 min to induce the formation of the β-sheet structure and insolubility in aqueous solution. The scaffolds were then immersed in water to extract the salt for 2 days. Generally the pore size of the scaffolds was slightly smaller than the original size of the granular NaCl particles used in the process, as we have previously reported.

Sterilization of the scaffolds was accomplished with two methods to control beta sheet secondary structure, and thus, crystallinity, since crystallinity directly impacts the degradation rate of the materials both in vitro and in vivo [8]. The scaffolds were cut into discs (12 mm diameter, 5 mm thick) and dried in a fume hood. The aqueous-derived silk scaffolds were sterilized with 70% EtOH for 2 days and the HFIP-derived scaffolds were autoclaved, in order to preserve the differences in crystallinity generated with the two different processing routes, yet accomplish the need for sterilization prior to cell seeding.

Degradation

The two study groups (aqueous- and HFIP-derived scaffolds) were incubated at 37°C in 10 ml solutions containing 2U/ml protease XIV in phosphate buffered saline (PBS) at pH 7.4 [28]. Solutions were replaced daily and at designated time points the scaffolds were rinsed in distilled water and dehydrated (60°C, 2hrs) for dry weight. Controls consisted of scaffolds studied under the same conditions but without enzyme. The mass retained was calculated by comparing dry weight remaining at that time point with the initial dry weight.

Isolation of hMSCs

Human bone marrow stem cell isolation and expansion followed our previously published protocols.[28] Bone marrow aspirates (25 ml, Lonza, 27 years old male, Walkersville, Inc., MD) were diluted in 75 ml of (1×) PBS. The cells were separated by density gradient centrifugation. Twenty ml aliquots of bone marrow suspension were overlaid onto a poly-sucrose gradient (1077 g/cm³, Histopaque, Sigma, St. Louis, MO) and centrifuged at 800 ×g for 30 min at room temperature. The cell pellet was resuspended in Minimum Essential Medium Eagle (α-MEM: Gibco BRL, Grand Island, NY) supplemented with 10% fetal bovine serum (FBS, Gibco BRL), 100 U/mL penicillin G (Gibco BRL) and 100 µg/mL streptomycin (Gibco BRL). Cell number and viability were determined using trypan blue exclusion. The resuspended cells were plated at a density of 1.5×10⁵ cells/cm² and placed in a 5% CO₂ incubator at 37°C. The culture medium was changed every other day. Cells were passaged three times before use.

In vitro culture

Passage 3 hMSCs (5×10⁶ cells/scaffold) were seeded onto prewetted (α-MEM, overnight) scaffolds (diameter×height; 12 mm × 5 mm). For cultivation in spinner flasks, the scaffolds (constructs) were threaded onto needles embedded in the stoppers of the spinner flask (two constructs on four needles per flask), as we have previously described [25]. Flasks were filled with 250 ml osteogenic medium and placed in a humidified incubator at 37°C/5% CO₂, with side arm vent caps used to permit gas exchange and stirred with a magnetic bar at 50 rpm. Medium was replaced at a rate of 50% every 3 days for 56 days. Osteogenic media consisted of α-MEM supplemented with 10% FBS, 0.1 mM nonessential amino acids, 50 µg/ml ascorbic acid-2-phosphate, 100 nM dexamethasone, 10 mM β-glycerolphosphate and 100 ng/ml BMP-2 in the presence of 100 U/ml penicillin, 100 µg/ml streptomycin, and 0.25 µg/ml fungizone. The scaffolds were retrieved for analysis at days 16 and 56, while media samples were saved at every 3 days for metabolic analysis (see below).

Scanning electron microscopy (SEM)

The cells and scaffolds were examined by SEM (Zeiss FESEM Supra55VP, Oberkochen, Germany). The samples were fixed for 24 h in 0.4% glutaraldehyde after fractured in liquid nitrogen using a razor blade and then dehydrated in a series of graded ethanol extractions prior to coating with gold/palladium for 3 min before SEM observation.

Biochemical analysis

For DNA analysis, the scaffolds were chopped with microscissors on ice. DNA content was measured using the PicoGreen assay (Molecular Probes) Samples (N=4) were extracted twice with 0.5 ml 5% trichloroacetic acid for total calcium content. Calcium content was determined by a colorimetric assay using ortho-cresolphthalein complexone (OCPC, Stanbio Laboratory, Boeme, TX). The calcium complex was measured spectrophotometrically at 575 nm. Alkaline phosphatase (ALP) activity was measured by biochemical assay from Stanbio Laboratory (Boeme, TX), based on conversion of p-nitrophenyl phosphate to p-nitrophenol, which was measured spectrophotometrically at 405 nm. The tissue engineered constructs were digested with pepsin solution (1 mg/mL of pepsin, pH 3.0) at 4°C for 48 h to determine total collagen content. Total collagen was measured as we have previously reported [30]. A dye solution (pH 3.5) was prepared with Sirius red dissolved in picric acid saturated solution (1.3%, Sigma) to a final concentration of 1 mg/mL. The digested samples were dried at 37°C in 96-well plates for 24 h and then reacted with the dye solution for 1 h on a shaker. The samples were then washed five times with 0.01 N HCl and the dye-sample complex in each well was resolved in 0.1 N NaOH and absorbance read at 550 nm (Versa MAX, Molecular Devices, Sunnyvale,

CA). Total collagen in each sample was extrapolated using a standard plot of bovine collagen (Sigma) in the range of 0–500 µg/mL.

Histology and immunohistochemistry

The samples (N=3 per group) of each of the two study groups were fixed with 10% neutral buffered formalin for 24 hrs. The samples were then embedded in paraffin wax and sectioned serially at 4 µm thick slices. The sections were stained with hematoxylin & eosin (H&E) for histological observation, and von Kossa for mineralization. All sections were evaluated with a Leica DMIL light microscope (Watzlar, Germany) and Leica Application Suite (v3.1.0) software. The wall thickness and area of the scaffolds in the sections were analyzed with ImagePRO Plus 6.0 (Media Cybernetics, Inc., MD). The wall thickness (N=40) was measured from junctions among neighboring pores. The area of silk scaffold (N=40) in the section was quantified from the area occupied by scaffold compared to total area in the H&E image. For type I collagen immunohistochemistry, sample sections were assessed using a diaminobenzidine tetrahydrochloride (DAB) detection kit (Ventana Medical Systems, Inc., Tucson, AZ) according to the manufacturer's instructions. Briefly, sections are digested with endopeptidase to unmask antigen binding sites, exposed to an endogenous peroxidase inhibitor, and incubated with primary collagen type I antibody (Biodesign, Saco, ME) at room temperature. Sections were then incubated with biotinylated IgG and exposed to horseradish peroxidase (HRP) labeled streptavidin Antibody complexes were visualized after the addition of a buffered DAB solution, which produce a dark brown precipitate that was detected by light microscopy. The sections were counterstained in hematoxylin and lithium carbonate and mounted. Negative controls were performed similarly in the absence of the primary antibody.

Real-time PCR

Fresh scaffolds (N=4 per group) were transferred into 2 ml plastic tubes and 1.0 ml of Trizol was added. Scaffolds were chopped with micro scissors on ice. The tubes were centrifuged at 12,000 g for 10 min and the supernatant was transferred to a new tube. Chloroform (200 µl) was added to the solution and incubated for 5 min at room temperature. Tubes were again centrifuged at 12,000 g for 15 min and the upper aqueous phase was transferred to a new tube. One volume of 70% ethanol (v/v) was added and applied to an RNeasymini spin column (Qiagen, Hilden, Germany). The RNA was washed and eluted according to the manufacturer's protocol. The RNA samples were reverse transcribed into cDNA using oligo (dT)-selection according to the manufacturer's protocol (High Capacity cDNA Archive Kit, Applied Biosystems, Foster City, CA). Collagen type Iα1 (ColIα1), ALP, bone sialoprotein (BSP) and osteopontin (OP) levels were quantified using the Mx3000 Quantitative Real Time PCR system (Stratagene, La Jolla, CA) for osteogenesis. All data analysis employed the Mx3500 software (Stratagene) based on fluorescence intensity values after normalization with an internal reference dye and baseline correction. Differences of gene expression were generated by using comparative Ct method ($Ct[\Delta\Delta]Ct$ comparison). Ct values for samples were normalized to the endogenous housekeeping gene. Hypoxia inducible factor-1α (HIF-1α transcript) was also examined to assess hypoxia condition. PCR reaction conditions were 2 min at 50°C, 10 min at 95°C, and then 50 cycles at 95°C for 15 s, and 1 min at 60°C. The data were normalized to the expression of the housekeeping gene, glyceraldehyde-3-phosphate-dehydrogenase (GAPDH) within the linear range of amplification and differences.[7] The GAPDH probe was labeled at the 5' end with fluorescent dye VIC and with the quencher dye TAMRA at the 3' end. Primer sequences for the human GAPDH gene were: forward primer 5'-ATG GGG AAG GTG AAG GTC G-3', reverse primer 5'-TAA AAG CCC TGG TGA CC-3', probe 5'-CGC CCA ATA CGA CCA AAT CCG TTG AC-3'. Primer sequences for the human collagen type Iα (ColIα1) gene were: forward primer 5'-CAG CCG CTT CAC CTA CAG C-3', reverse primer 5'-TTT TGT ATT CAA TCA CTG TCT TGC C-3', probe 5'-CCG

GTG TGA CTC GTG CAG CCA TC-3'. Probes for ALP, HIF-1 α , BSP and OP were purchased from Assay on Demand (Applied Biosciences, Foster City, CA).

Metabolite analysis

Spent medium samples collected at each media change (every three days) were analyzed for glucose and lactate concentrations using the methods of Trinder [31] and Loomis [32], respectively. Amino acids were quantified by HPLC (Alliance 2690, Waters, Milford, MA) using gradient elution [33] and fluorescence-based detection following pre-column derivatization of primary or secondary amines with 6-aminoquinolyl-N-hydroxysuccinimidyl-carbamate [34]. All metabolite data were normalized by the corresponding cell sample DNA content, which was determined with a fluorescence-based assay using the Hoechst dye.

Statistical analysis

Statistical differences were determined using a Mann-Whitney U test (Independent t-test, SPSS). A statistical significance was assigned as * $P < 0.05$, ** $P < 0.01$ and *** $p < 0.001$, respectively.

Results

Enzyme scaffolds degradation test

The silk scaffolds processed in aqueous conditions and sterilized in 70% EtOH (study group: aqueous scaffolds) degraded more rapidly with the protease XIV treatment compared to the silk scaffolds processed in organic solvent (HFIP) and sterilized in by autoclaving (study group: HFIP scaffolds). The remaining mass at day 7 was around 5% of the original mass for the aqueous scaffolds and around 93% for the HFIP scaffolds (Fig. 1).

SEM

Analysis of fractured surfaces by SEM showed that all of the pores were occupied with new tissue after 56 days. The amount of mineralized nodules appeared to increase in both study groups, while dense mineralized ECM was observed in the more rapidly degrading (aqueous-derived) scaffolds after 56 days (Fig. 2).

Scaffolds degrading in cell culture

After 56 days of osteogenic culture, the average wall thickness of the aqueous scaffolds (7.2 ± 0.7 μ m) was significantly less than that of the HFIP scaffolds (10 ± 1.5 μ m) (Fig. 3a and 3b). Images of H&E stained sections showed that the area occupied by the aqueous scaffolds decreased significantly by 24%, whereas the area occupied by the HFIP scaffolds remained nearly unchanged (Fig. 3c).

Histology and immunohistochemistry

Histological evaluation with von Kossa revealed similar extents of matrix mineralization in both aqueous and HFIP scaffolds after 16 days. Positive staining (black) in the aqueous scaffolds was observed after 56 days. The intensity of the stain for the aqueous scaffolds was qualitatively higher than in the HFIP scaffolds (Fig. 4a). Immunohistochemistry demonstrated that type I collagen expression (dark brown) increased in the aqueous-derived scaffolds compared to the HFIP-derived scaffolds after 56 days (Fig. 4b).

Biochemical analysis

Cell proliferation was determined by measuring total DNA content in the scaffolds after 16 and 56 days of culture. After 56 days, cell numbers in the aqueous-derived scaffolds

significantly decreased compared to the HFIP-derived scaffolds ($p < 0.01$) (Fig. 5a). ALP activity as a marker of early osteoblastic differentiation displayed activity in both groups at day 56 and was significantly decreased when compared to the levels at day 16 (Fig. 5b). To determine mineralized matrix deposition in the scaffolds a calcium dissolution assay was used. Mineralized matrix deposition after 56 days in the aqueous-derived scaffolds was significantly higher than in the HFIP-derived scaffolds ($p < 0.001$). Calcium deposition in the aqueous-derived silk scaffolds increased 10-fold after 56 days of culture ($p < 0.001$) in comparison to the day 16 data, while calcium deposition in the HFIP-derived silk scaffolds increased 3.5-fold after 56 days of culture ($p < 0.05$) in comparison to the day 16 data (Fig. 5c). Total collagen content indicated significantly higher collagen synthesis ($8.28 \pm 2.8 \mu\text{g}/\text{DNA}$) in the aqueous-derived scaffolds at 56 days compared to $0.79 \pm 0.2 \mu\text{g}$ of collagen per DNA in the HFIP-derived scaffolds (Fig. 5d). This result is consistent with the collagen staining reported above.

Gene expression

Transcript levels of osteogenic markers such as $\text{Coll}\alpha 1$, ALP, OP and BSP were analyzed by real-time PCR. After 16 days, ALP and $\text{coll}\alpha 1$ transcript levels were significantly higher in the aqueous scaffolds, whereas BSP and OP levels were similar in the two scaffold groups. After 56 days, expression of ALP in the aqueous scaffolds was 2.6-fold higher than that in the HFIP scaffolds (Fig. 6a). At this later time point, BSP and OP transcripts were not statistically different between the two groups. Compared to day 16, expression levels of both genes were significantly increased (Figs. 6b and 6d). $\text{Col}\alpha 1$ transcript level in the aqueous-derived scaffolds was more highly expressed at day 16 ($p < 0.05$) (Fig. 6c). HIF-1 α indicated increased transcript levels with time in both study groups, while the values in the aqueous-derived scaffolds were significantly higher than the HFIP-derived scaffolds at days 16 and 56 ($p < 0.01$) (Fig. 6e).

Metabolic analysis

At the 56 day time point, the rate of glucose consumption significantly increased by 10-fold in the aqueous scaffolds compared to the HFIP scaffolds (Fig. 7a). The rate of lactate synthesis also increased significantly in the aqueous scaffolds compared to the HFIP scaffolds (Fig. 7b). The relative increase in lactate synthesis was more pronounced than glucose consumption, leading to a greater ratio of lactate produced to glucose consumed for the aqueous scaffolds (1.66) compared to the HFIP scaffolds (1.03) (Fig. 7). Cells cultured in the aqueous scaffolds consumed more proline and lysine from the media compared to the cells in the HFIP scaffolds. In addition, cells in the aqueous scaffolds synthesized more glutamine (GLN) at day 16, and more glutamate (GLN) at day 56 (Fig. 8).

Discussion

Biomaterials play a critical role as mediators of cell function for tissue regenerative processes by instructing cells, providing mechanical support or delivering therapeutics to promote tissue regeneration. The rate and extent to which biomaterials degrade and are subsequently replaced by native extracellular matrix is a critical factor in successful clinical repairs, impacting the quality and rate of regeneration [17,35,36]. Bone repairs represent a major orthopedic clinical demand, yet there is currently little consideration for the relationships between scaffold degradation rate and bone repair needs. Enhanced osteogenesis has been demonstrated to be in part related to decreased degradation of collagen matrix at the bone fracture site [37], and degradation rate is an important scaffold feature related to tissue formation and replacement [20]. While some biomaterial scaffolds may provide immediate defect stabilization, slow integration can lead to failure over time. On the other hand, rapid degradation may result in mechanical destabilization prior to full osteogenesis. Slow degradation can facilitate transport throughout the 3D scaffolds for a longer period of time than rapidly degrading scaffolds, to

foster improved homogeneity of new tissue formation and to avoid decreased cell function [38].

A critical aspect of the materials design features for the present study was to generate scaffolds with the same polymer but with different rates of degradation. Towards this goal, silk-based scaffolds were prepared using two different processing methods in order to modulate degradation rates, building off our understanding of processing-structure relationships with this protein when formed into 3D sponge structures [24,28]. We have previously shown that scaffolds prepared using an aqueous process degrade to completion between 3 and 6 months *in vivo*, while those prepared using an organic solvent (HFIP) persist more than 1 year [8]. We have also found different degradation rates between these aqueous- and HFIP-derived silk scaffolds *in vitro*. The aqueous-derived scaffolds (8 wt%) were rapidly degraded by protease XIV (4 U/mL) with 10% of the original mass remaining after 24 hrs. The HFIP-derived scaffolds (8 wt%) degraded more slowly, with 70% of the original mass remaining after 21 days [28]. These results demonstrated that it is possible to modulate the degradation rates of the silk-based scaffolds based solely on the processing conditions (as opposed to chemical modification). Since autoclaving during scaffold sterilization can impact crystallinity and/or water content in the scaffolds [39], we utilized two different methods of sterilization in the present study, 70% EtOH treatment and autoclaving.

The different degradation rates of the two groups of scaffolds prepared in the present study was demonstrated with protease XIV treatment (Fig. 1). Importantly, the two processing procedures to generate the two study groups did not change other factors relevant to the degradation rate, such as the chemistry or morphology of the scaffolds. The aqueous (42.4%) and HFIP (42.7%) scaffold groups had similar crystalline β -sheet contents based on FTIR analysis, as well as similar pore sizes based on SEM (710~850 μ m) (S. Figs. 1 and 2). Biodegradation processes vary greatly, and the mechanisms are complex, involving physical, chemistry and biological factors [40]. Silk degradation suggested that pore size did not correlate with degradation rate, nor did the nature of the initial concentration of fibroin in prior study [28]. Aqueous- and HFIP- derived scaffolds in phosphate buffer without protease showed no degradation [28]. In contrast, silk degrades enzymatically and the physicochemical, mechanical and biological properties of silk have been investigated with respect to enzymatic degradation [41]. Larger beta sheet crystals or more compact noncrystalline domains result in lower rates of degradation and thus remodeling [8,28]. The different degradation rates between the two groups of scaffolds were shown biologically, based on the response in osteogenic cell culture (Fig. 3). The differences in structure responsible for the differences in biological outcomes in the present study may be due to a combination of the above subtle differences in crystalline content such as crystal size and distribution, as well as differences in the noncrystalline regions of the protein matrix. The elucidation of the source of these structure-biological differences will require more detailed mechanistic insight. Most importantly for the current study, the degradation rates were significantly different (fast vs. slow), yet the morphology (pore size distribution) and chemistry of the two scaffold groups were not different.

The overall relationships between bone regeneration and scaffold degradation are depicted in Fig. 9. This model was developed based on known metabolic pathways to describe bone remodeling. Cellular metabolism and scaffold degradation are used to describe bone remodeling with fluxes representing expected pathways of extracellular matrix synthesis leading to the formation of new bone. This model is the basis for interpretation of the data from the present study, as well as establishing a path forward for refining these relationships towards future goals of establishing predictive tools to match biomaterials to specific repairs and needs in regenerative medicine. There are several models that address the appearance of new extracellular matrix (ECM); however, none of these models describe bone regeneration in the context of cellular metabolism related to scaffold degradation [42,43].

Highly mineralized ECM was found in the aqueous systems based on SEM analysis, von Kossa staining and type I collagen staining, as well as calcium and collagen assays. DNA content and ALP activity decreased at day 56 compared to day 16 (Fig. 5a and 5b). This tendency could be explained by the processes involved in bone development. Cell growth and expression of the osteoblastic phenotype is generally defined in three phases: 1) proliferation accompanied with the formation of extracellular matrix; 2) matrix maturation accompanied by down-regulation of proliferation and up-regulation of ALP expression; 3) mineralization marked by further decrease of proliferation and a decline of ALP activity [44]. ALP activity is generally considered an early stage marker for osteoblast phenotype and an important indicator of differentiation and mineralization [45]. Decreased cell content with time is due to apoptotic behavior of mature osteoblasts residing in mineralized nodules [46]. In our previous study we also showed this same pattern during osteogenesis using silk scaffolds [25]. The constructs cultured in spinner flasks supported hMSC proliferation during the first 16 days of culture (Fig. 5a). The higher calcium deposition was at the end of the culture period (Fig. 5c). Similar trends were observed in cell behavior in the cultures with fast/slow silk scaffolds, however, this effect was accelerated in the more rapidly degrading silk scaffolds. In addition, both OP and BSP also modulate the structure of the mineralized matrix *in vitro*. OP is responsible for cell attachment at bone remodeling sites and for regulation of crystal formation and growth because of the ability to bind hydroxyapatite [47,48]. BSP increases nucleation of hydroxyapatite crystals and is a marker for osteogenic differentiation and bone formation [49,50]. In the present study, ALP and *Colla1* gene expression were higher, and BSP and OP transcript levels increased, in the more rapidly degrading aqueous scaffolds in comparison to the HFIP system. Thus, higher levels of extracellular matrix and osteogenic transcript levels were found from cells grown in the more rapidly degrading aqueous scaffold study group. These results suggest that the aqueous scaffold group promoted more osteogenic differentiation and mineralized tissue formation than the HFIP or more slowly degrading scaffold group.

To characterize the effects of the different degradation rates on cellular response, we performed a series of analyses on biosynthetic and metabolic functions. Based on these analyses, correlations were drawn between metabolic pathway activities and osteogenic differentiation. These correlations have previously been suggested [51-53], but not explicitly investigated. Glucose and lactate were analyzed as indicators of glycolytic activity. Glucose is the primary nutrient for most cell types and is mainly metabolized via glycolysis. Under normoxic conditions, the glycolytic product pyruvate is further oxidized by the TCA cycle. When the supply of oxygen is limited, pyruvate can not be fully oxidized and is converted into lactate. Known as the Warburg effect, some cancer cells utilize increased metabolism of glucose into lactate to compensate for reduced oxidative phosphorylation under hypoxic conditions. Under these conditions, glutamine becomes a major nutrient. In the absence of glutamine, cells may adopt a quiescent-like state, reducing glucose consumption and ceasing proliferation [54]. In the present study, increases in glucose consumption and lactate production both correlated with accelerated scaffold degradation (Fig. 7). These changes also correlated with elevated HIF-1 α expression, suggesting a limited supply of oxygen in the aqueous scaffolds (Figs. 6e and 7). Oxygen limitation is also consistent with the elevated lactate to glucose ratio of ~ 2 [55,56].

Several previous studies have also noted a negative correlation between oxygen availability and glucose metabolism or cell differentiation. In tissue engineered cartilage, Heywood and coworkers found an inverse relationship between oxygen consumption and glucose utilization by chondrocytes. Depriving glucose, and thus reducing glycolytic flux, significantly increased the oxygen consumption, consistent with the Crabtree phenomenon [57]. Hirao and coworkers observed that lowering the oxygen tension (from 20 to 5%) promoted osteoblastic differentiation and matrix mineralization [58]. Studies in transgenic mice have shown that the oxygen sensitive HIF-1 α pathway is a critical mediator of skeletal regeneration *in vivo* [59].

As documented in a recent review, HIF-1 α directly contributes to the regulation of oxygen and glucose consumption [60]. The expression of important enzymes involved in glucose and pyruvate metabolism, including glucose transporters and lactate dehydrogenase, are transcriptionally regulated by HIFs [61,62]. Our data showed increased HIF-1 α gene expression in the more rapidly degrading aqueous scaffolds (Fig. 5d). Based on these results, we inferred that highly mineralized ECM formed by cells growing in more rapidly degrading scaffolds could generate hypoxic conditions, leading to anaerobic metabolism as reflected in rapid glucose consumption and increased lactate synthesis.

HPLC analysis was used to quantify amino acids relevant to bone matrix formation, including proline (PRO), lysine (LYS), glutamate (GLU), and glutamine (GLN). PRO and LYS are directly involved in collagen synthesis [63,64], whereas GLU and GLN are metabolic substrates. PRO, a unique proteogenic secondary amino acid, has its own metabolic system with special features [65]. The metabolism of GLN and PRO are interrelated. These amino acids can interconvert with glutamate and ornithine via the mitochondrial pathway involving pyrroline-5-carboxylate (P5C) [66]. In addition, PRO-rich tyrosine kinase 2 regulates the differentiation of early osteoprogenitor cells and bone formation [67]. LYS is an essential amino acid that can promote the metabolism of glucose and may play a significant role in osteogenesis [68]. LYS has been shown to promote osteogenesis *in vitro* and differentiation and proliferation of undifferentiated mesenchymal stem cells *in vivo* [69]. In the present study, PRO and LYS were likely consumed for collagen biosynthesis, which was elevated in the aqueous scaffolds compared to the HFIP scaffolds.

In addition to oxygen limitation and HIF-1 α activation, rapid degradation of the silk scaffold could promote osteoblastic differentiation through a substrate-dependent mechanism. Accelerated proteolytic degradation of the silk proteins could enrich the amino acid content of the cellular microenvironment to a greater extent. Amino acids, particularly PRO and LYS, constitute major residues of the collagen chains that facilitate the triple helix formation. The hydroxylation of these amino acids is a critical step in the regulation of collagen self-assembly into functional ECM [70]. Optimal collagen deposition is important during bone defect repair [71]. In a recent study, Tsuji and coworkers found that addition of LYS to the culture medium enhanced the osteogenic differentiation of bone marrow derived stem cells on hydroxyapatite (HA) [69]. In this regard, amino acid substrates derived from rapid degradation of the silk scaffold proteins could positively influence new ECM formation by providing the building blocks for collagen biosynthesis, by allosterically activating the biosynthetic enzymes, or both. Furthermore, both of these substrate-dependent mechanisms could act synergistically with transcriptional activation of biosynthetic enzymes mediated by HIF-1 α .

GLU and GLN were determined for glutamate metabolism because extracellular GLN conversion to GLU and coupled transporter activity can regulate energy metabolism and cell signaling involved in regulating cellular function [56]. GLU is a key molecule in cellular metabolism and affects both osteoblast and osteoclast phenotypes, with potential for therapeutic manipulation of glutamate signaling to enhance bone formation [72]. Another study also reported that GLU had a physiological function in the skeleton with strong evidence for a regulatory role in osteoblast and osteoclast differentiation and function *in vitro* [73]. GLN is another source of metabolic energy for cells, feeding directly into the Krebs cycle through the process of glutaminolysis. It is also an important building block for protein production and a variety of other cellular processes. Under some conditions, glutaminolysis can significantly contribute to cellular metabolism [74]. Aqueous scaffolds in this study supported the higher synthesis of GLN and GLU which are main parameters for control of glutamate metabolism. This result is consistent with reported result that glutamine synthesis was significantly increased under hypoxic conditions [54]. Therefore, this metabolism may be influenced during osteogenesis.

A biomaterial used as a bone substitute should be a temporary support for natural bone remodeling [75]. The material must degrade in a controlled fashion into non-toxic products that the body can metabolize or excrete via normal physiological mechanisms [76]. Thus, considerable efforts have been made to produce rapidly resorbable bone substitute biomaterials which stimulate enhanced bone formation. For instance, the impact of rapidly resorbable calcium phosphates and bone cements on the expression of bone-related genes and proteins by human bone-derived cells, with comparison to tricalcium phosphate (TCP) has been reported [77,78].

The data for cellular metabolism reported in the present study may be particularly relevant to bone tissue engineering using degradable scaffolds. The metabolic model reported (Fig. 9) for bone regeneration related to scaffold degradation provides a basis for the prediction of cell behavior when seeded on biomaterials or tissue-engineered matrices. In the present results, collagen and mineralized ECM synthesis by the cells were significantly greater when grown on the more rapidly degrading scaffold versus slow degrading scaffolds. It seems like cause by differently composed culture conditions (high amino acid synthesis and low oxygen condition) in the rapid degrading scaffold. However, further investigation is required to clarify the effect of key factors such as exogenous amino acids and hypoxic conditions. Eventually, in tissue-engineering applications, controlling the rates of new tissue synthesis to match degradation rates of the matrix could lead to more successful repairs, integration and tissue function. Further, these approaches could be tailored to patient-specific needs, to match biomaterial degradation rate to implant site, patient age and related measures of cell function and metabolism at the site of repair.

Conclusions

Attempts to match biomaterial features to bone in-growth rate and tissue regeneration are generally based on the choice of the biomaterial, and in some cases on pore size. However, there are many features of biomaterials that can impact bone outcomes, including scaffold chemistry, morphology and structure. These features can impact cell function (e.g., metabolism) and thus rates of remodeling and new tissue formation. In the present study, scaffold features in terms of degradation rate were correlated to osteogenesis in vitro, as well as to cell metabolism. The results supported the hypothesis that different degradation rates of scaffolds impact bone tissue formation, and this was validated with a variety of phenotypic and genotypic measures. Major findings included: (a) highly mineralized bone matrix formed in scaffolds designed to degrade more rapidly, with many measures of osteogenesis significantly higher in the more rapidly degrading scaffolds than in the more slowly degrading scaffolds, (b) metabolic analysis confirmed the rate differences with the two scaffold types, with the more rapidly degrading scaffolds exhibiting higher glucose consumption and lactate synthesis in comparison to the more slowly degrading scaffold system. Initial relationships between scaffold degradation rate, metabolism and osteogenic outcomes is provided (Fig. 9), with refinements needed to work towards predictive models in the future for improve patient-specific bone tissue regeneration.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Figure 1. Enzyme degradation of silk scaffolds in vitro. Mass of scaffolds remaining from aqueous scaffolds (●) and HFIP scaffolds (■) degraded by protease XIV (2 U/ml).

Figure 2. SEM images of hMSCs on rapid and slow degrading scaffolds cultured in spinner flasks for 56 days. Mineralized nodules (M). Scale bar = 50 μ m.

Figure 3. Degradation of scaffolds during osteogenesis. (a) Hematoxylin and eosin (H&E) staining, (b) The wall thickness from junctions among neighboring pores and (c) the area of silk scaffold in the section from the area occupied by silk scaffolds compared to total area. Arrow and pound symbols indicate the degraded and remaining scaffold structures, respectively. Scale bar = 100 μ m.

Figure 4. Histology. (a) Deposition of mineralized matrix on scaffolds, von Kossa (dark brown, arrow) (b) Immunohistochemistry for type I collagen, brown color (arrow). Star symbol indicates scaffold structure. Scale bar = 100 μ m.

Figure 5. Chemical characterization. (a) DNA content at days 16 and 56, (b) ALP activity per DNA, (c) calcium content per DNA, (d) total collagen content per DNA. Data are mean ± standard deviation, from 4 samples, statistical differences (*P<0.05, **P<0.01 and ***p<0.001).

Figure 6.

Transcript levels related to osteogenic differentiation markers (a) ALP, (b) BSP, (c) Colla1 and (d) OP and to metabolism (e) HIF-1 α , quantified by real-time RT-PCR and normalized to GAPDH. Data are mean $\pm$ standard deviation from N=4, statistical differences (*P<0.05, **P<0.01 and ***p<0.001).

<i>Ratio Lac/Glu</i>	Day 16	Day 56
Aqueous derived silk scaffold	0.88	1.74
HFIP derived silk scaffold	1.08	1.32

Table. 1 Lactate synthesis rate/ Glucose consumption rate for measuring metabolic activity

Figure 7. Metabolic analysis of culture media at days 16 and 56 for (a) glucose consumption rate and (b) lactate synthesis rate. Data are mean $\pm$ standard deviation for N=4, statistical differences (*P<0.05, **P<0.01 and ***p<0.001).

Figure 8.

Amino acid analysis in culture media by high performance liquid chromatography at days 16 and 56. Amino acids, (a) proline, (b) lysine for collagen biosynthesis and proline metabolism and (c) glutamine, (d) glutamate for glutamate metabolism, were selected among the 20 amino acids. Data are mean $\pm$ standard deviation for N=4, statistical differences (* $P < 0.05$, ** $P < 0.01$ and *** $p < 0.001$).

Figure 9.

Cellular metabolism-scaffold degradation model describing bone remodeling with fluxes representing expected pathways leading to the formation of new bone. (a) Model for rapidly degrading scaffold, and (b) model for slow degrading scaffolds. Solid arrows: metabolic flux. Dashed arrows: signal transduction. Colors: red – inhibition; green - activation