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Quantitative Characterization of Mineralized Silk Film Remodeling during Long-Term Osteoblast-Osteoclast Co-Culture

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Abstract

The goal of this study was to explore quantitative assessments of mineralized silk protein biomaterial films by co-cultures of human mesenchymal stem cell-derived osteoblasts and human acute monocytic leukemia cell line-derived osteoclasts during long-term culture (8 to 32 weeks). The remodeled films were quantitatively assessed using three different techniques during this extended cultivation to provide more comprehensive insight into the impact of co-cultures on surface remodeling. Scanning electron microscopy (SEM) with three dimensional surface reconstructions was used to quantitatively determine various surface morphological features and measures of roughness indicative of remodeling by the cells. Additionally, reconstructed surfaces were converted to depth images for Fourier analysis to quantify the potential fractal organization of biomineralization. The long-term remodeled films were also imaged using confocal reflectance microscopy and micro-computed tomography (micro-CT) to further quantify morphological changes. Films remodeled in co-culture demonstrated increased roughness parameters, fractal organization, and volume compared to films remodeled by osteoblasts alone. The combination of these techniques to quantify remodeling of mineralized protein films shows promise for quantifying processes related to mineralized surfaces.

Introduction

As the population ages, increased understanding of osteoporosis as well as improved treatment options are essential. Tissue-engineered disease models circumvent pragmatic and ethical problems with animal models and may present a path toward these goals. However, in order to develop *in vitro* models to study the impact of cells over the extended time frames involved in chronic diseases such as osteoporosis, it is first important to establish relevant long-term cultures that include appropriate cell types, matrices and modes to quantify the system. Development of quantifiable surrogate endpoints which are representative of disease states is essential for such studies.

Structural information is a particularly important outcome when characterizing bone formation and remodeling from co-cultures of osteoblasts and osteoclasts, as biochemical measures of deposited calcium are insufficient to describe the remodeling process. In this

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study three imaging techniques were used to assess remodeling of the film structures. For scanning electron microscopy (SEM) surface reconstruction, eucentric SEM images were captured from two different tilt angles, and surface metrology software was used to generate a three dimensional model of the biomaterial surface [1]. The chief advantage of this technique is access to both high resolution and wide field of view. Depending on the desired features, surfaces can be reconstructed from millimeters to nanometers. However, samples must be dried and sputter coated prior to imaging, preventing longitudinal monitoring of the same samples throughout a study. In confocal reflectance imaging, the intensity of the light reflected at biomaterial interfaces with different refractive indices is recorded [2]. The advantages of confocal reflectance microscopy include imaging live cultures in the hydrated state, while a drawback is the lack of specificity to mineralization and limited spatial resolution relative to SEM. Microcomputed tomography (micro-CT) allows a large number of X-ray images to be consolidated into a 3D model of the sample [3]. Micro-CT measures whole samples which eliminates sampling errors which may be present with techniques that measure only a small area, however, micro-CT resolution is generally about 5 to 10 microns, and thus is limited when compared to SEM resolution. In the future, nano-CT may be preferable to micro-CT for this application. Additionally, micro-CT works best for samples of similar length, width, and height. Films, which have one dimension that is much smaller than the other two, can be difficult to image, but staining solutions can be used to enhance contrast. The utility of these outcomes for monitoring long-term mineralization *in vitro* has not been systematically compared previously.

The goal of this study was to assess analytical tools that would provide quantitative measures of surface remodeling, as a prelude to future options to exploit such *in vitro* tissue models and methods towards the study of chronic bone-related diseases, such as osteoporosis. Previous studies have demonstrated that silk biomaterials support long-term tissue engineering; silk sponges supported adipose tissue engineering for six months [4,5]. Additionally, silk sponges have been used for bone tissue engineering *in vitro* and *in vivo* in various animal models, as well as to study cancer metastasis and osteoarthritis [6–11]. In the present study, silk films were used because of their accessibility for surface metrology. While three-dimensional scaffolds are more relevant to disease states, the films represent a compromise to permit a quantitative assessment of imaging modes, while moving a step towards 3D long-term culture. Throughout long cultures cells remodel the films to establish local three-dimensional environments, providing a start towards a 3D-type system.

In the present study, SEM-based surface metrology, confocal reflectance, and micro-CT were used to assess biomaterial remodeling by human mesenchymal stem cell (hMSC)-derived osteoblasts, THP-1 human acute monocytic leukemia cell line-derived osteoclasts, and co-cultures of the two cell types at 8, 16, 24, and 32 weeks of culture in order to compare the benefits and limitations of each of these imaging techniques. hMSC-derived osteoblasts have been widely used in bone tissue engineering, and hMSCs have been demonstrated to be suitable for long-term culture [12,13]. Once differentiated to macrophages, THP-1 cells can be differentiated to osteoclasts and have been demonstrated to form degradation pits on silk biomaterials [14,15]. We have previously measured calcium deposition and alkaline phosphatase activity in co-cultures of hMSC osteoblasts and THP-1 osteoclasts in studies of up to twelve weeks, suggesting that such co-cultures are suitable for long-term study [16,17]. For *in vitro* tissue studies involving extended cultivation times where mineralization is involved, the results provide a guide to the most appropriate techniques or combinations to consider depending on the nature of the quantitative information desired.

Materials and Methods

Silk film preparation

Aqueous silk solution was prepared as described previously [18]. Briefly, cocoons of *Bombyx mori* were cut to pieces approximately 1.5 cm² and boiled for 30 minutes in water containing 0.02 M Na₂CO₃, and then rinsed thoroughly with water to remove sericin. The remaining silk fibroin was then dried and dissolved in 9.3 M LiBr (Sigma Aldrich, St. Louis, MO) solution at 60°C for 4 hours. This solution was dialyzed in distilled water using a Slide-A-Lyzer dialysis cassette (MWCO 3,500, Thermo Fisher Scientific, Rockford, IL) for 2 days resulting in an 8% silk solution. Silk films approximately 100 µm thick were cast onto polystyrene from aqueous silk solution and allowed to dry overnight. Beta sheet formation was induced by water annealing at room temperature for six hours [19]. Films were sterilized by autoclaving. Sterilized films were incubated overnight in medium prior to cell seeding.

Cell culture

Unless otherwise noted, cell culture reagents were purchased from Life Technologies (Grand Island, NY). hMSCs were isolated from bone marrow aspirate from a male donor under 25 years old (Lonza, Walkersville, MD) as described previously [20]. Aspirate was combined with hMSC proliferation medium (minimal essential medium (MEM) α with 10% fetal bovine serum (FBS), 1% antibiotic/antimycotic, 1% non-essential amino acids (NEAA)) and cultured at 37°C with 5% CO₂ in a humidified environment. Flasks were rocked every day to allow hMSCs to adhere and media was added every 3–4 days until hMSCs reached 80% confluence. hMSCs were used at passage 1. THP-1 cells (ATCC, Manassas, VA) were maintained in proliferation medium (Roswell Park Memorial Institute (RPMI) medium 1640 supplemented with 10% FBS, 1% antibiotic/antimycotic, and 1% NEAA) prior to seeding. Following seeding, all cultures were maintained in the same medium, a half and half mixture of RPMI 1640 and MEM α supplemented with 10% FBS, 1% antibiotic/antimycotic, 1% NEAA, 100 nM dexamethasone (Sigma Aldrich, St. Louis, MO), 10 mM B-glycerol phosphate (Sigma Aldrich, St. Louis, MO), and 0.05 mM ascorbic acid (Sigma Aldrich, St. Louis, MO) (for osteoblast differentiation, as described previously [21]), and 40 ng/ml phorbol 12-myristate 13-acetate (PMA) (Sigma Aldrich, St. Louis, MO) and 10 ng/ml receptor activator of nuclear factor kappa-B ligand (RANKL) (for osteoclast differentiation, as described previously [15]) with medium changes every 3–4 days. Cells were seeded at a density of 15,000/cm² in a 20 µl drop and incubated for 2 hours to allow attachment. For co-cultures, hMSCs and THP-1 cells were seeded in equal number for the same total number of cells as the monoculture. Films were incubated in ddH₂O overnight at 4°C in order to remove cells prior to analysis at 8, 16, 24, or 32 weeks.

SEM

Samples were dried and then coated for 120 seconds at 18 mA using the Emitech sputter coater SC7600 (Quorum Technologies, Laughton, England). Samples were imaged using a Zeiss EVO MA10 SEM with the SE1 detector (Oberkochen, Germany). From each sample four different fields of approximately 100 µm by 150 µm were imaged. Imaged fields were well-spaced over the area of the film, and every technically suitable field encountered was imaged in order to avoid bias. Eucentric images were taken from tilt angles of 0 degrees and 8 degrees at 2000X magnification.

Surface metrology

MeX software (Alicona, Graz, Austria) was used for surface metrology analysis of SEM images. 3D surfaces were generated from eucentric image pairs with an eight degree tilt

differential and area analysis was performed using a reference plane generated from all points to identify the underlying film surface. Average height, ten point height, and developed interfacial ratio are reported. The average height of the surface is relative to the reference plane. The ten point height is the average of the heights of the five highest peaks plus the average of the absolute values of the depths of the five lowest points. This is a robust measure of the peak-peak height (the distance between the highest and lowest points on the surface). The developed interfacial ratio is the percentage increase in area of the reconstructed surface compared with a plane of the same size.

Confocal reflectance

Confocal reflectance imaging was performed with illumination at 800 nm from a Ti:sapphire laser through a 40x NA 1.1 water-immersion objective. Average laser power was approximately 45 mW. Field of view was 200 μm x 200 μm , with a scan resolution of 0.38 μm per pixel. Imaged fields were well-spaced over the area of the film, and every technically suitable field encountered was imaged in order to avoid bias. Images were captured at 1.5 μm increments along the z-axis. Image stacks ranged from 30 μm to 180 μm , depending on the flatness of the surface being imaged. An average intensity z-projection was created from each image stack in Matlab for Fourier analysis.

Fourier analysis

Both SEM depth images exported from MeX software and average intensity z-projection images from confocal reflectance microscopy were analyzed using established Fourier-based techniques [22,23]. Images were cropped to a square image size, and a 2D power spectral density (PSD) map was obtained through a discrete Fourier transform in Matlab. The 2D PSD was radially sampled to produce a single PSD curve as a function of spatial frequency [22]. Because self-affine fractals exhibit a PSD that decays over all spatial frequencies according to an inverse power law, a power law function, $f(x)=\alpha x^\beta$, was fit to each PSD curve [22]. To ensure all frequency ranges were fit with equal weight, PSD and frequency data were resampled with logarithmic spacing between points prior to fitting. The power law exponent of the fit was taken as the degree of clustering of mineralization, and the goodness of fit to the power law function was taken as the level of fractal organization, as shown in Figure S1.

Micro-CT

Films were stained in an iodine solution (1% iodine metal with 2% potassium iodide in water, diluted to 10% in water immediately before use) as described previously [3]. Films were then dried, physically stabilized in floral foam, and scanned using an X-Tek HMXST225 machine (Nikon Metrology, Brighton, MI) at 60 kV and 170 μA . Samples were reconstructed and a threshold was set to exclude floral foam using Volume Graphics Studio Max 3D software (Heidelberg, Germany). The volume of the reconstructed sample above the threshold was recorded.

Statistical analysis

All values are presented as mean $\pm$ SD. Statistical significance was determined by Tukey's HSD post-hoc ANOVA using R: A Language and Environment for Statistical Computing or JMP 10 and presented as * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$. For SEM, 4 spots of each of 4 films were imaged for each condition at each time point. For confocal reflectance, 4–11 locations on each of 4 films were imaged for each condition at each time point. For micro-CT, 3 to 4 whole films were imaged for each condition at each time point.

Results

Overview

hMSC osteoblasts, THP-1 osteoclasts, and co-cultures of the two cell types were grown on silk films for 8, 16, 24, or 32 weeks (Figure 1). Cells were monitored microscopically and remained confluent on films throughout 32 weeks of culture. Film remodeling was evaluated by SEM-based surface metrology using MeX software, confocal reflectance, and micro-CT.

Time zero films

Films were imaged by SEM, confocal reflectance, and micro-CT at time zero before cells were seeded (Figure 2). Films appeared microscopically flat when examined by SEM. Confocal reflectance images also showed smooth films with some visible reflectance artifacts from the casting process. Micro-CT images showed smooth films with some macroscopic curvature.

SEM

Representative SEM images of remodeled films are shown in Figure 3. At 8 weeks, small deposits of mineral were evident on films remodeled by osteoblasts, and larger deposits were evident on films remodeled in co-culture. By 16 weeks, films remodeled in co-culture and those remodeled by osteoblasts had different appearances; while osteoblast-remodeled films were still characterized by discrete deposits of mineralization, films remodeled by co-cultures exhibited large stretches of continuous extracellular matrix (ECM) deposits. At week 24 the ECM deposition continued to increase in the co-culture group, while the osteoblast group was visually similar between week 16 and week 24. At 32 weeks the mineralization of films remodeled in co-culture appeared similar to those remodeled in co-culture for 24 weeks.

SEM-based surface metrology

The average height, ten point height, and developed interfacial ratio are shown in Figure 4. Average height was not significantly different between time points for films remodeled by either osteoblasts or osteoclasts, while the average height of films remodeled by co-cultures increased significantly between 8 and 16 weeks ($p = 0.0044$) and between 16 weeks and 24 weeks ($p < 0.0001$). The average height of films remodeled by co-cultures was significantly higher than that of films remodeled by osteoblasts or osteoclasts ($p < 0.0001$).

For the ten point height, an average of five peak-peak heights, differences between time points in films remodeled by osteoblasts or osteoclasts were not significant. The ten point height of films remodeled in co-culture increased significantly between 8 and 16 weeks ($p < 0.0001$) and between 16 and 24 weeks ($p < 0.0001$), and decreased significantly between 24 and 32 weeks ($p = 0.0416$). Differences between the cell type groups were all significant, with films remodeled by osteoclasts having the lowest ten point height ($p < 0.0001$ vs. co-culture films and $p = 0.0094$ vs. osteoblast films) and films remodeled by co-cultures having the highest ten point height ($p < 0.0001$ vs. osteoblast films).

The developed interfacial ratio quantifies the increase in surface area as a result of roughness. Differences between time points for films remodeled by osteoblasts or osteoclasts were not significant. The developed interfacial ratio of films remodeled in co-culture increased significantly between week 8 and week 24 ($p = 0.0003$), and the developed interfacial ratio of films remodeled by co-cultures was significantly higher than that of films remodeled by osteoblasts or osteoclasts ($p < 0.0001$).

Fourier analysis of SEM images

3D surface reconstructions were exported as depth images and power law functions were fit to corresponding PSD curves (Figure S2). Films remodeled by co-cultures had the highest power law exponent, while films remodeled by osteoclasts had the lowest, which indicates the co-cultures produced mineralization that was highly clustered, while the osteoclasts produced less clustering (Figure 5). Each group was significantly different than the other two groups, but there were no significant differences within cell-type groups between time points (osteoclast vs. osteoblast films $p < 0.0001$, osteoclast vs. co-culture films $p < 0.0001$, osteoblast vs. co-culture films $p = 0.0009$). The goodness of fit of the PSD function to the power law model suggested that co-culture films exhibited significantly more fractal-like organization than osteoclast or osteoblast cultures. Osteoblast films also demonstrated a significantly higher goodness of fit than osteoclast films (osteoclast vs. osteoblast films $p < 0.0001$, osteoclast vs. co-culture films $p < 0.0001$, osteoblast vs. co-culture films $p = 0.0034$). Differences between time points within cell types groups were not significant.

Confocal reflectance

Confocal reflectance images of remodeled silk films were obtained at 8, 16, 24, and 32 weeks (Figure 6). At early time points, films remodeled by osteoclasts were visually distinct from films remodeled by osteoblasts and co-cultures. PSD curves from films remodeled for 32 weeks are shown in Figure S3. The mineralization clustering factor was highest in the co-culture group ($p < 0.0001$ vs. osteoclast films and $p = 0.0036$ vs. osteoblast films) (Figure 7). The mineralization clustering factor of osteoblast films and osteoclast films did not vary significantly between time points, but co-culture remodeled films had a significantly higher mineralization clustering factor at the 32 week time point compared with the 16 week time point ($p = 0.0027$) and 24 week time point ($p = 0.0024$). The level of fractal organization was also highest for co-culture films ($p = 0.0024$ vs. osteoblast films, $p < 0.0001$ vs. osteoclast films). The level of fractal organization of osteoclast films was significantly higher at week 24 ($p = 0.0081$) and week 32 ($p = 0.0163$) compared to week 8.

Micro-CT

Whole remodeled films were imaged by micro-CT at 8, 16, 24, and 32 weeks (Figure 8). By 24 weeks some films remodeled by osteoclasts showed areas of thinning, while macroscopic ECM deposits were apparent on films remodeled in co-culture. Film volumes after applying a threshold to the reconstructions are shown in Figure 9. Co-culture films had the largest volumes ($p = 0.0194$ vs. osteoblast films and $p = 0.0005$ vs. osteoclast films) while osteoclast films had the smallest volumes. Osteoblast film volume at week 32 was significantly lower than osteoblast film volume at week 8 ($p = 0.0337$). Osteoclast film volume did not significantly vary throughout the experiment. Co-culture film volume was significantly increased at week 32 compared to week 16 ($p = 0.0266$).

Discussion

Use of SEM, confocal reflectance microscopy, and micro-CT techniques provided a multi-faceted approach to understanding remodeling of silk films by osteoblasts and osteoclasts throughout long-term culture. While most studies of osteoblasts and osteoclasts in co-culture are run for less than two weeks, in this study we maintained the cultures for 32 weeks in order to establish long-term suitability of our model and measure long-term remodeling, which is essential in the study of chronic disease states. However, to have relevance, appropriate quantitative analytical tools are required to assess bone remodeling outcomes.

SEM-based surface metrology demonstrated increasing ECM deposition between 8 weeks and later time points in the co-culture group, and increased ECM deposition in the co-culture

group compared with osteoblast and osteoclast groups. We calculated the average height, ten point height, and developed interfacial ratio parameters to assess surface remodeling. Ten point height is a robust measure of peak-peak height while the developed interfacial ratio represents the increase in surface area due to roughness. While average height and ten point height means in the osteoblast group were higher than those in the osteoclast group differences were not significant because films remodeled by osteoblasts had highly variable average height and ten point height as a result of the sporadic nature of ECM deposition on films remodeled by osteoblasts.

Developed interfacial ratio means were very similar between films remodeled by osteoblasts and films remodeled by osteoclasts. Films remodeled by osteoblasts were characterized by very smooth surfaces punctuated by deposits of mineral, while films remodeled by osteoclasts exhibited uniform roughness due to degradation by osteoclasts. While the surfaces of films remodeled by osteoclasts appeared very different from those of films remodeled by the osteoblasts, the overall increase in surface area as a result of roughness was similar on average. Developed interfacial ratios of films remodeled in co-culture were large but highly variable as a result of the large and variable ECM deposits on the films.

Fourier analysis has been used to analyze deposits of mineralization on biomaterials [24,25]. However, in those studies, mineralization was produced by treating films with calcium chloride and sodium phosphate solutions, and the PSD derived from SEM images lacked a scale invariant power law decay over a broad range of spatial frequencies that is indicative of fractal organization. Self-affine fractal behavior is common in nature, including in trabecular bone [26]. While parameters like average height, ten point height, and developed interfacial ratio are useful for characterizing the surface, Fourier analysis provided confirmation that mineralization deposited on our films is consistent with a fractal nature. Additionally, the mineralization clustering factor and developed interfacial ratio provide complementary surface information. The mineralization clustering factor confirmed that even though films remodeled by osteoblasts had similar developed interfacial ratios as films remodeled by osteoclasts, the arrangement of the roughness was very different between the two groups.

Confocal reflectance has been used to study various tissues *in vivo* and *in vitro* [27–30]. Contrast arises from differences in refractive index, scattering, and absorption [2]. Fourier analysis of confocal reflectance images yielded similar results to SEM-based Fourier analysis, but while all three film types had significantly different power law exponents and goodness of fit by SEM-based analysis, osteoblast and osteoclast films were not significantly different by confocal reflectance-based Fourier analysis. This may have resulted in part from the difficulty acquiring a broad range of image intensities within the limited dynamic range of the detector. Additionally, some reflectance occurs at the surface, whether the surface is the silk film, deposited collagen, or deposited mineralization, and shape and organization of the substance also affect reflectance measurements.

Micro-CT demonstrated quantifiable macroscopic differences between remodeled films. Because the aspect ratio and relatively sparse nature of mineralization resulted in significant overlap between X-ray permeability of silk films and floral foam, iodine staining was used to enhance contrast. Iodine staining has been used extensively as a simple, effective stain for micro-CT that rapidly permeates tissues [3,31,32]. Silk films as well as deposited ECM were successfully stained, enabling discrimination between groups based on the volume of the reconstruction above the threshold. Additionally, staining enabled visualization of macroscopic changes to the remodeled surfaces.

Overall, SEM-based surface reconstructions were an extremely useful technique. High levels of significance were found between osteoblast, osteoclast, and co-culture films for standard surface characterization measures as well as Fourier analysis. While SEM images used in this study were of relatively low magnification, collection of images at a variety of magnifications would further enhance this technique. Confocal reflectance microscopy-based Fourier analysis largely corroborated SEM-based Fourier analysis, however, improvements to this technique may be necessary to enhance sensitivity. Finally, micro-CT was chiefly useful as a means to visualize macroscopic differences at the whole-film level. While differences in film volumes were significant, these differences were less pronounced than those measured with the other techniques and limited by the resolution of micro-CT. In the future, nano-CT may be a more suitable method for measuring mineralization *in vitro*.

While the different techniques address different resolution of surface features, among the study groups the co-cultures were found to have increased ECM deposition. Long-term co-cultures of osteoblasts and osteoclasts are not common, and when conducted, spatial characteristics of remodeling have not been measured [33,34]. Therefore, it is unknown whether the differences between remodeling by osteoblasts and co-cultures with osteoclasts is unique to the cell types used in this study or representative of a more general effect.

There are several possible explanations for the differences in remodeling behavior by the co-cultures. *In vivo*, osteoblast and osteoclast differentiation, survival, and activity are tightly coupled, and some of these regulatory mechanisms may increase deposition of mineralization in co-cultures [35–38]. In particular, resorption of bone matrix by osteoclasts releases growth factors that trigger localized deposition of bone matrix by osteoblasts [39]. Additionally, while microscopic inspection and measurement of remodeling demonstrate successful differentiation of osteoblasts and osteoclasts, residual undifferentiated THP-1 monocytes, THP-1 macrophages, and undifferentiated hMSCs may remain, all of which may be involved in the secretion of molecules that regulate osteoblast activity.

While osteoclasts and macrophages secrete molecules that increase osteoblast activity, surface differences may account for some of the differences between films remodeled by osteoblasts and films remodeled by the co-cultures. Films remodeled by osteoblasts were generally flat except for the deposits of mineral, but films remodeled by osteoclasts were roughened as a result of surface degradation by osteoclasts as shown in Figure 4. Increased differentiation and activity of osteoblast monocultures has been demonstrated on rougher surfaces [40–43]. While most of these studies focused on titanium, rougher silk-elastin biomaterials also enhance osteoblast differentiation [44]. Future studies on mechanically roughened silk films, enzymatically pre-degraded silk films, and silk films previously partially degraded by osteoblasts compared with films remodeled by direct and indirect co-cultures could help to clarify the roles of secretory molecules and mechanical properties in terms of the increased mineral deposition on films remodeled by the co-cultures.

Conclusions

Silk biomaterials supported long-term co-cultures to at least 32 weeks. SEM-based surface metrology, confocal reflectance, and micro-CT contributed to an understanding of the dynamics of remodeling on a quantitative basis. SEM-based surface metrology was particularly useful, demonstrating increased roughness parameters, mineral clustering and fractal organization with high levels of significance throughout 32 weeks of culture on films remodeled by co-cultures of osteoblasts and osteoclasts compared with films remodeled by osteoblasts alone. Confocal reflectance-based Fourier analysis also demonstrated increased mineral clustering and fractal organization in co-culture films, and micro-CT demonstrated increased volume of co-culture films. Future directions for this work include the use of other

cell sources of osteoblasts and osteoclasts, as well as studies to isolate the mechanisms responsible for observed significantly increased ECM deposition in the co-cultures. These directions in terms of co-cultures, systems and analytical tools are anticipated to provide future options in the development of chronic tissue models to address bone-related disease, such as osteoporosis.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. Experimental schematic. Silk films were seeded with osteoblasts, osteoclasts, or a co-culture of the two cell types. Film remodeling was assessed by SEM-based surface metrology, confocal reflectance, and micro-CT after 8, 16, 24, and 32 weeks of culture.

Figure 2.

SEM, micro-CT, and confocal reflectance images of films at time zero, prior to cell seeding. Films appear microscopically flat by SEM with a scan resolution of $0.15\ \mu\text{m}$ per pixel. Micro-CT reveals smooth films with some macroscopic curvature at a resolution of approximately $10\ \mu\text{m}$, and confocal reflectance microscopy reveals smooth films with some artifacts from casting and drying with a scan resolution of $0.38\ \mu\text{m}$ per pixel at $1.5\ \mu\text{m}$ increments along the Z-axis.

Figure 3. SEM images of remodeled film surfaces at 8, 16, 24, and 32 weeks. ECM deposition is particularly pronounced on co-culture films.

Figure 4.

Average height, ten point height, and developed interfacial ratios of silk films remodeled by osteoblasts, osteoclasts, or co-cultures at 8, 16, 24, and 32 weeks. Average height is relative to the reference plain, ten point height is a robust measure of peak height, and developed interfacial ratio is the increase in surface area as a result of roughness. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

Figure 5.

Power law exponent and goodness of fit of SEM-based reconstructions of films remodeled by osteoblasts, osteoclasts, or co-cultures at 8, 16, 24, and 32 weeks. The power law exponent quantifies the level of clustering of mineralization, while the goodness of fit quantifies the degree to which the surface exhibits fractal properties. ** $p < 0.01$, *** $p < 0.001$.

Figure 6. Average projection of confocal reflectance from remodeled films at 8, 16, 24, and 32 weeks. Osteoclast films are visually distinct at early time points, but film appearance across groups is more similar at later time points.

Figure 7. Power law exponent and goodness of fit of confocal reflectance microscopy-based reconstructions of films remodeled by osteoblasts, osteoclasts, or co-cultures at 8, 16, 24, and 32 weeks. The power law exponent quantifies the level of clustering of mineralization, while the goodness of fit quantifies the degree to which the surface exhibits fractal properties. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

Figure 8. Micro-CT of remodeled films at 8, 16, 24, and 32 weeks. Co-culture films at 24 and 32 weeks exhibit macroscopically visible ECM deposition.

Figure 9. Volume of reconstructed silk films determined by micro-CT with a threshold to exclude surrounding material. * $p < 0.05$, *** $p < 0.001$.