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Low-temperature Electrospun Silk Scaffold for *In Vitro* Mucosal Modeling

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Abstract

Electrospinning is often used to create scaffolding as a biomimetic of the extracellular matrix of tissues. A frequent limitation of this technique for three-dimensional tissue modeling is poor cell infiltration throughout the void volume of scaffolds. Here, we generated low-temperature electrospun silk scaffolds and compared these to conventional electrospun silk scaffolds in terms of mechanical properties, void volume, cell infiltration, cell viability and potential to support mucosal models under three-dimensional culture conditions. Low-temperature electrospun silk scaffolds supported fibroblast attachment and infiltration throughout the volume of the scaffolds, while conventional electrospun scaffolds exhibited limited cell infiltration with fibroblasts attaching exclusively to the seeding surface of the scaffolds. The porosity of low-temperature electrospun scaffolds was 93% compared to 88% of conventional electrospun silk scaffolds. Uniaxial tensile testing showed a 3.5 fold reduction in strength of low-temperature electrospun silk compared to the conventional in terms of peak stress and modulus, but no significant change in strain at break. Mucosal modeling with fibroblast-keratinocyte or fibroblast-carcinoma co-cultures showed similar results, with cell infiltration occurring only in low-temperature electrospun scaffolds. Cell viability was confirmed using live/dead staining after 21 days in culture. Furthermore, low-temperature electrospun silk scaffolds were able to support keratinocyte differentiation, as judged by involucrin immunoreactivity. The low-temperature electrospun silk scaffold that we have developed eliminates the limitation of electrospun silk scaffolds in terms of cell infiltration and, therefore, can potentially be used for a wide range of tissue engineering purposes ranging from *in vitro* tissue modeling to *in vivo* tissue regeneration purposes.

Keywords

low-temperature electrospinning; cryogenic electrospinning; silk; cell infiltration; fibroblasts; keratinocytes

INTRODUCTION

Electrospinning is a commonly used biomimetic technique for creating extracellular matrix analogues that can be used for three-dimensional *in vitro* cell culture as well as for implantable tissue constructs. Many natural and synthetic polymers have been electrospun for the purpose of simulating native tissue cell-matrix interactions^{1–7}. One natural material that is frequently used for tissue engineering purposes is silk fibroin, due to its biocompatibility and mechanical properties⁸, as well as structural similarities to collagen⁹. Electrospun silk has been used for various tissue engineering applications including *in vitro* keratinocyte-fibroblast co-culture with excellent cell adhesion to the surface of the scaffolds¹⁰.

One major drawback to the application of electrospun scaffolds is limited cell infiltration and therefore limited three-dimensional cell-cell and cell-matrix interactions, as well as limited vascular ingrowth potential upon implantation of such constructs due to restricted void space between fibers^{11, 12}. Possible solutions to increasing cell infiltration into electrospun scaffolds include electrostatically spraying cells into a nascent scaffold during the electrospinning process¹³. While this method is effective at integrating cells throughout the entire volume of the scaffold, vascular ingrowth potential remains poor due to lack of adequate void space for cell migration¹¹. Another approach to increase porosity and therefore increase cell infiltration is the inclusion of sacrificial fibers that can be dissolved after scaffold electrospinning is complete^{14, 15}. Minimal cell infiltration has been shown with this method¹⁵. The cotton-ball electrospinning technique has also been applied to improve cell infiltration with positive results for one week of *in vitro* cell culture¹⁶. Low-temperature electrospinning also known as cryogenic electrospinning is a promising technique that addresses the issue of increasing void volume between electrospun fibers and therefore facilitating cell infiltration throughout the entire volume of the electrospun scaffolds^{12, 17, 18}. Cryogenic electrospinning uses ice crystals throughout the growing scaffold to prevent formation of highly compacted fibers during the electrospinning process. The ice crystals are then removed via freeze-drying of the scaffolds, leaving large void spaces for cell infiltration. The resulting scaffolds support cell infiltration *in vitro* and vascularization *in vivo*^{12, 17, 19, 20}.

Although electrospun silk has been utilized for various tissue engineering purposes, poor cell infiltration has been a consistent limitation for using these types of scaffolds in biomimetic applications requiring true three-dimensional cell-scaffold interactions. In the present work, we have used cryogenic electrospinning of silk to generate scaffolds that support growth of fibroblast-epithelial co-cultures in an *in vitro*, three-dimensional model.

METHODS

Scaffold Fabrication

Silk Extraction—Silk extraction from *Bombyx mori* silkworm cocoons was performed using a previously reported method¹⁵. Silk cocoons were boiled in 0.02M Na₂CO₃ solution for 30 min, washed and then dissolved in 9.3M lithium bromide (LiBr) solution. The silk-LiBr solution was dialyzed against 18.2MΩ-grade water with 6 changes over 3 days in order to remove LiBr from the solution. The silk solution was then centrifuged to remove remaining impurities, and the purified silk solution lyophilized.

Conventional Electrospinning—Conventional electrospinning of silk²¹ was performed by dissolving freeze-dried silk in 1,1,1,3,3,3-hexafluoro-2-propanol (HFP) at a concentration of 12% (w/v). A syringe of this solution was placed in an electrostatic field with the applied voltage of 25kV to an 18 gauge blunt needle, while an aluminum collecting mandrel

remained grounded 20cm away from the needle. The collecting mandrel was a hollow cylinder with a 4cm outer diameter and 11cm in length with a wall thickness of 2mm. The solution was dispensed from the syringe at a rate of 11ml/h into the electrostatic field while electrospun fibers accumulated on the rotating (500rpm) and translating (2cm/s over 7 cm) mandrel at ambient temperature in an atmosphere of 33% humidity. Humidity inside the electrospinning chamber was monitored with a humidity meter (Fisher Scientific, Pittsburg, PA) and adjusted with an evaporative humidifier (Vornado, Andover, KS).

Cryogenic Electrospinning—Cryogenic electrospinning was performed based on previously described methods for synthetic polymers^{12, 17–20}. The parameters for conventional electrospinning were applied to cryogenic electrospinning with the exception that the temperature of the collecting mandrel was maintained below 0°C with dry ice occupying the entire inside volume of the collecting mandrel. The mandrel was pre-chilled with dry ice for 1 hour prior refilling with fresh dry ice and immediate electrospinning for 30 minutes. The cryogenically electrospun silk scaffold was then freeze-dried immediately in order to prevent thawing of the collected ice.

Cell Culture

Human telomerase reverse transcriptase (hTERT)-immortalized human foreskin fibroblasts (BJ-hTERT) were a gift from Dr. S. Holt (Dept. of Pathology, VCU). These cells were propagated in DMEM (Thermo Fisher, Ashville, NC) with 10% (v/v) calf serum (Thermo Fisher, Ashville, NC), 100 Units/mL of penicillin and 100µg/mL streptomycin (Thermo Fisher, Ashville, NC), 3% 1X 199 medium (Sigma-Aldrich, St. Louis, MO), and 1mM sodium pyruvate (Thermo Fisher, Ashville, NC). HN12 human head and neck squamous cell carcinoma cells were maintained as previously described²². The spontaneously immortalized human oral keratinocyte cell line NOKSI (a gift from Dr. J.S. Gutkind, NIDCR, Bethesda, MD) and hTERT-immortalized HFK-398 human foreskin keratinocytes²³ were maintained in defined serum-free medium (K-SFM; Invitrogen, Carlsbad, CA). Epithelial-fibroblast co-culture experiments were performed in K-SFM at the air-liquid interphase, using standard procedures²⁴.

Fluorescently-labeled cells were generated by nucleofection as described previously²². Briefly, BJ-hTERT cells were nucleofected with a pCEFL-YFP plasmid that directs expression of yellow fluorescent protein (YFP). HN12 and NOKSI cells were similarly nucleofected with pCEFL-mCherry to generate cells with red fluorescence. Cell populations expressing high levels of fluorescent proteins were isolated by selection in 400µg/ml G418 followed by ring cloning.

Scaffold disks were prepared using 10mm diameter tissue biopsy punches from electrospun silk mats. Disks were placed into 48 well plates, disinfected with 70% ethanol, and washed with PBS prior to cell seeding. Ethanol treatment causes β -sheet formation and was critical to prevent subsequent solubility in aqueous solutions²¹. Fibroblasts were trypsinized, washed, re-suspended and 1×10^6 cells added per disk. In co-culture experiments, fibroblasts were cultured on the scaffolds for one week prior to seeding of 2×10^5 NOKSI, 2×10^5 HFK-398 or 1×10^5 HN12 cells on the same side of the scaffolds as previous seeding of the fibroblasts. After epithelial cell attachment, scaffolds were raised to the air-liquid interface as per standard organotypic culture methodology, by placing the scaffolds in the upper chamber of 0.4µm pore Transwell® inserts (Corning, Lowell, MA) and adding medium to the bottom chamber²⁴, such that the lower surface of the scaffold was in contact with medium and the upper surface was exposed to air.

Scanning Electron Microscopy

Scanning electron microscopy (SEM) was performed on dry scaffolds to evaluate the morphology of the scaffolds resulting from the two electrospinning methods. Dry samples were gold sputter coated and imaged with a Zeiss EVO 50 XVP scanning electron microscope (Nano Technology System Division, Carl Zeiss Microimaging Inc., Thornwood, NY). Average fiber width data were obtained from the SEM images of the scaffolds using a custom image-processing program as previously described²⁵ and independently confirmed using ImageTool3.0 (Shareware provided by UTHSCSA) as previously described²¹.

Mechanical Testing

Uniaxial tensile testing of electrospun scaffolds was performed as previously described²¹. Briefly, each scaffold type was dehydrated in 70% ethanol for 30 minutes and allowed to air dry for 2 hours. Dog-bone shaped samples (overall length of 20 mm, 2.67 mm at its narrowest point, gauge length of 7.5 mm, n=10) were punched from the two types of scaffolds perpendicular to the direction of mandrel rotation. Uniaxial testing was then performed using an MTS Bionix 200 testing system with 100 N load cell (MTS Systems, Eden Prairie, MN). Samples were tested to failure at the rate of 10 mm/min (1.33min^{-1} strain rate). TestWorks version 4 was used to calculate peak stress, modulus and strain at break, as described previously²¹.

Porosity

Porosity was determined using the liquid intrusion method²⁶. Briefly, ethanol pre-treated then dried scaffold disks (n=10) were weighed and then submerged in water overnight. The scaffolds were then removed from water and reweighed. Porosity was calculated as the ratio of volume of intruded water to the sum of the volume of the intruded water and the volume of the scaffold. Densities of water and silk fibroin of 1g/cm^3 and 1.43g/cm^3 , respectively, were used to determine the volumes²⁷.

Fluorescence Microscopy

For fluorescence microscopy, samples were fixed in cold methanol for 20 min, washed 3 times in PBS, flash frozen, embedded in Neg50 frozen section medium (Thermo Fisher, Asheville, NC), cryosectioned at $10\mu\text{m}$ and mounted on glass slides. Samples were counterstained with 4',6-diamidino-2-phenylindole (DAPI), mounted in Vectashield Hard Set (Vector Laboratories, Burlingame, CA) and examined using a Zeiss Axiovert 200M fluorescence microscope (Carl Zeiss Microimaging, Inc, Thornwood, NY). Scaffold thickness was also measured from the cryosections under phase contrast for each scaffold (n=10).

Analysis of Cell Viability

Samples were stained with 5-chloromethylfluorescein diacetate (CMFDA) for viable cells and ethidium homodimer-1 (EthD-1) for dead cells, as described previously²⁸. After 21 days in culture, disks were incubated with $10\mu\text{M}$ CMFDA and $5\mu\text{M}$ EthD-1 for 40 minutes and then fixed, cryosectioned and imaged as described above. Viability was calculated by counting the percentage of viable cells per field of view from five randomly chosen fields of view per sample from three samples per condition.

Immunofluorescent Staining

Samples were fixed in cold methanol, permeabilized in 0.1% Triton X-100, blocked in 5% bovine serum albumin (BSA) for one hour at room temperature, then incubated with either anti-Ki-67 antibody (clone B56; Dako, Carpinteria, CA), or anti-pan-keratin antibody (clone AE1/AE3; Thermo Fisher, Asheville, NC), or anti-involucrin (clone SY5; Thermo Fisher,

Ashville, NC) antibody diluted 1:100 in 5% BSA overnight at 4°C. Samples were then washed three times in phosphate buffered saline with 0.1% Tween for 5 minutes each. Anti-mouse FITC-conjugated secondary antibody (Vector Laboratories, Burlingame, CA) was applied at a dilution of 1:500 for one hour at room temperature and then, after subsequent washes, samples were frozen, cryosectioned and imaged as described above.

Statistical Analysis

The mean porosity measurements, mean percent viability values and mean mechanical properties between cryogenic and conventional scaffolds were compared using a one tailed, paired student *t*-test with $p < 0.05$ considered to be statistically significant. All quantitative data are presented with the standard error of the mean (s.e.m.).

RESULTS

Morphology of Scaffolds

Scaffold morphology was evaluated by SEM in order to visualize the structure of the scaffolds. As shown in Figure 1, conventional electrospun silk scaffolds exhibited randomly oriented, densely packed, non-woven fibers, whereas cryogenic electrospun silk scaffolds exhibited randomly oriented non-woven fibers with qualitatively more open structure. In order to quantify difference between the openness of each type of scaffold, porosity analysis was performed. The porosity was determined to be $88 \pm 0.6\%$ and $93 \pm 0.08\%$ for conventional and cryogenic electrospun scaffolds, respectively ($p < 0.001$). Fiber widths were determined to be $2.58 \pm 0.06 \mu\text{m}$ and $3.61 \pm 0.36 \mu\text{m}$ for the cryogenic and conventional scaffolds respectively. Scaffold thickness was measured to be $497 \pm 6 \mu\text{m}$ and $533 \pm 7 \mu\text{m}$ for cryogenic and conventional scaffolds respectively.

Uniaxial tensile testing

As shown in Figure 1C, there were statistically significant differences in mechanical properties between cryogenic and conventional electrospun silk scaffolds in terms of peak stress and modulus. Cryogenic electrospun silk was shown to be 3.5 fold weaker than the conventional electrospun silk. However there was no significant difference between the strain at break for both scaffolds ($p = 0.0863$).

Fibroblast infiltration of scaffolds

In order to evaluate the potential for cell infiltration throughout the volume of the scaffolds, BJ-hTERT fibroblasts were seeded on cryogenic electrospun and conventional electrospun silk scaffolds and maintained in culture for up to 21 days. Cell distribution is shown in Figure 2, which shows DAPI staining of transverse cryosections of fixed scaffolds. Conventionally electrospun silk scaffolds supported attachment of BJ-hTERT fibroblasts on the seeding surface with no apparent cell infiltration to the interior of the scaffolds. In contrast, cryogenically electrospun scaffolds supported a wide cell distribution throughout the entire depth of the scaffold.

Co-culture studies

Co-culture experiments were performed to show the potential of using the novel scaffold for multiple *in vitro* 3D culture applications, such as mucosal modeling of either normal growth or tumor cell invasion. BJ-hTERT/YFP fibroblasts were seeded onto cryogenic or conventional electrospun scaffolds and allowed to grow for one week prior to the addition of mCherry-transfected epithelial cell lines onto the surface of the scaffold disks, in order to allow for separate epithelial and connective layer formation mimicking epidermal and

dermal cell composition respectively. Co-culture systems were maintained for up to 21 days prior to fixation, cryosectioning and microscopic analyses.

As shown in Figure 3, BJ-hTERT and HN12 cells were found to have infiltrated the entire volume of the cryogenic electrospun scaffolds. The corresponding conventional electrospun scaffolds did not support cell infiltration, with cell attachment only to the superficial/seeding surface of the scaffolds even after 14 days of co-culture.

NOKSI cells form a multi-cell layer on the seeding surface of the cryogenic electrospun scaffolds with fibroblasts distributed throughout the scaffolds, as shown in Figure 4. In contrast, attachment of both NOKSI and BJ-hTERT fibroblasts to the conventional electrospun scaffolds is restricted to the surface of the scaffolds with no apparent infiltration of the scaffold interior by the fibroblasts. NOKSI cells show anti-keratin immunoreactivity in the cryogenic scaffold (Fig. 5A). Ki67 was robustly expressed, indicating the proliferative nature of these cells (Fig. 5C). However, expression of involucrin, a marker of epithelial terminal differentiation, was negligible (Fig. 5E), suggesting that NOKSI cells may be unable to differentiate in this system.

In order to determine whether cryogenic silk scaffolds are capable of supporting epithelial differentiation, co-culture of HFK-398 cells with BJ-hTERT fibroblasts was carried out under the same conditions as NOKSI co-cultures described above. As shown in Figure 6, whereas the epithelial cells on cryogenic silk scaffolds also showed high expression of keratin, in contrast to NOKSI cultures expression of the proliferation marker Ki67 was low, and involucrin expression was high, consistent with cell cycle exit and entry into a terminally differentiated state. Taken together, these data suggest that cryogenic silk scaffolds are able to support the terminal differentiation of keratinocytes that are competent for differentiation, such as HFK-398.

Viability of cells in electrospun silk scaffolds

Viability analysis of cells cultured on the scaffolds was performed, to confirm that the cell culture conditions were adequate to support long-term experiments. Live/dead analysis was performed for cells cultured on cryogenic and conventional electrospun scaffolds as shown in Figure 7A–H. Cell viability on cryogenic scaffolds was found to be significantly higher (Fig. 7I) than cells cultured on conventional scaffolds. As shown in Figure 7I, monoculture and co-culture systems consistently resulted in more than 90% viability on the surface and throughout the volume of cryogenic scaffolds compared to less than 90% viability on conventional scaffolds after three weeks of culture.

DISCUSSION

Low temperature, or cryogenic, electrospinning has been previously reported to be an effective technique for inducing cell infiltration into synthetic electrospun scaffolds^{12, 17–20}. The current report demonstrates the application of this technique for the first time to the natural polymer, silk. Cell distribution throughout the entire volume of the electrospun silk scaffold was achieved only using the cryogenic electrospinning technique, whereas conventional electrospun silk did not exhibit the same structural properties and no cell infiltration was observed. Fibroblast infiltration throughout the electrospun silk scaffolds was only observed in cryogenically electrospun scaffolds. The three dimensional cell culture conditions also allowed for variation in cell type distribution with the invasive HN12 cells infiltrating the entire volume of the scaffolds, whereas the immortalized keratinocytes formed multi-cell layers on the surface of the scaffolds at the air-liquid interface (Fig. 3A&B). Poor fibroblast attachment and limited keratinocyte infiltration were observed in co-culture experiments with conventional electrospun silk scaffolds, with no formation of

distinct cell layers (Fig. 3C&D). Co-culture systems of cryogenic electrospun silk scaffolds seeded with fibroblasts and NOKSI cells exhibited two distinct layers: a deeper fibroblast layer and a more superficial epithelial layer more reminiscent of dermal and epidermal structures, respectively (Fig. 4B). Immunofluorescent staining for keratin revealed low, evenly distributed keratin expression in the epithelial cells, confirming their lineage (Fig. 5A). The NOKSI cells showed high expression of the proliferation marker Ki67 (Fig. 5C) even after three weeks in air-liquid interface co-culture, but extremely low or undetectable levels of the terminal differentiation marker involucrin (Fig. 5E). Thus, NOKSI cells may show blocked terminal differentiation. In order to investigate further whether cryogenic scaffolds support epithelial differentiation, we conducted similar co-culture experiments with the hTERT-immortalized HFK-398 keratinocytes which have previously been shown to undergo terminal differentiation in organotypic culture²³. Similar to NOKSI cells, HFK-398 cells were immunoreactive with a pan-keratin antibody (Fig. 6A). However, after three weeks of air-liquid interface culture, Ki67 expression in HFK-398 cells was minimal and involucrin expression was prominent, features that are consistent with epithelial differentiation (Figs. 6C&E). Therefore, our data suggest that terminal differentiation of epithelial cells is possible on cryogenic electrospun silk scaffolds, and this system may be useful for *in vitro* mucosal modeling. In addition, co-culture systems of cryogenic scaffolds seeded with fibroblasts and lymph node metastasis-derived HN12 cells resulted in the tumor cells occupying and sharing the entire scaffold volume with the fibroblasts, raising the possibility that the silk scaffolds may also show utility for *in vitro* investigation of tumor cell invasion. Furthermore, all cells that we have tested have remained viable for at least three weeks, with higher viability observed on cryogenic scaffolds, possibly due at least in part to increased cell-cell and cell-matrix interactions in three dimensions compared to two dimensions²⁹.

Fluorescent labeling of cells was used to facilitate identification of cell types in co-culture experiments. Silk auto-fluorescence in the red region of the spectrum makes it difficult to identify mCherry labeled cells, therefore DAPI was also used to counterstain cell nuclei. Electrospun silk exhibited no auto-fluorescence in the ultra-violet or blue regions of the spectrum. Although silk also fluoresces in the green region, the intensity of the YFP signal was much greater than that of the scaffold, allowing a clear distinction of YFP labeled cells. A major advantage of using fluorescently labeled cells is the ability to differentiate between the cell types in co-culture systems. A disadvantage of using fluorescently labeled cell lines is the possible variation in protein expression between labeled and original cell lines. However, no notable differences in cell proliferation, attachment or viability were observed between labeled and corresponding unlabeled cell lines (unpublished observations); therefore, in these studies the labeled cells seeded on scaffolds are predicted to behave in a similar fashion to unlabeled cell lines.

We found that maintaining relative humidity above 30% was a critical requirement for creating electrospun silk scaffolds capable of inducing cell infiltration, a similar result to that found with other cryogenic electrospun scaffolds^{12, 17, 18}. Cryogenic electrospun silk scaffolds that were manufactured at lower humidity levels did not support cell infiltration and did not appear to differ appreciably from conventional electrospun scaffolds, as judged by SEM analysis (unpublished observations). The cryogenic electrospun silk fibers were measured to have narrower widths than conventional electrospun silk fibers. This observation might be attributable to a possible change in the electric field during the electrospinning process due to ice accumulation on the mandrel, as well as diameter change of the collecting mandrel. Another critical element of cryogenic electrospinning of silk is maintaining the scaffold frozen until the sample is lyophilized. Any melting of the ice crystals dissolves the electrospun silk, which does not become insoluble in aqueous solution until after dehydration and ethanol treatment.

A common technique to increase porosity of electrospun scaffolds is to introduce a sacrificial polymer into the electrospinning process. The sacrificial polymer, such as poly(ethylene oxide), is mixed in the electrospinning solution with the polymer of interest, such as silk. The solution of both polymers is electrospun conventionally and then the sacrificial polymer is dissolved to leave a scaffold composed only of the desired material¹⁴. Although this procedure does result in increased void volume, minimal cell infiltration has been demonstrated using this approach¹⁵. Thus, cryogenic electrospinning of silk would appear to be advantageous. The porosity increase between cryogenic and conventional electrospinning remains consistent from polymer to polymer as demonstrated by previous studies and confirmed by this study¹⁸. Another recent method for increasing porosity of electrospun scaffolds is the cotton-ball electrospinning method¹⁶. Cell infiltration does appear to increase with this method compared to conventional electrospinning. However, these studies were carried out over a 7-day period¹⁶, whereas experiments in the present study show high cell viability after 21 days in culture.

In the present study, we performed uniaxial tensile testing to characterize the cryogenic electrospun silk further, and found a 3.5-fold decrease in strength of the scaffolds compared to conventional electrospun silk. Although mechanical strength did decrease with increased porosity, the decrease for silk was considerably less than the 40-fold reduction in tensile strength reported for cryogenically electrospun poly(D,L- lactide) when compared to conventional electrospun poly(D,L-lactide)¹². Cellular infiltration appears to be comparable between the cryogenic electrospun silk and poly(D,L-lactide).

In this work, we have demonstrated the potential of cryogenic electrospinning as an improvement over conventional electrospinning for true three-dimensional cell culture on a natural polymer. To our knowledge, we report the first electrospun silk scaffold with enhanced cell infiltration and cell viability as a result of increased void volume throughout the scaffolds, which has been a limitation of previous studies²¹. We have successfully demonstrated epithelial cell viability in co-culture with fibroblasts, as well as terminal differentiation of keratinocyte cultures, and suggest that this new scaffold system can, potentially, be used for many applications ranging from *in vitro* three-dimensional cell culture for tissue modeling as well as *in vivo* applications for tissue regeneration and reconstruction.

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Figure 1. SEM of cryogenic electrospun silk (A) and conventional electrospun silk (B). (C). Uniaxial tensile testing results of cryogenic and conventional electrospun silk. Bar = s.e.m.

Figure 2.
BT-hTERT fibroblasts on cryogenic electrospun scaffolds after 14 and 21 days of culture (A, B), and on conventional electrospun scaffolds after 14 and 21 days (C, D).

Figure 3. HN12/mCherry and BJ-hTERT/YFP co-culture on cryogenic electrospun scaffolds at (A) 7 and (B) 14 days of co-culture, and on conventional electrospun scaffolds at (C) 7 and (D) 14 days of co-culture. Note autofluorescence of the silk scaffold in the red channel.

Figure 4. NOKSI/mCherry and BJ-hTERT/YFP co-culture on cryogenic electrospun scaffolds at (A) 7 and (B) 14 days of co-culture, and on conventional electrospun scaffolds at (C) 7 and (D) 14 days of coculture. Note autofluorescence of the silk scaffold in the red channel.

Figure 5.

Immunofluorescent staining of BJ-hTERT and NOKSI for keratin expression on (A) cryogenic and (B) conventional scaffolds; for Ki67 expression on (C) cryogenic and (D) conventional scaffolds; and for involucrin expression on (E) cryogenic and (F) conventional scaffolds. Immunofluorescent staining was performed after 21 days of co-culture at the air-liquid interface (28 days total culture).

Figure 6. Immunofluorescent staining of BJ-hTERT and HFK-398 for keratin expression on (A) cryogenic and (B) conventional scaffolds; for Ki67 expression on (C) cryogenic and (D) conventional scaffolds; and for involucrin expression on (E) cryogenic and (F) conventional scaffolds. Immunofluorescent staining was performed after 21 days of co-culture at the air-liquid interface (28 days total culture).

Figure 7.

Viability analyses of cells on electrospun silk scaffolds after 21 days of culture. BJ-hTERT on (A) cryogenic and (B) conventional scaffolds; BJ-hTERT and HN12 on (C) cryogenic and (D) conventional scaffolds; BJ-hTERT and NOKSI on (E) cryogenic and (F) conventional scaffolds; BJhTERT and HFK-398 on (G) cryogenic and (H) conventional scaffolds. (I) Percent viability of cells after 21 days of culture on electrospun silk scaffolds. Bar = s.e.m.