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Simultaneous Delivery of Highly Diverse Bioactive Compounds from Blend Electrospun Fibers for Skin Wound Healing

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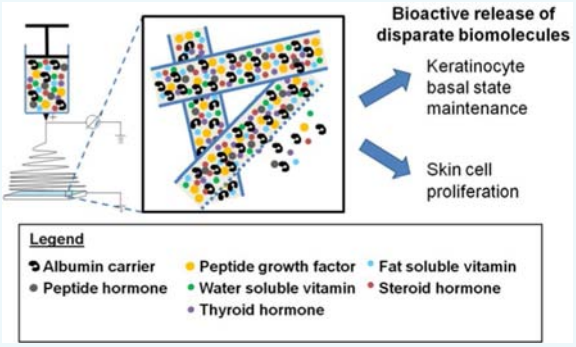
ABSTRACT: Blend emulsion electrospinning is widely perceived to destroy the bioactivity of proteins, and a blend emulsion of water-soluble and nonsoluble molecules is believed to be thermodynamically unstable to electrospin smoothly. Here we demonstrate a method to retain the bioactivity of disparate fragile biomolecules when electrospun. Using bovine serum albumin as a carrier protein; water-soluble vitamin C, fat soluble vitamin D3, steroid hormone hydrocortisone, peptide hormone insulin, thyroid hormone triiodothyronine (T3), and peptide epidermal growth factor (EGF) were simultaneously blend-spun into PLGA-collagen nanofibers. Upon release, vitamin C maintained the ability to facilitate Type I collagen secretion by fibroblasts, EGF stimulated skin fibroblast proliferation, and insulin potentiated adipogenic differentiation. Transgenic cell reporter assays confirmed the bioactivity of vitamin D3, T3, and hydrocortisone. These factors concertedly increased keratinocyte and fibroblast proliferation while maintaining keratinocyte basal state. This method presents an elegant solution to simultaneously deliver disparate bioactive biomolecules for wound healing applications.



INTRODUCTION

The retention of bioactivity from disparate biomolecules such as proteins, growth factors, hormones, vitamins, and steroids released from biomaterials is one of the crucial factors for successful tissue engineering and modulation. In this regard, electrospinning strategies to prepare bioactive scaffolds, most frequently for the delivery of proteins (e.g., growth factors or cytokines)[1] have been evaluated extensively, especially in blend/emulsion electrospinning.[2] However, this technology has come under scrutiny as proteins are known to easily lose their activity upon chemical or physical processing during scaffold fabrication and storage.[1] We previously reported that electrospinning of collagen or co-spinning of collagen-synthetic polymers with highly volatile fluoroalcohols such as 1,1,1,3,3,3-hexafluoro-2-propanol (HFP) results in its denaturation, resulting in yield of effectively gelatin fibers.[3] Additional damage might be done by the violent emulsification process (i.e., high speed stirring, ultrasonication, or homogenization of the protein/polymer mixture prior to electrospinning). Moreover, an emulsion of water-soluble and nonsoluble molecules is believed to be thermodynamically unstable and can cause disruptions during the spinning process.[4] To circumvent these

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limitations, more technically sophisticated strategies have been designed since 2003, such as coaxial electrospinning, core−shell electrospinning and noncovalent adsorption techniques (reviewed in Jiang et al.[4]). However, these technologies have intrinsic issues of reproducibility and scalability. Here, we demonstrate that a simple blend/emulsion electrospun scaffold can still be a viable approach to achieving bioactive release of highly disparate biomolecules. Considering the physiology of serum transport of highly diverse molecules we identified albumin as their common transport carrier.[5,6] Albumin, a major protein of ∼66 kDa in blood plasma, consists of a repeating pattern of three largely helical domains stabilized by disulfide bonds. Albumin has been described as a highly stochastically configured molecule with a loop-link structure that permits its rapid expansion, contraction, and flexion. This high degree of plasticity allows albumin to accommodate binding ligands of diverse affinities and sizes within a time span of ∼0.1−0.3 s.[7] Unlike other proteins, albumin can conformationally recover from structural changes induced by almost any condition including disulfide bond reduction, except a combination of heat and strong alkalinity.[7] We therefore selected native bovine serum albumin (BSA) purified by the Cohn method as a carrier protein and protectant of biomolecules incorporated during the electrospinning process. As electrospun scaffolds are fabricated as a mesh, they are particularly suitable for use in wound coverage and skin regeneration.[8] We have previously proposed a combination of factors for use in skin regeneration[9] composing of six disparate biomolecules that would need to be delivered from a wound care scaffold: C - vitamin C (Mw: 290); H - hydrocortisone (Mw: 363); I - Insulin (Mw: 5.8 kDa); T - triiodothyronine (T3) (Mw: 650.97); E - epidermal growth factor (EGF) (Mw: 6 kDa); and D - 1,25-dihydroxyvitamin D3 (VD3) (Mw: 416.64) with the acronym CHITED. The components represent biomolecules of different class, size, solubility, and sensitivity to light/oxygen. We were able to incorporate them in an electrospun poly(DL-lactide-*co*-glycolide) (PLGA)-collagen scaffold. The resultant nanofiber scaffold mats released CHITED over 12 h. We demonstrated that each of the six biomolecules was bioactive after release from the scaffold, and the combined release of biomolecules CHITE (i.e., CHITED excluding vitamin D3), stimulated human dermal fibroblast and keratinocyte proliferation. This effective technique harnesses the properties of albumin as a physiological transport system for delivering disparate biomolecules under blend/emulsion electrospinning. Bioactive CHITE blend/emulsion electrospun nanofiber scaffolds hold great potential for future skin wound healing applications.

RESULTS AND DISCUSSION

Fabrication and Morphology of CHITE-PLGA-Collagen Electrospun Fibers. Blend electrospun scaffolds containing BSA as a carrier protein were fabricated by dissolving aqueous BSA with or without CHITE into predissolved PLGA-Collagen HFP mixture just prior to electrospinning (Figure 1A). The blending of an aqueous biomolecule mixture into an organic solvent resulted in the formation of a milky emulsion that when electrospun resulted in the formation of large beads such as those reported previously by Ji et al.[10] In fact, Ji et al. reported an inability to electrospin scaffolds containing only polycaprolactone and BSA mixed at a ratio of 3:1. We have previously tried various ratios of organic to aqueous solution and found that 10:1 is an optimum ratio for incorporation of aqueous solutions without compromising spinnability of the

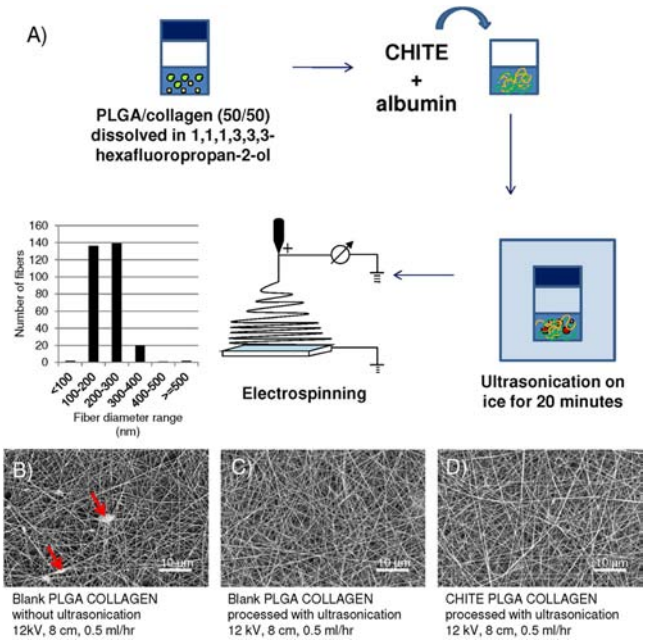

Figure 1. Fabrication process of blend electrospun PLGA-Collagen nanofiber scaffold with biomolecules CHITE incorporated. (A) PLGA-Collagen is predissolved in HFP and mixed at room temperature until homogeneous. The cocktail of biomolecules CHITE with albumin is then added to the PLGA-Collagen solution, forming an emulsion. The emulsion is ultrasonicated on ice prior to electrospinning to facilitate even mixing and dispersion of the emulsion droplets. Resultant fibers have an average diameter of 210 ± 62 nm (n = 300). (B) Scanning electron micrograph of a blank PLGA-Collagen scaffold (i.e., without CHITE, with albumin) fabricated without ultrasonication reveals multiple irregularly sized beads (red arrows) in its structure. Scanning electron micrograph of (C) blank PLGA-Collagen scaffold and (D) CHITE-PLGA-Collagen scaffold fabricated with ultrasonication showing bead-free morphology.

scaffolds and bead formation. Beads may contribute to decreased mechanical strength of the scaffold[11] and altered release profiles, but can be largely eliminated by ultrasonication of the emulsion. It was observed that fibers obtained without ultrasonication contained beads ranging between 1 and 5 μm in diameter (Figure 1B, red arrows) while ultrasonication facilitated the fine mixing of the emulsion resulting in bead-free fibers with an average diameter of 210 ± 62 nm (n = 300) (Figure 1C and D). The use of a water bath on ice prevents biomolecules from denaturation from heat produced during sonication.

Degradation Properties of Scaffold. In order to understand the degradation behavior of the scaffold, scaffolds electrospun onto glass coverslips were immersed in pH 7.4 phosphate buffered saline (PBS) in a simulated physiological condition of 37 °C with 5% carbon dioxide atmosphere. The ultrastructure of the air-dried degrading fibers was then studied at fixed time points using scanning electron microscopy (Figure 2A). The scaffold appeared to have enlarged round pores 1 day after soaking but retained most of its fiber morphology. By day 7, first degradation "kinks" appeared on the surfaces of the fibers that eventually progressed to breaks in the fibers at day 28. This is most likely attributed to the degradation of the collagen component of the fibers, as the PLGA used has an expected degradation time of 3−4 months.[12] When attenuated total reflectance-fourier transform infrared (ATR-FTIR) was used to determine the chemical composition on the surface of

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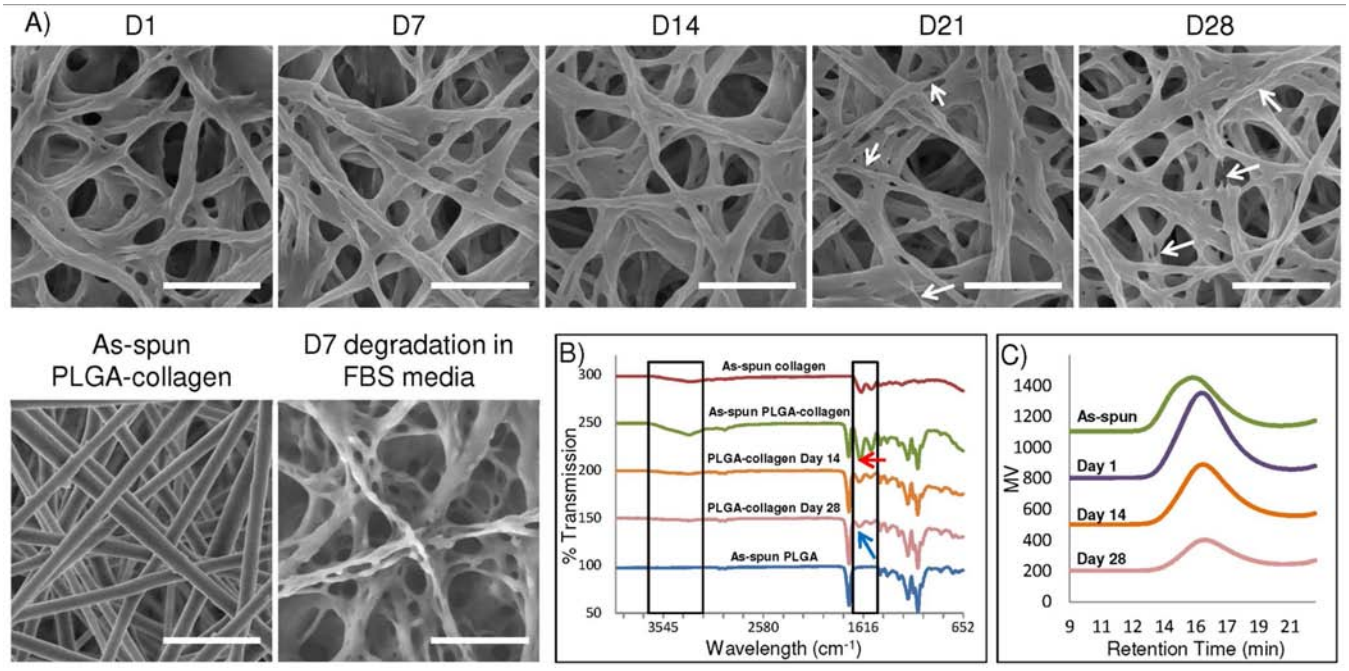

Figure 2. Degradation profile of blank scaffolds. (A) Scanning electron micrograph of blank scaffolds incubated in pH 7.4 PBS at 37 °C (top row) for 1, 7, 14, 21, and 28 days. The white arrows indicate points where degradation has caused fiber breakage. As-spun PLGA-collagen scaffold is shown in the scanning electron micrograph on the bottom left while the same scaffold incubated for 7 days in 10% FBS supplemented DMEM media is shown on its right. The scale bars represent 1 μm in length. (B) ATR-FTIR spectrum of hydrolytically degraded scaffolds. The black box crops out regions where a peak is expected from amide bonds. A reduction in amide peak was observed from as-spun scaffolds (red arrow) to scaffolds incubated for 28 days (blue arrow) indicating loss of amide bond contributing collagen on the surface of the scaffolds. (C) GPC profiles comparing the hydrolytic degradation of PLGA-collagen scaffolds. Scale bars indicate 1 μm.

the fibers, the amide peaks contributed by collagen is greatly diminished by day 14 while the aliphatic carbonate esters peak contributed by PLGA remains strong even at day 28 of degradation (Figure 2B). An examination of the molecular weight (MW) using gel permeation chromatography (GPC) shows a gradual but slight decrease in MW (from 44 480 ± 1310 as-spun to 35 380 ± 510 1 day after to 32 590 ± 520 after 28 days) of PLGA throughout the 28 days (Figure 2C).

In the body, the scaffold is expected to degrade faster in the presence of collagenases and metalloproteinases secreted by macrophages and phagocytes.[13] This is evident from the faster degradation when the scaffold was submerged in Dulbecco's Modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS) that contains viable enzymes (Figure 2A bottom right).

Release Profile of CHITED. The release profiles of incorporated CHITED were investigated by measuring the released cytokines using specific enzyme-linked immunosorbant assays (ELISA) for individual factors (Figure 3). The total volume of release buffer was extracted at each time point and replaced with fresh buffer. This simulates a sink environment that is most comparable to in vivo conditions where the plasma half-life of the cytokines is only several minutes to hours.[14] A sustained release profile typical of a diffusion-based release that plateaus at around 12 h was observed with slight variations on release percentage and half-time. Up to 97% of hydrocortisone and EGF was released within 8 h of the study. T3 and insulin on the other hand achieved a total release of around 80% during the period of our study. Vitamin C showed a slower release of factors, releasing around 55% of its incorporated factors 4 h into the study and another 30% in the next 4 h. VD3 had around 30% of the incorporated amount being released in

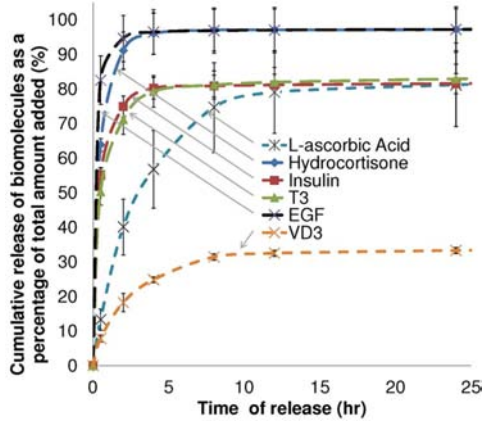

Figure 3. Cumulative release profile of CHITED biomolecules from PLGA-Collagen nanofiber scaffolds immersed in 1% BSA supplemented PBS at 37 °C shaking at 100 rpm over 24 h. Data are shown as mean ± standard deviation ($n \geq 3$).

around 12 h (Figure 3). The variations were probably due to differences in how the individual biomolecules interact with the polymers used as a base material for the scaffold. Other contributing factors include the water solubility of the individual factors and their molecular weight which would affect diffusion of the factors out of the scaffold's polymer matrix. In particular, VD3 had very low release percentage compared to the other biomolecules. This is probably due to the chemical fragility of VD3 toward oxidation and photodenaturation.[15] While a 12 h release profile is relatively quick, long-term stimulation of cells with growth factors may not be necessary. As shown in Tan et al.,[16] a 4 h stimulation with

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transforming growth factor-β1 was as effective in inducing myofibroblast transformation of fibroblasts as a 4 day stimulation due to an autocrine stimulation of transforming growth factor-β1 by fibroblasts after short pulses of external stimulation. Short term burst release may also be better as a long term stimulation of mitogenic factors such as that during inflammation have been linked to malignant transformation in cells.[17,18] The rapid degradation behavior of the scaffold also suggests an effective burst delivery system to initiate cell migration and proliferation while at the same time making way for the local increase of cell mass.

Bioactivity of CHITED Released. The bioactivity of incorporated factors post-fabrication was investigated with fibers containing only one factor of interest to ensure biological specificity in the living cell systems. Novel assays were designed to test the bioactivity of insulin and vitamin C released while commercially available systems were used to test the bioactivity of the other four factors. A transwell system was used in all assays to ensure that only released factors were assessed. The bioactivity of hydrocortisone, T3, and VD3 was assayed using engineered nonhuman mammalian reporter cells that express the designated human receptor protein. Ligands, if bioactive, bind to the receptor resulting in the transcription of the luciferase gene. The luminescence detected is proportional to the concentration of bioactive ligands. To dispel concerns that bioactivity of ligands might be significantly lost during chemical or physical processing of blend electrospinning, we first assessed the bioactivity of VD3 released from a PLGA-Collagen Scaffold (fabricated without BSA). As seen in Figure 4A, only ~20% of VD3 released from PLGA-Collagen scaffolds remained bioactive. However, the addition of BSA to the blend electrospun emulsion significantly doubled the retention of VD3 bioactivity to ~40%.

Next, we assessed changes in bioactivity of VD3 released at an early time point (0 to first hour) to that at a later time point (1st to 12th hour) (Figure 4B). VD3, being a light and oxygen sensitive biomolecule, has a rather short half-life. When added directly in media its bioactivity decreased to ~40% after 1 h of incubation at 37 °C. With the incubation time extended to 12 h, its bioactivity further decreased to 20%. A similar decrease in bioactivity of VD3 released from PLGA-Collagen-BSA scaffold was observed in these two periods. Nonetheless, no further loss in bioactivity of VD3 was observed when compared to VD3 added directly in media and incubated for the same length of time prior to assessment.

Bioactive hydrocortisone and bioactive T3 were also released from the PLGA-Collagen-BSA scaffold as seen in Figure 5B and D. The luminescence detected corresponds to a bioactivity of 33% for hydrocortisone and 35% for T3. Using a well-established bioactivity assay for EGF, we observed that EGF released from the scaffold remained mitogenic, significantly increasing human dermal fibroblast (HDF) proliferation 48 h post stimulation (Figure 5E).

Vitamin C is an important cofactor for prolyl hydroxylases which catalyze the post-translational hydroxylation of proline in procollagen. When added in cell culture, vitamin C increases the in vitro secretion of Col I into the culture medium.[19] This characteristic of vitamin C was used in this self-devised system using HDF. Silver staining of SDS-page gels for pepsin stable collagens showed that there was increased collagen secretion in cells grown in the presence of vitamin C containing scaffolds (Figure 5A). To investigate the functionality of insulin, a system that involves the differentiation of bone-marrow derived

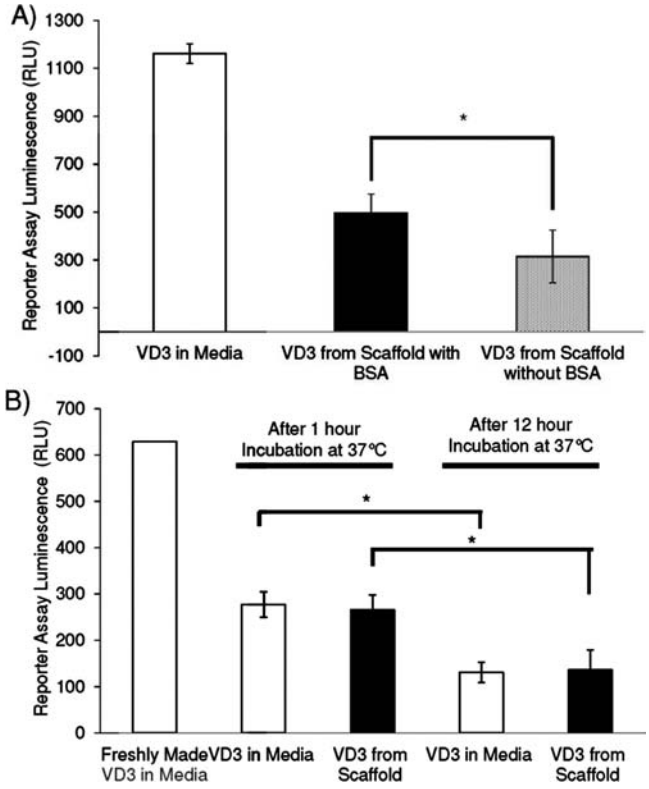

Figure 4. (A) Bioactivity of 150 nM VD3 released from PLGA-Collagen scaffolds with or without BSA assessed over a period of 22 h using a transgenic reporter assay. VD3 released from scaffolds with BSA retains ~40% bioactivity (black bar) compared to VD3 freshly added to media (white bar). VD3 released from scaffolds without BSA have a significantly lower bioactivity of ~20% (gray bar). (B) Bioactivity of 75 nM VD3 released from PLGA-Collagen scaffolds in the 1st hour or between the 1st to 12th hour. Bioactivity of VD3 released from the scaffold over these two periods remain as bioactive as that added directly in media and incubated for the same time prior to assessment. Concentration of VD3 released from scaffolds is determined from ELISA curve in Figure 3. Data shown as mean ± standard deviation ($n \geq 3$) and * indicated a significant difference when a one-way ANOVA analysis was performed ($p < 0.05$).

mesenchymal stem cells (MSCs) into adipocytes was used. Being a mesenchymal lineage progenitor, MSCs will differentiate down the fat lineage if given the right in vitro cues. Insulin is a crucial regulator in the differentiation cocktail for adipogenic differentiation of MSCs and was previously shown to act through the insulin growth factor I receptor.[20−22] Hence, MSCs exposed to an insulin supplemented adipogenic cocktail have an enhanced adipogenic differentiation potential, resulting in increased intracellular lipid accumulation, when compared to a non-insulin supplemented adipogenic cocktail. Using this assay, we observed that MSCs differentiated in the presence of insulin-containing scaffolds had a 2-fold increase in intracellular lipid accumulation (Nile red staining) when compared to the negative control (Figure 5C). Insulin remained bioactive after electrospinning.

Concerns have been frequently expressed over the functional bioactivity of factors included in blend electrospinning systems due to the direct contact of hydrophilic factors with organic solvents. Special systems such as coaxial electrospinning[23,24] and post-electrospinning conjugation[25] have been designed to address these issues. However, the blend emulsion system represents a simpler fabrication process with high consistency[26]

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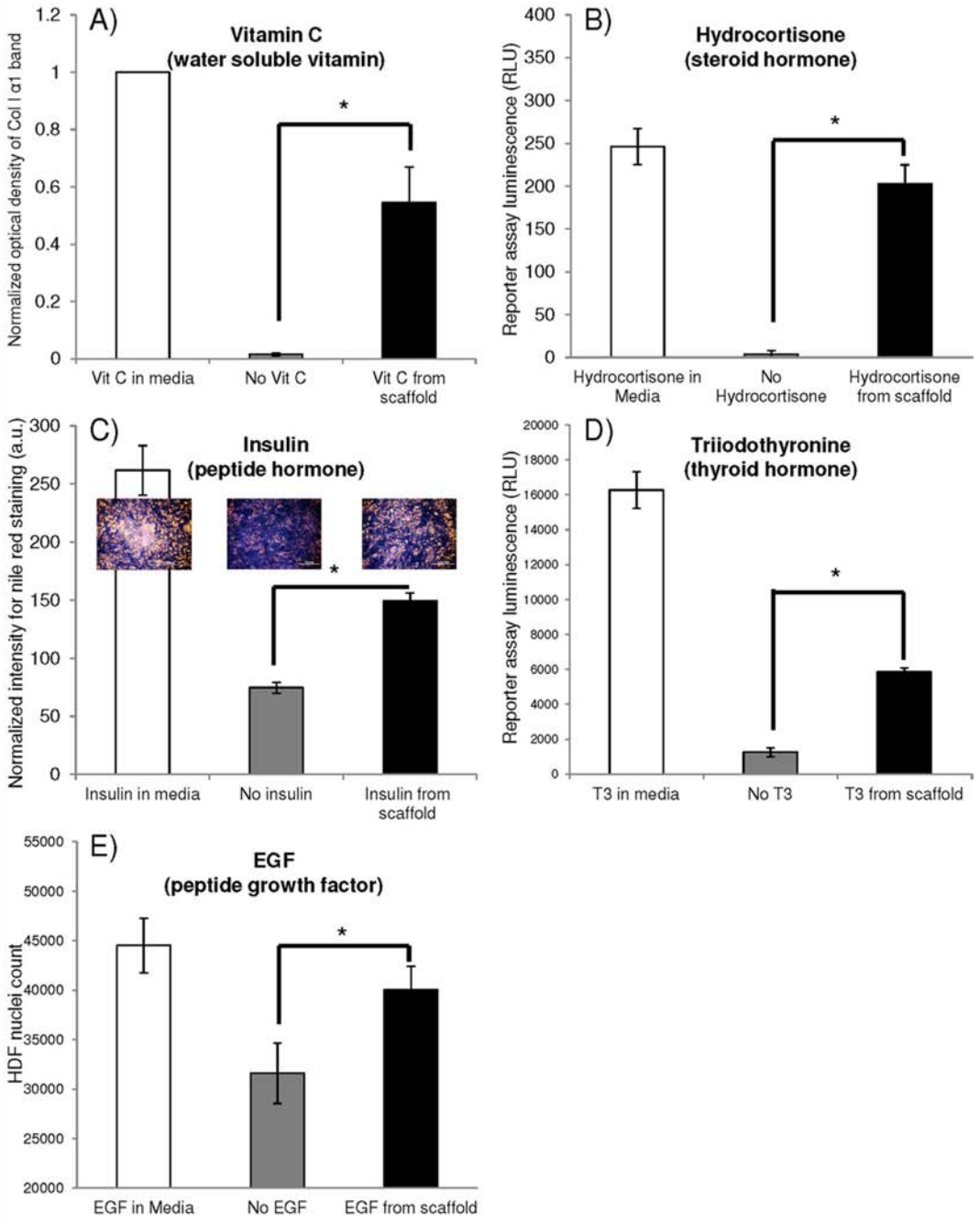

Figure 5. In vitro bioactivity assays of individual CHITE biomolecules released from scaffolds. (A) Bioactive vitamin C released from scaffold increases collagen I secretion by HDF. Bioactive (B) hydrocortisone and (D) T3 released from scaffold results in luminescence detected in transgenic reporter assays. (C) Bioactive insulin released from scaffold increases intracellular lipid production in human MSCs. (E) Bioactive EGF released from scaffold potentiates HDF proliferation. Data are shown as mean ± standard deviation ($n \geq 3$) and * indicated a significant difference when a one-way ANOVA analysis was performed ($p < 0.05$).

which is an important attribute for upscaling. While the bioactivity/viability of one or two growth factors have been tested in their controlled release systems,[23,27] this is to the best of our knowledge the first time that the bioactivity of a wide variety of biomolecules released from a single system has been evaluated. We investigated the bioactivity post-fabrication of six biomolecules with distinctly diverse chemical properties and sizes, proposed previously to aid in skin regeneration.[9] There was a water-soluble molecule vitamin (vitamin C), a steroid hormone (hydrocortisone), a large molecule thyroid hormone (T3), a small peptide hormone (Insulin), a small peptide growth factor (EGF), and a fat soluble vitamin (VD3). These factors are susceptible to oxidation or denaturation to different extents. The biomolecules were blend electrospun with BSA added as a carrier protein. Use of serum albumin in pharmacological formulations was reported to enhance the stability and extend the half-life of peptide or protein based drugs in circulation.[28−30] Serum albumin binds to and transports important ligands such as fatty acids, metal ions, steroids, and amino acids.[7] The presence of sulfur containing amino acids in albumin confers antioxidant properties; albumin scavenges free radicals and functions as a metal chelator to

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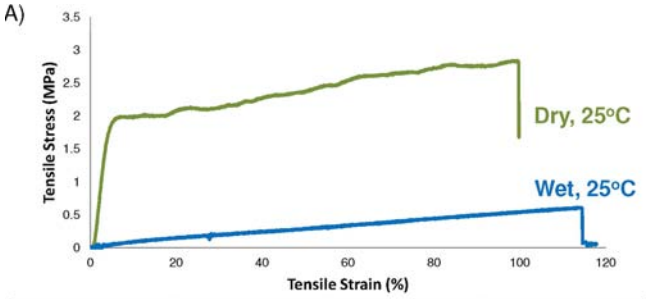
protect surrounding proteins from denaturation through oxidation.[31] It was verified that all the included factors withstood the relatively harsh processing conditions and maintained functionality in cellular assays demonstrating that the system is robust enough to deliver a wide range of biomolecules while maintaining their bioactivity.

Mechanical Properties of Scaffold. The scaffold's tensile mechanical properties at room temperature were assessed using an Instron tensile tester according to ASTM standard D3039/D3039 M (Standard Test Method For Tensile Properties of Polymer Matrix Composite Materials). The scaffold had a breaking strain of 96 ± 10% when dry and the ultimate stain increased to up to 117 ± 9% when wet (Figure 6A). This



	Dry, 25°C, Scaffold	Wet, 25°C, Scaffold	Human Skin
Tensile Modulus (MPa)	54± 9	0.5± 0.05	15-150
Ultimate Tensile Stress (MPa)	2.8± 0.29	0.6± 0.07	5-30
Ultimate Strain (MPa)	96± 10	117.00± 8.90	35-115

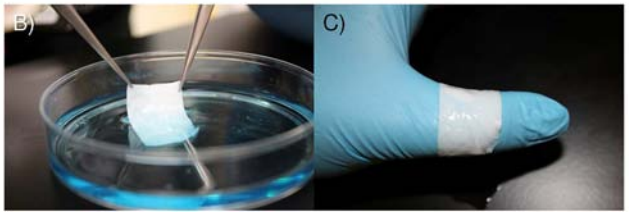


Figure 6. Mechanical properties of the scaffold. (A) Stress−strain curve of dry and wet PLGA-Collagen blend electrospun scaffolds at room temperature. Table compares the scaffold's tensile modulus, ultimate tensile stress, and ultimate strain to that of normal human skin. Photo shows that the wet scaffold (B) can be easily handled using forceps and (C) is able to conform to the finger contours.

ultimate strain of the scaffold lies in the upper range of that of normal human skin (35−115%).[32,33] This high breaking strain indicates that the scaffold is a compliant and stretchable mesh. The scaffold has an ultimate tensile stress of 2.8 ± 0.29 MPa when dry and it becomes more fragile with a reduced ultimate tensile stress of 0.6 ± 0.07 MPa. Nonetheless, the scaffold could still be easily handled with a pair of forceps when wet, suitable for surgical use (Figure 6B). The pliability of the wet scaffold is demonstrated by wrapping it around the contours of a finger (Figure 6C).

CHITE Scaffold Increases Skin Cell Proliferation and Maintains Keratinocyte Basal State. Vitamin C, hydrocortisone, insulin, T3, and EGF (collectively known as CHITE) have been previously shown to facilitate keratinocyte proliferation and viability.[34−38] The response of keratinocytes and fibroblasts was investigated in vitro by allowing the supplemented scaffold to release CHITE while sitting in a transwell. This is to emulate the wound situation where cells may not be in direct contact with the scaffold. The CHITE scaffold stimulated the proliferation of keratinocytes and HDFs significantly. Five days post-exposure to CHITE scaffold, both keratinocytes and HDFs registered up to 2 times more cells per well then when exposed to blank scaffolds or no scaffold (Figure 7).

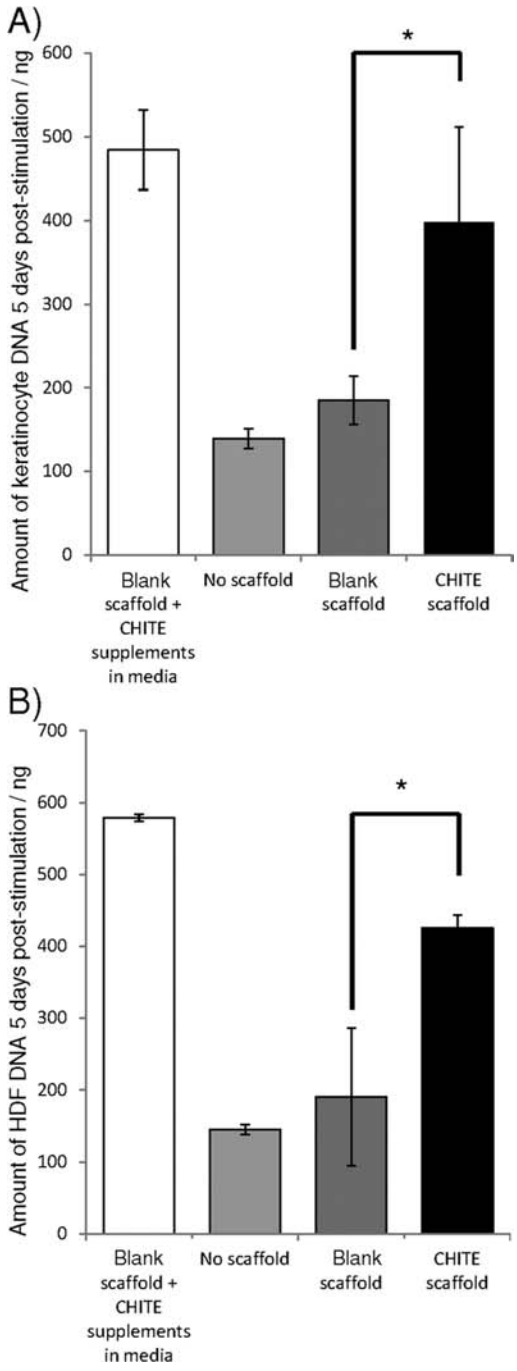


Figure 7. Proliferation profile of skin cells in the presence of CHITE scaffolds. Total DNA amount of (A) primary keratinocytes and (B) HDF grown 5 days in exposure to CHITE scaffolds. Data are shown as mean ± standard deviation ($n \geq 3$) and * indicated a significant difference when a one-way ANOVA analysis was performed ($p < 0.05$).

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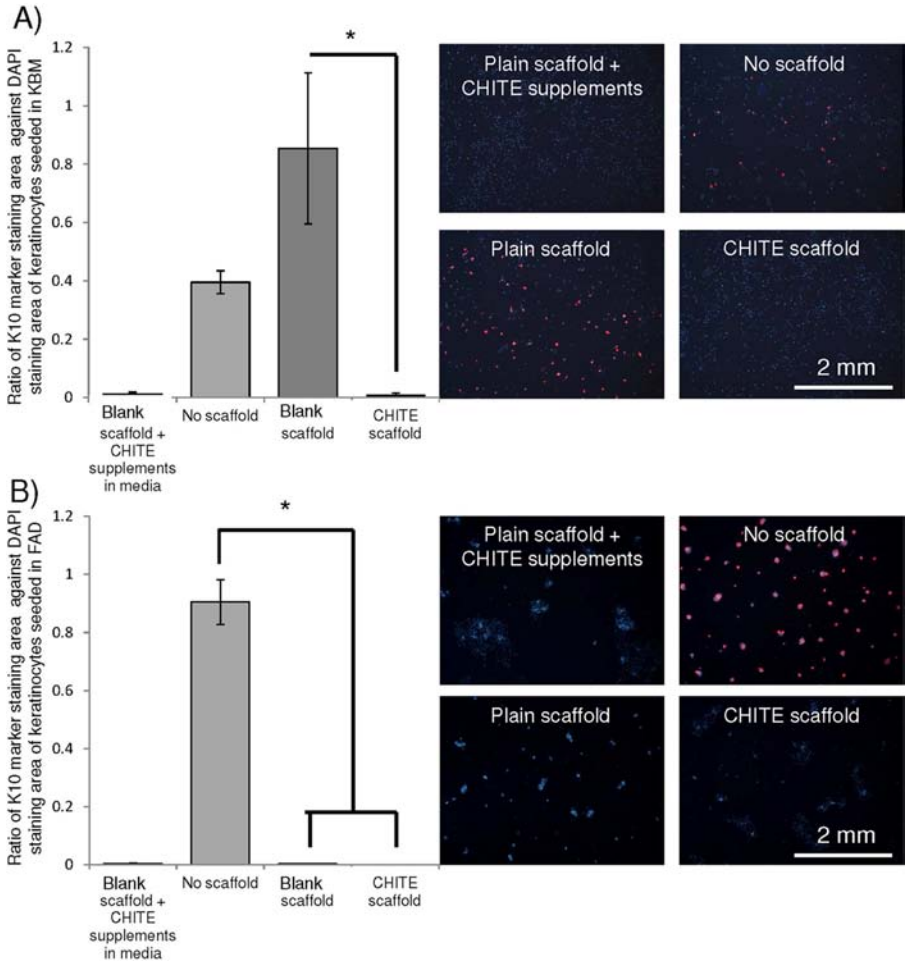

Figure 8. Differentiation status of keratinocytes seeded in the presence of the CHITE scaffolds. Keratinocytes seeded on tissue culture plate in low calcium, differentiation discouraging keratinocyte basal media (KBM) with/without CHITE scaffold over a period of 5 days. (Left) Quantification of the area of cytokeratin 10 (K10) staining (red) against 4′,6-diamidino-2-phenylindole (DAPI) staining (blue). (Right) Representative microscope images of keratinocytes (4× magnification) under different conditions. Similar assays were performed with (A) KBM formulated to promote keratinocyte proliferation and (B) FAD media formulated to promote keratinocyte differentiation. Data are shown as mean ± standard deviation ($n \geq 3$) and * indicated a significant difference when a one-way ANOVA analysis was performed ($p < 0.05$).

Keratinocytes exposed to the scaffolds were assessed for their differentiation state in keratinocyte basal media (KBM) and it was observed that fewer cells expressed cytokeratin 10 intermediate filaments (K10) in the presence of CHITE scaffolds as compared to blank scaffolds (Figure 8). K10 is a marker for suprabasal keratinocytes committed to terminal differentiation. The absence of K10 is a good indicator that the CHITE scaffold supports a more naïve state of keratinocytes.[39] As differentiated keratinocytes have limited proliferation capacity, naïve keratinocytes are crucial during wound healing. The ability of CHITE to stimulate proliferation of skin cells is particularly beneficial for the initial stages of wound healing when keratinocytes and fibroblast from the wound edges proliferate in order to provide coverage for lost epithelial and dermal tissue. Interestingly, when the assay was repeated in FAD media containing physiological calcium, which induce terminal differentiation of keratinocytes, it was observed that the presence of any scaffold, with or without CHITE, suppressed the expression of K10.

CONCLUSION

We demonstrated a method of retaining the bioactivity of fragile biomolecules with varying chemical classes, sizes, solubilities, and sensitivity to light and oxygen, when added into blend electrospun scaffolds. We further established the feasibility of a system that simultaneously delivers disparate bioactive compounds we have previously optimized for skin tissue engineering. The release of this group of biomolecules effectively enhances the proliferation of skin cells crucial for wound healing. These findings are significant because of the widely perceived view that the technique of blend/emulsion electrospinning (a) destroys the bioactivity of proteins during the violent emulsification and (b) is thermodynamically unstable to be electrospun smoothly for an emulsion of water-soluble and nonsoluble molecules. We demonstrate for the first time that a wide range of biomolecules can in fact be blend electrospun smoothly together and even retain their bioactivity upon release. This simple and reproducible fabrication method allows disparate biomolecules, which are essential ingredients of many differentiation cocktails, to be blended together and electrospun effectively creating bioactive scaffolds that maintain the growth capacity of cells and bioactive lineage directing scaffolds. This method presents an elegant solution to overcome technical difficulties in delivering disparate biomolecules simultaneously, opening up possibilities

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of creating various lineage inducting or tissue instructive substrates/scaffolds for tissue engineering applications.

EXPERIMENTAL PROCEDURES

Fabrication of Blended Electrospun Nanofiber Scaffolds. Poly(DL-lactide-*co*-glycolide) (PLGA 75:25) copolymer at a molecular weight of 90 000 to 126 000 (Sigma-Aldrich, St. Louis, MO) and bovine atelocollagen powder (CosmoBio, Japan) were mixed at a ratio of 1:1 (w:w) overnight at room temperature at 10% (w/v) concentration in 1,1,1,3,3,3-hexafluoro-2-propanol (HFP). Prior to electrospinning, CHITED biomolecules were added (see Table 1) into 3.3% (w/v) of bovine serum albumin (BSA) (Sigma-Aldrich, St. Louis, MO) dissolved in phosphate buffered saline. L-Ascorbic acid phosphate magnesium salt (Vitamin C) was purchased from WAKO, Japan, while hydrocortisone, insulin, 3,3,5-triiodo-L-thyronine sodium (T3), EGF, and 1α,25-dihydroxyvitamin D3 (VD3) were purchased from Sigma-Aldrich, St. Louis, MO. The cocktail of biomolecules were added into the PLGA−collagen solution, vortexed briefly, and ultrasonicated for 20 min on ice, and then loaded into a polypropylene syringe attached to a flat-beveled 27G needle. With the aid of a syringe pump (KD-Scientific, USA), the solution was dispensed at a feeding rate of 0.5 mL/h at a humidity of 50−60% and temperature of 24 °C. Electrospinning voltage was applied to the needle at 12 kV DC, using a high voltage power supply (Gamma High Voltage Research, Ormond Beach, FL). The electric field generated by the surface charge caused the drop emerging at the tip of the needle to distort into a Taylor cone. Once the electric potential at the surface charge exceeded a critical value, the electrostatic forces would overcome the solution surface tension and a thin electrified jet of solution would erupt from the surface of the cone. The electrospinning process was carried out for 2−3 h depending on the amount of nanofibers needed. The resultant CHITED-PLGA-Collagen scaffold (CHITED scaffold) was collected on an aluminum foil plate located 8 cm away from the needle tip. After solvent evaporation, the CHITED scaffold coated aluminum foil was placed in a vacuum desiccator overnight to remove any remaining HFP solvent. Blank scaffolds were fabricated with the same polymer−albumin mixture but in the absence of biomolecules.

Table 1. Composition of CHITED Loaded into PLGA-Collagen Scaffold

composition of CHITED	mass per mg of fiber (ng)
C - L-Ascorbic Acid Phosphate Magnesium Salt	9396
H - Hydrocortisone	68
I - Insulin	849
T - 3,3′,5-Triiodo-L-thyronine	0.12
E - Epidermal growth factor	1.70
D - Vitamin D3	71

Scanning Electron Microscopy. Scanning electron microscopy (SEM) micrographs in Figure 1 were obtained with a JSM-5800LV and JSM-6510 scanning electron microscope (JEOL, Tokyo, Japan). Micrographs in Figure 2 were taken with a MERLIN FE-SEM (ZEISS, Jena, Germany) to observe the morphology of electrospun nanofibrous membrane. The diameter range of the fabricated nanofibers was measured on the basis of SEM images, using image analysis software (*ImageJ*, n = 300).

Degradation Studies of Scaffold. PLGA-Collagen nanofiber scaffold mat was fabricated as described above on 15-mm-diameter cover glasses and had an average weight of 1.2 mg. Margins of the scaffolds were fixed to the coverslip using a medical grade silicone glue (Bluestar Silicones, Lakeside, USA) to prevent contraction. Scaffolds were then placed into 24 well tissue culture plates (Cellstar, Greiner Bio-one, North-America) and were ethylene oxide sterilized. Sterile metal rings of 15 mm (outer diameter) were used to hold down cover glasses during culture. 0.5 mL of pH 7.4 phosphate buffered saline or low glucose DMEM supplemented with 10% FBS, 100 units/mL penicillin, and 100 μL/mL streptomycin was added into each well and culture plates were kept in a standard cell incubator in a humidified atmosphere at 37 °C in 5% CO_2. Media change was carried out on day 4 and during every collection time point. At days 1, 7, 14, 21, and 28, the scaffolds were taken out, washed with distilled water, and placed in a drybox.

Completely dried samples were sputter-coated with gold and imaged using FE-SEM (Zeiss, Germany). Attenuated Total Reflectance-Fourier Transform Infrared (ATR-FTIR) spectra were obtained with a Frontier FTIR machine (PerkinElmer, MA, USA) to characterize the chemical composition of blended NFS. Gel permeation chromatography (GPC) analysis was carried out on the tetrahydrofuran (THF) soluble PLGA portion of the scaffold with a Waters (MA, USA) e2695 separations module equipped with two Styragel HR columns (weight range: 500−30 000 and 5000 to 600 000) and a Waters 2414 refractive index detector. THF was used as eluent at a flow rate of 0.30 mL/min at 40 °C. Monodispersed polystyrene standards were used to obtain a calibration curve.

Determination of Release Profile. Ten milligrams of CHITED supplemented scaffolds were completely immersed in 5 mL of phosphate buffered saline (PBS) with 0.1% w/v BSA in Falcon tubes placed in a 37 °C shaking water bath for 24 h. The tubes were constantly shaken at 100 rpm. After 0.5, 2, 4, 8, 12, and 24 h, all buffer was collected and replaced with fresh buffer. Release kinetics of different biofactors were monitored with a Cayman Ascorbate Assay Kit 700420 (Cayman Chemical, USA), Cortisol ELISA kit (R&D Systems, Minneapolis, USA), Insulin (Bovine) ELISA (ALPCO Diagnostics, Salem, NH), T3 ELISA (Calbiotech, Spring Valley, CA), Quantikine Human EGF (R&D Systems, Minneapolis, USA) system, and a 1,25-Dihydroxy Vitamin D EIA (Immunodiagnostic Systems, United Kingdom), respectively.

Cell Culture. Human neonatal foreskin dermal fibroblasts (HDF) (ATCC #PCS-201-010 VA, USA) and human adult bone marrow derived mesenchymal stem cells (MSCs) (Lonza, USA) were expanded in high glucose and low glucose DMEM, respectively, both containing 10% FBS, 100 IU/mL penicillin, and 100 μg/mL streptomycin (GIBCO, Invitrogen Corporation, USA). HDFs and MSCs were used at passage 8−9. Normal pooled adult human epidermal keratinocytes (Promocell, Heidelberg, Germany) were expanded in fully supplemented Keratinocyte Growth Media (KGM) (Promocell) prior to use at passage +4 to +5. KGM is made up of keratinocyte basal media (KBM) supplemented with bovine pituitary extract (0.004 mL/ml), epidermal growth factor (recombinant human) (0.125 ng/mL), insulin (recombinant human) (5 μg/mL), hydrocortisone (0.33 μg/mL), epinephrine (0.39 μg/mL), transferrin, holo (human) (10 μg/mL), $CaCl_2$ (0.06 mM). Cell cultures were incubated in a carbon dioxide incubator with 5% CO_2 and 95% atmospheric air at a temperature of 37 °C.

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Bioactivity Assays. Bioactivity assays were performed on scaffolds containing single biofactors to avoid biological interference. For sterilization, weighed scaffolds were placed flat under a biological safety cabinet germicidal UV lamp that has an average intensity of 100 mW per cm^2 in a sterile environment for 1 h, then flipped and UV sterilized for another hour. Unless otherwise described, reporter assay cells were seeded into 24 well plates and 1 mL of culture medium was added. Transwell inserts (Greiner ThinCert 24 well, 0.4 μm pore size, Greiner Bio-one, North-America) containing scaffolds were then lowered into the culture medium until fully immersed. The biofactor released from the scaffold in the top compartment was expected to diffuse through the pores of the transwell into the lower chamber to stimulate reporter cells.

EGF Bioactivity. Bioactivity of EGF was assessed by its effect on the proliferation of HDF using a protocol adapted from Promega (Madison, USA).[40] 10 000/well HDFs were seeded in 24 well plates and allowed to adhere overnight in HDF expansion medium. Then, HDFs were serum-starved in blank HG DMEM for 24 h. Scaffolds containing 15 ng of EGF were then loaded in transwell inserts and lowered into the wells. For the negative control, the EGF-containing scaffold was substituted with a blank scaffold. For the positive control, a blank scaffold was loaded and 15 ng EGF was directly supplemented into 0.5 mL culture medium. After 2 days of proliferation HDFs were fixed in 4% methanol-free formaldehyde (10 min; room temp). Nuclei of fixed HDFs stained with 4′,6-diamidino-2-phenylindoldilactate (DAPI) were counted using adherent fluorescent cytometry as previously described.[41]

Hydrocortisone, T3, and VD3 Bioactivity. Hydrocortisone, T3, and VD3 Reporter assays (Indigo Biosciences Inc., PA, USA) were used to measure the respective bioactivities. Briefly, reporter cells were dispensed into 96 well plates and allowed to adhere for 6 h. Thereafter, transwells (Corning 96 well, 8 μm pore size) with scaffolds containing 0.016 ng of hydrocortisone, 13 ng T3, or 7.7 ng VD3 were loaded into the wells containing 100 μL of media. Negative controls were using a blank scaffold, and for positive control, a blank scaffold was loaded and the equivalent amount of hydrocortisone, T3, or VD3 was directly supplemented into the culture medium. Following a 22 h incubation with the scaffolds, the culture media was discarded and fresh Luciferase Detection Reagent was added. Binding of the ligand of interest to the receptors on the reporter cells will cause expression of luciferase which catalyzes the oxidation of luciferin. Luminescence from luciferin was quantified using a Pherastar Microplate reader (BMG LABTECH GmbH, Germany) and measured against a calibration curve.

Insulin Bioactivity. The preserved bioactivity of insulin was assessed in a MSCs adipogenic differentiation assay using a standard induction cocktail lacking insulin but delivering this factor via the scaffold. MSCs were seeded at an initial density of 25 000 cells/well in 24-well plates (CellStar, Gibco). Adipogenic differentiation was stimulated upon confluence via three cycles of induction/maintenance. Each cycle consists of 4 days exposure to induction medium followed by 3 days of culture in basal medium.[42] During the induction cycle, transwells loaded with scaffolds containing 2 μg of insulin were lowered into each well. The induction medium contains HG DMEM (Gibco, Invitrogen Corporation, USA) supplemented with 10% FBS, 100 units/mL penicillin, 100 μL/mL streptomycin, 3-isobutyl-1-methylxanthine (0.5 mM), dexamethasone (10^{-7} M), and indomethacine (0.2 mM) (Sigma-Aldrich, USA). For the positive control, transwells loaded with blank scaffold were immersed in insulin (1.75 μM) supplemented induction media. For the negative control, transwells loaded with blank scaffold was immersed in insulin-deprived induction media. During the maintenance cycle, transwells containing scaffolds were removed and induction medium was changed to basal medium (high glucose DMEM/10% FBS, antibiotics). After three cycles of induction/maintenance, monolayers were rinsed with PBS, fixed in 4% methanol-free formaldehyde, and stained with Nile Red (Sigma-Aldrich; 0.05 mg/mL in water; 1 h) to assess cellular lipid content. The extent of adipogenic differentiation was quantified by adherent fluorescent cytometry assessing area of Nile Red fluorescence normalized to nuclei count (DAPI).[42]

Vitamin C Bioactivity. HDFs were seeded in tissue culture dishes at a density of 50 000 cells/24 well plate well and allowed to adhere overnight in high glucose DMEM/10% FBS, and antibiotics. On the second day, culture media was changed to high glucose DMEM/0.5% FBS, antibiotics. Thereafter, transwells containing scaffolds containing 50 μg of μM L-Ascorbic Acid Phosphate Magnesium Salt were lowered into each well. For the positive control, transwells were loaded with blank scaffold and immersed in vitamin C supplemented media (0.5 mL of 100 μg/mL of vitamin C). For the negative control, transwells were loaded with blank scaffold and immersed in vitamin C-free induction media. 72 h after loading the scaffold, culture medium was pepsin digested to remove noncollagenous proteins and then subjected to SDS-PAGE (5% resolving/3% stacking polyacrylamide gels.[43] Protein bands were stained with the SilverQuest kit (Invitrogen). Densitometric analysis of wet gels was performed on collagen α1(I) bands with a GS-800 Calibrated Densitometer and Quantity One v 4.5.2 image analysis software.

Quantitative Assessment of Bioactivity. The percentage of bioactivity of hydrocortisone, VD3, and T3 released from the scaffold has been examined with the commercially available INDIGO's cell-based nuclear receptor assays. A titration of the agonist (hydrocortisone, VD3, or T3) was directly added in media as recommended by the manufacturer's protocol. These concentrations were plotted against the luminescence intensity obtained for each dose. A standard curve, i.e., concentration vs luminescence, was obtained. The mass of hydrocortisone, VD3, or T3 loaded into the scaffolds was calculated such that upon release the concentrations of these agonist would fall within the working range of the standard curve. In this study, the concentrations released from the scaffold at specific time points (1, 12, and 22 h) were identified from the ELISA cumulative release curve shown in Figure 3. With the luminescence intensity, the bioactive concentration detected can be read off from the standard curve.

As such the percentage bioactivity of the agonist is calculated as

$$\frac{\text{Bioactive Concentration detected}}{\text{Concentration released from scaffold}} \times 100\%$$

Mechanical Testing. Tensile tests were carried out in accordance to ASTM: D3039/D3039 M (Standard Test Method for Tensile Properties of Polymer Matrix Composite Materials) with a 5848 microtester (Instron, Norwood, MA) at a stroke rate of 1 mm/min and with a 10 mm gauge length. Nanofiber scaffolds were cut into bone-shaped samples with a width of 4 mm and a length of 20 mm. The ultimate tensile stress of the PLGA-collagen scaffold was determined from the

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maximum force sustained by it before failure per unit cross sectional area, while the ultimate tensile strain of the scaffold was determined from the maximum elongation of the scaffold before failure. Young's modulus was determined from the ratio of the uniaxial stress over the uniaxial strain.

CHITE Scaffold's Biological Effect on Keratinocytes and Fibroblasts. Primary keratinocytes or human dermal fibroblasts were seeded in expansion media at 10 000 cells per 24 well plate well and allowed to attach overnight. The next day, the cells were serum-starved in basal media (Keratinocyte basal media, KBM containing only $CaCl_2$ (0.06 mM) for keratinocytes and blank HG DMEM for fibroblasts) for 24 h. Transwells containing CHITE scaffolds pre-sterilized using ethylene oxide gas were then lowered into HDF or keratinocytes cultures in basal media. Negative controls contained blank scaffold. Positive controls contained blank scaffolds, which were immersed in medium supplemented with CHITE: 50 μg/mL L-Ascorbic Acid Phosphate Magnesium Salt, 0.4 μg/mL hydrocortisone, 5 μg/mL insulin, 1 nM T3, and 10 ng/mL EGF. Five days later, the cells were lysed with a proteinase K, Triton X-100 buffer, and its DNA content was quantified with PicoGreen (Invitrogen).

K10 Staining for Terminally Differentiating Keratinocyte Cells. Primary keratinocytes were seeded in KGM at 10 000 cells per 24 well plate well and allowed to attach overnight. The next day, the cells were serum-starved in basal media comprising either KBM or FAD (a 3:1 mixture of high glucose DMEM and Ham's F12 media) for 24 h. Transwells containing ethylene oxide sterilized CHITE scaffolds were lowered into the keratinocyte cultures in basal media. Five days later, the cells were washed thrice with PBS and fixed with paraformaldehyde for 20 min. The cells were permeabilized with 0.25% Triton X-100 in PBS for 10 min and stained with mouse anti K10 primary antibody (1:200) (ThermoScientific) and Alexafluor-594 secondary antibody. Five non-overlapping random fields of view were taken per well using an Olympus epifluorescence microscope with DAPI as a counter stain. Images were thresholded and the area of staining was quantified using *ImageJ*.

Statistical Analysis. All experiments were done in triplicate and in at least two independent runs. Data presented are expressed as mean ± standard deviation of the mean and statistical differences were determined by one-way ANOVA. Post hoc analysis was carried out using Bonferroni correction. $p < 0.05$ was considered statistically significant.

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Notes

The authors declare no competing financial interest.

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ABBREVIATIONS

ATR-FTIR, attenuated total reflectance-fourier transform infrared; ANOVA, analysis of variance; BSA, bovine serum albumin; CHITED, C-vitamin C, H-hydrocortisone, I-insulin, T-triiodothyronine, E-epidermal growth factor, D-vitamin D3; CHITE, CHITED excluding vitamin D3; DAPI, 4′,6-diamidino-2-phenylindole; DMEM, Dulbecco's modified eagle's medium; EGF, epidermal growth factor; ELISA, enzyme-linked immune assay; FAD, a 3:1 mixture of high glucose DMEM and Ham's F12 media; FBS, fetal bovine serum; GPC, gel permeation chromatography; HDF, human dermal fibroblast; HFP, 1,1,1,3,3,3-hexafluoro-2-propanol; K10, cytokeratin 10; KBM, keratinocyte basal media; KGM, keratinocyte growth media; MSCs, mesenchymal stem cells; PBS, phosphate buffered saline; PLGA, poly(DL-lactide-*co*-glycolide); SEM, scanning electron microscopy; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis; T3, triiodothyronine; VD3, vitamin D3

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