International Journal of Bioprinting

RESEARCH ARTICLE

Developing a bioink for single-step deposition and maturation of human epidermis

Ruth Jinfen Chai[1], Wan Ling Wong[1], Cyrus Weijie Beh[1,2]*

[1]Molecular Engineering Lab, Institute of Molecular and Cell Biology, Singapore 138673, Singapore

[2]Institute of Bioengineering and Bioimaging, Singapore 138669, Singapore

(This article belongs to the *Special Issue: Related to 3D printing technology and materials*)

Abstract

Patients with severe burns, which cause extensive damage to their skin, require rapid intervention to prevent life-threatening hypothermia, infection, and fluid loss. Current treatments typically involve surgical excision of the burned skin and reconstruction of the wound with the aid of skin autografts. However, there is a lack of donor site in the most severe cases. While alternative treatments such as cultured epithelial autografts and "spray-on" skin can allow much smaller donor tissues to be used (and hence reduce donor site morbidity), they present their own challenges in terms of fragility of the tissues and control of the cell deposition, respectively. Recent advances in bioprinting technology have led researchers to explore its use to fabricate skin grafts, which depend on several factors, including appropriate bioinks, cell types, and printability. In this work, we describe a collagen-based bioink that allows the deposition of a contiguous layer of the keratinocytes directly onto the wound. Special attention was given to the intended clinical workflow. For example, since media changes are not feasible once the bioink is deposited onto the patient, we first developed a media formulation designed to permit a single deposition step and promote self-organization of the cells into the epidermis. Using a collagen-based dermal template populated with dermal fibroblasts, we demonstrated by immunofluorescence staining that the resulting epidermis recapitulates the features of natural skin in expressing p63 (stem cell marker), Ki67 and keratin 14 (proliferation markers), filaggrin and keratin 10 (keratinocyte differentiation and barrier function markers), and collagen type IV (basement membrane protein involved in adherence of the epidermis to the dermis). While further tests are still required to verify its utility as a burn treatment, based on the results we have achieved thus far, we believe that our current protocol can already produce donor-specific model for testing purposes.

Keywords: Human skin equivalents; Bioprinting; *In vitro* models; Tissue regeneration; Skin; Tissue engineering

***Corresponding author:**
Cyrus Weijie Beh
(beh_cyrus@ibb.a-star.edu.sg)

Citation: Chai RJ, Wong WL, Beh CW, 2023, Developing a bioink for single-step deposition and maturation of human epidermis. *Int J Bioprint*, 9(4): 738. https://doi.org/10.18063/ijb.738

Received: September 09, 2022

Accepted: September 30, 2022

Published Online: April 26, 2023

1. Introduction

According to the World Health Organization (WHO), burns are a global public health issue that is estimated to cause 180,000 deaths yearly[1]. In 2004, more than 11 million people were burned seriously enough to require medical attention[1]. Patients with severe burns suffer extensive damage to their skin that requires immediate intervention to prevent

hypothermia, infection, and fluid loss. Treatment for these patients is typically through the use of split-thickness skin grafts (STSG), where healthy skin is harvested from the patient and applied on the burn wound[2]. In order to maximize wound coverage and improve graft "take," slits are often cut into flattened grafts to create a mesh-like structure, which results in poorer esthetic outcome for the patient since the mesh pattern may be visible after healing. Furthermore, STSG is not an option in cases where autologous donor sites are unavailable due either to extensive injury or underlying conditions[3].

While off-the-shelf dermal replacement templates (DRT) can be used to recreate a functional (if incomplete) dermis[3], restoring the epidermis requires the use of patient cells. Autologous cell sheets known as cultured epithelial autografts (CEAs) can be grown *in vitro*, and applied onto a DRT or native dermis for partial thickness wounds[4]. However, CEAs require a large number of cells[5]; the resulting skin tends to be fragile[5-7]; and are vulnerable to infection[6]. An alternative approach is to utilize so-called "spray-on" skin, where a skin biopsy is enzymatically treated to release the cells, which are then sprayed onto the wound bed[8,9]. While it is easier to achieve conformance to body contours with a cell spray than a CEA, the cell solution can flow away from the site of application when deposited on large wounds with physiological topographies and orientations[10]. Since the cell suspension is clear, determining adequate cell coverage can also be challenging (L. Téot, personal communication, May 9, 2022).

In recent years, the field of bioprinting has progressed significantly, with many groups working on direct printing of cell-laden bioinks[10-16]. Consequently, bioprinting has been utilized to create 3D skin constructs containing dermal and epidermal components[17-24]. This typically involves a multi-step process, where different components are printed at different times, and matured under different conditions over several days. While most bioprinted skin constructs are intended for *in vitro* testing purposes[17-19,24], some have taken it a step further, and implanted the constructs onto animal models[20-23]. Although these implants have shown promise, they also share the challenges associated with CEA, such as poor conformance to body contours, and long and costly culture before use. The long-term graft stability is also unproven in most cases, and the requirement for sophisticated bioprinters limits their availability[25]. Fortuitously, the unique superficial position of skin allows *in situ* printing to be performed[10,15,16,26,27]. Furthermore, the ability of keratinocytes to self-organize into functional epidermis means that it is possible to deliver the cells without extensive spatial control. Therefore, when used in combination with a dermal template, a simple hand-held bioprinter is sufficient to deposit the cell-laden bioink onto the wound[10,15].

Here, we describe the development of a serum-free bioink formulation that is designed to be deposited directly onto a wound bed with a suitable dermal layer using such a bioprinter (Figure 1). Since media change is difficult once the bioink is deposited on the patient, the bioink is designed to require only a single deposition step, and promote self-organization of the cells into the epidermis layers with no further intervention. To arrive at this formulation, we first utilized the robust N/TERTs immortalized human keratinocyte cell line to help us narrow down the suitable formulations and culture conditions for creating a reconstituted human epidermis (RHE) model. Using the knowledge gained from the N/TERTs, we developed a media formulation that achieves a balance between proliferation and differentiation, triggering differentiation as the bioink dehydrates. By adding bovine collagen type I to our formulation, we improved control of the deposition process, since the gelled bioink will not flow away from the site of application. Finally, using a collagen-based dermal template, we demonstrate the formation of various epidermal structures from primary keratinocytes by immunofluorescence. We believe that this bioink has great potential to be used in skin bioprinting, as a new treatment option for burns.

2. Materials and methods

2.1. Materials

Cell culture reagents Dulbecco's Modified Eagle Medium/Nutrient Mixture F-12 (DMEM/F-12), 0.05% trypsin-EDTA and TrypLE™ Express were purchased from Gibco (USA). CnT-Prime epithelial proliferation medium (CnT-PR), CnT-Prime epithelial 3D airlift medium (CnT-PR-3D) and CnT-Prime Fibroblast Proliferation medium (CnT-PR-F) were bought from CELLnTEC (Switzerland). Primary human epidermal keratinocytes and KGM-Gold Keratinocyte Growth Medium (KGM) were purchased from Lonza (Switzerland). Rat tail collagen type I solution from Corning (USA) and lyophilized bovine collagen type I from Symatese (France) were used. Human dermal fibroblast (HDF/TERT164) cell line was purchased from Evercyte (Austria) while immortalized cell line N/TERT was a gift from Associate Professor Hao Li, Amy (National University of Singapore, Singapore).

Primary and secondary antibodies used for immunofluorescence staining are listed in Table 1.

2.2. Preparation of bovine collagen and bioink

Lyophilized bovine collagen type I was dissolved in sterile 5 mM acetic acid (Merck, USA) on a multi-rotator (Grant-Bio, UK) at room temperature. The acidic collagen solution (0.1% w/v) was then kept at 4°C until further use. Bioink

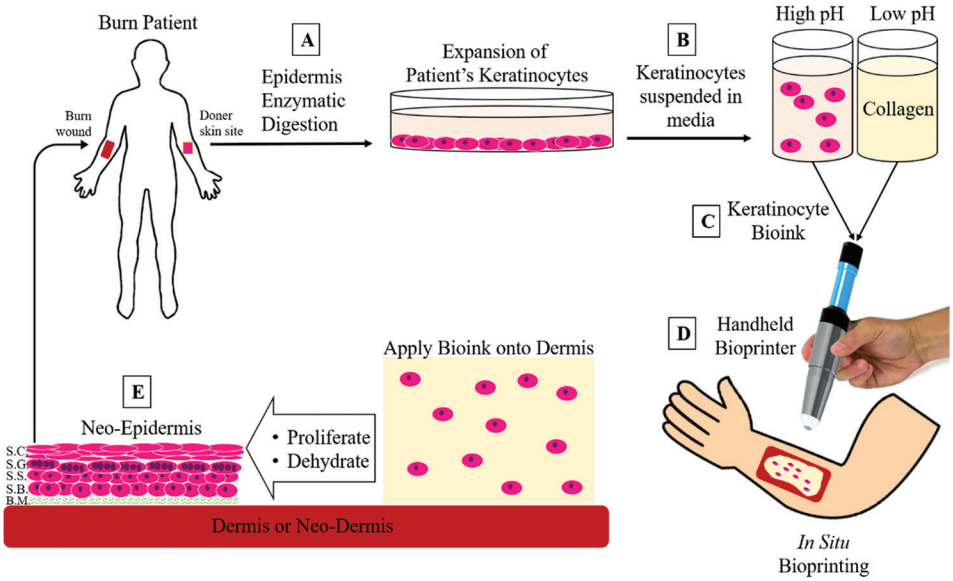

Figure 1. Overview of the steps involved in bioprinting the epidermis. (A) Keratinocytes will be harvested from a piece of healthy skin from the patient and expanded in culture. (B) When sufficient keratinocytes are obtained, they are placed in a cartridge containing media with NaOH. The other cartridge contains collagen dissolved in acetic acid. (C) On mixing of components from both cartridges, collagen gelation occurs when the pH is neutral. (D) A handheld bioprinter is used to dispense the bioink onto the patient's wound, (E) where the keratinocytes would proliferate and differentiate in culture to form a neo-epidermis. Abbreviations: S.C., stratum corneum; S.G., stratum granulosum; S.S., stratum spinosum); S.B., stratum basale; B.M., basement membrane. Modified figure from Materials Today: Proceedings, International Conference of Additive Manufacturing for a Better World (AM Conference) 2022[42].

Table 1. List of antibodies used in immunofluorescence staining.

Antibody	Brand (region)
Primary antibodies	
Filaggrin (ab81468)	Abcam (UK)
Keratin 10 (DKO.M7002)	DAKO (Denmark)
Collagen type IV (CIV22)	DAKO (Denmark)
Ki67 (514520)	Invitrogen (USA)
Keratin 14 (ab7800)	Abcam (UK)
p63 (ab735)	Abcam (UK)
Secondary antibodies	
Alexa Fluor 488	Invitrogen (USA)
Alexa Fluor 568	Invitrogen (USA)
Alexa Fluor 647	Invitrogen (USA)

was prepared with a 1:1 mixture of collagen solution (0.1% w/v) and neutralization solution consisting of primary keratinocytes and sterile 5 mM sodium hydroxide (NaOH, Sigma, USA). Mixing of these two components was done on ice to prevent pre-mature gelation of the collagen solution.

2.3. Cell culture

N/TERT keratinocytes were cultured in CnT-PR medium and culture medium was changed every 2 days. Cells were trypsinized with TrypLE™ Express and routinely passaged in tissue culture flasks when they reach 80% confluency. Primary human keratinocytes were cultured in KGM, and cells no later than passage 3 were used in all experiments. HDFs were cultured in DMEM/F-12 medium supplemented with 10% fetal bovine serum (FBS, Hyclone, USA). Media were changed every 2 days, and cells were routinely passaged at 80% confluency using 0.05% trypsin-EDTA. All cell cultures were kept in 37°C incubator (ESCO, Singapore) with 5% CO_2 before being used in experiments.

2.4. Cell viability

Viability assays of N/TERT keratinocytes and primary keratinocytes in various media were performed.

2.4.1. Viability of N/TERT keratinocytes in PDα medium

N/TERT keratinocytes (5000 cells/well) were seeded on 24-well plate in PDα medium (A*STAR RSC, Singapore) and CnT-PR medium. Cells were cultured for 72 h and cell viability was then measured by performing CellTiter-Blue® Cell Viability Assay (Promega, USA) with excitation wavelength at 555 nm and emission wavelength at 585 nm.

2.4.2. Viability of primary keratinocytes in high pH environment

Before mixing the two-part component of the bioink, primary keratinocytes were suspended in chilled PDβ

media (A*STAR RSC, Singapore) containing NaOH. Viability of primary keratinocytes in such a high pH environment over an hour was determined. Briefly, 5 mM NaOH was added to primary keratinocytes suspended in PDβ medium and left on ice for t = 0, 5, 15, 30, 45 and 60 min. At the end of each time point, 5 mM acetic acid was added to the NaOH-cell suspension for neutralization. They were then seeded in 24 well-plate (1.8×10^5 cells/well) and more PDβ medium was added to each well before incubating them at 37°C overnight. The next day, cell viability was measured using CellTiter-Blue® Cell Viability Assay with excitation wavelength at 555 nm and emission wavelength at 585 nm.

2.4.3 Viability of primary keratinocytes extracted from collagen

To determine whether encapsulation of primary keratinocytes in collagen would have any effect on the proliferative properties of the cells, the following collagenase protocol was utilized to extract the primary keratinocytes. Briefly, primary keratinocytes (5.3×10^5 cells) were encapsulated in collagen and cultured for 24 h in PDβ medium. After 24 h, PDβ media were removed and replaced with 1 mL of warmed 0.5 mg/mL collagenase (Merck, USA) solution and incubated at 37°C for 45 min. Collagenase solution was removed and collected into a 15 mL tube. 0.25% trypsin-EDTA was used to disassociate cells that were adhered to the plate. The collagenase and trypsin-EDTA solutions were neutralized with 10% FBS and spun down at 200 G for 5 min. Cell pellet were resuspended in 1 ml of PDβ followed by cell counting using Trypan blue solution (Gibco, USA).

2.5. Proliferation of keratinocytes in various media

To determine proliferation of primary keratinocytes in PDβ media over 24 h, primary keratinocytes (5.3×10^5 cells) were seeded in 6-well plate in PDβ. The next day, cells were trypsinized, and cell count was performed using Trypan blue solution.

To compare proliferation of N/TERT keratinocytes and primary keratinocytes in PDα medium, cells (2×10^4 cells/well) were seeded on 24-well plate in PDα medium and cultured for 2 days. Thiazolyl Blue Tetrazolium Blue solution (MTT, Sigma, USA) was added to the culture media to a final concentration of 0.3 mg/mL. Cells were grown in the media for 3 h at 5% CO_2 and 37°C, and then the media were removed. Acidified isopropanol was used to dissolve the formazan crystals and the absorbance was measured at 570 nm wavelength. Bright field images of the cells at the end of incubation period were also taken with Nikon Eclipse Ti-U inverted microscope (Nikon, Japan).

2.6. Reconstituted human epidermis (RHE) using immortalized N/TERT keratinocytes and primary keratinocytes

N/TERT-RHEs were generated using the following established protocols. Briefly, N/TERT keratinocytes were seeded at 3×10^5 cells per polycarbonate cell culture insert (24-well size insert, 0.47 cm^2, Thermo Scientific, USA) in CnT-PR medium. After 48 h, cultures were switched to CnT-PR-3D medium for 24 h, then cultured at air-liquid interface and harvested at various time points for downstream analysis.

For single medium N/TERT-RHEs generation, N/TERT keratinocytes were seeded at 3×10^5 cells per polycarbonate cell culture insert in PDα. The PDα media were refreshed after 24 h, N/TERTs were cultured for a further 48 h submerged, and then cultured at air-liquid interface and harvested at various time points for downstream analysis. For primary keratinocyte-RHE generation with single medium, the protocol remains the same except that primary keratinocytes were used instead of N/TERT keratinocytes.

2.7. Formation of human dermal fibroblast (HDF) dermis

HDFs (4.67×10^5 cells/mL) were mixed with 10 mg/mL of rat tail collagen type I and seeded into 24 mm polyester (PET) membrane inserts (Corning, USA). A silicone disk was used to create an indent in the middle of the dermis layer after gelation at 37°C. The indent serves as a basin to contain and prevent overflow of additional HDF and primary keratinocytes that will be seeded later. The HDF-dermis was cultured in CnT-PR-F medium for 2 days, followed by the addition of a monolayer of HDF (3.91×10^5 cells/mL) seeded into the indent. The dermis was cultured for a further 10 days with alternate days of CnT-PR-F medium changes.

2.8. Human skin equivalent (HSE) generation

Two different types of skin equivalents were generated. HSE refers to primary keratinocytes that were seeded onto HDF-dermis to test the differentiative properties of PDβ medium. Collagen-HSE (c-HSE) was generated by depositing the bioink of primary keratinocytes suspended in bovine collagen onto HDF-dermis.

2.8.1. Formation of human skin equivalent (HSE)

Primary keratinocytes (5.31×10^6 cells/insert) were seeded into the indent of the HDF-dermis and cultured in PDβ medium. PDβ media were refreshed after 24 h, keratinocytes were cultured for a further 48 h and then cultured at air-liquid interface for the next 7 days before fixation and staining. Media were refreshed on alternate days.

2.8.2. Formation of collagen-human skin equivalent (c-HSE)

Primary keratinocytes (5.31×10^6 cells/insert) were first suspended in chilled PDβ media containing NaOH and then mixed with chilled bovine collagen type I, and a small volume of this bioink was then deposited onto the indent of HDF-dermis (Figure 2). The bioink was allowed to gel at 37°C for 30 min, and PDβ medium was added to the inserts. The next day, medium was refreshed, and the inserts were cultured submerged for another 48 h before being cultured at the air-liquid interface for another 7 days before fixation and staining. Media were refreshed on alternate days.

2.9. Hematoxylin and eosin staining

Histological studies were performed on the HSE and c-HSE generated. Samples were fixed in 10% neutral buffered formalin (NBF) for paraffin embedding. Paraffin sections were stained with standard hematoxylin and eosin (H&E) protocol to observe cell morphology. Images were obtained on a Zeiss Axio Imager Microscope (Zeiss, Germany).

2.10. Immunohistochemistry

Immunofluorescence (IF) staining was used to detect keratinocyte proliferation and differentiation. For IF staining of primary keratinocytes, cells were briefly fixed with 4% paraformaldehyde (4% PFA) for 15 min, rinsed with phosphate-buffered solution (1×) (PBS, 1st Base, Singapore), blocked with 10% donkey serum (Sigma, USA) and incubated at 4°C overnight with primary antibodies. Slides were then rinsed and incubated with secondary antibodies and Hoechst (Sigma, USA) for 60 min, rinsed with PBS and mounted with Prolong-Gold Anti-fade reagent (Invitrogen, USA). Confocal images were captured on the Zeiss LSM700 (Zeiss, Germany).

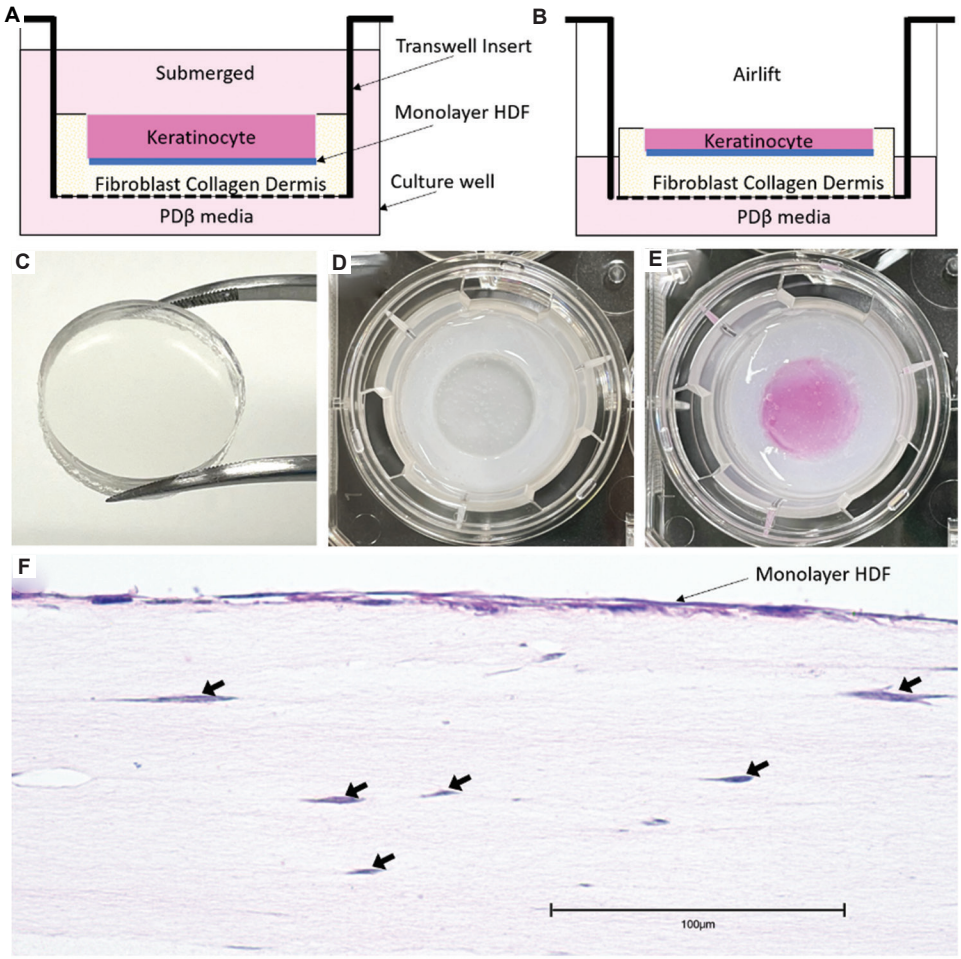

Figure 2. Dermis formation. (A) Schematic diagram showing HSE construct cultured submerged in PDβ media initially, followed by (B) culturing at airlift interface for epidermis maturation. (C) Silicone disk used to create an indent in the collagen. (D) Transwell insert containing the transparent disk and gelled opaque collagen. (E) Media (pink) contained within the indent on the collagen dermis. (F) H&E stained dermis showing monolayer of HDF (dark purple) lining the indent surface and HDF (arrows) within the collagen dermis. Scale bar: 100 μm.

For IF staining of paraffin sections, standard dewaxing and rehydration steps were performed. Antigen retrieval was performed using citrate buffer pH6 (Novus Biologicals, USA) and 2100 Antigen Retriever (ProteoGenix, France), followed by Proteinase K (Sigma, USA) digestion for 15 min at room temperature. Sections were rinsed with PBS and blocked with donkey serum (Sigma, USA), and then incubated with primary antibodies overnight at 4°C. Next, the slides were rinsed in PBS-T (0.05% Tween-20, BioBasic, Singapore) and incubated with secondary antibodies and Hoechst (Sigma, USA) for 60 min, and then rinsed with PBS and mounted with Prolong-Gold Antifade reagent (Invitrogen, USA). Confocal images were captured on the Zeiss LSM700 (Zeiss, Germany).

2.11. Statistical analysis

Statistical analyses were performed using GraphPad Prism version 9.4.1 software (GraphPad Software, USA). Two-tailed unpaired *t*-test (Welch's *t*-test) was conducted. $P < 0.05$ was considered statistically significant. Analysis of data from cell viability of primary keratinocytes in high pH environment required a one-way analysis of variance (ANOVA) test.

3. Results

3.1. Bioink media formulation PDα for immortalized N/TERT keratinocytes

The protocol for making reconstructed human epidermis (RHE) typically requires culturing in two different types of media, namely, a proliferative media (e.g., CnT-PR, K-SFM) followed by a differentiative media (e.g., CnT-PR-3D)[28]. However, media changes are not feasible for cells applied onto patient skin. Hence, we developed the PDα media specifically for N/TERT keratinocytes to form RHE without requiring media switching. We demonstrated that there was no significant difference in the viability of N/TERT keratinocytes cultured in PDα media compared to commercial proliferative CnT-PR media after 2 days, indicating that cell proliferation rate was the same in both medium (Figure 3A). N/TERT keratinocytes were then used to make RHEs to determine the differentiative properties of PDα medium compared to CnT-PR-3D medium. H&E stained sections of N/TERT keratinocytes RHE made using the PDα medium was comparable to CnT-PR-3D medium, with keratinocytes differentiating to form the stratified epidermis layers (Figure 3B). Finally, in Figure 3C, to further characterize the RHEs formed and check for the stemness and differentiative capabilities of PDα medium on N/TERT keratinocytes, IF staining was used to detect protein expression of p63, filaggrin (FLG) and keratin 10 (K10). p63 stains for stem cells, which play important roles in skin renewal, producing new cells that continually push older cells outward toward the uppermost layer, where they are eventually shed. On the other hand, keratinocyte differentiation markers FLG and K10 demonstrate that the epidermis has stratified and matured appropriately. In particular, FLG plays an important role in the barrier function of the skin, which helps prevent water loss and infection.[29,30] The above markers were detected in both PDα and CnT-PR-3D RHEs at day 7 and day 18 post airlift, indicating that RHEs made using the newly formulated PDα media were comparable to the two-step method.

3.2. Bioink media formulation for primary keratinocytes

While we were able to achieve good RHEs with N/TERT, it is well-known that immortalized cells can behave differently from primary cells, which will be needed for clinical applications. However, primary keratinocytes were unable to differentiate and form proper RHEs in PDα media (Figure S1 in Supplementary File). As such, using PDα as a starting point, we developed the PDβ media for primary keratinocytes. Since the formation of RHE is a time-consuming process, we also used morphological cues in 2D culture to accelerate the optimization process (Figure S2 in Supplementary File). First, we established that primary keratinocytes were able to significantly proliferate ($P = 0.0245$) over 24 h when cultured in PDβ media (Figure 4A). Next, when cultured at low density in PDβ media for 24 h, primary keratinocytes expressed stem cell marker p63 (green) and proliferative marker Ki67 (red) (Figure 4B), with very few cells expressing keratinocyte differentiative marker K10 (green, Figure 4C). The results in Figure 4A–C indicate that primary keratinocytes cultured at low density in PDβ media maintained a proliferative phenotype. On the other hand, Ki67 expression decreased while K10 expression increased at high cell density (Figure S3 in Supplementary File), suggesting that the cells can transition to a differentiated phenotype when they have proliferated sufficiently to reach the requisite density. This transition would thus allow us to accomplish the desired cell expansion and differentiation in the *in situ* printed bioink.

In addition to the media component, the bioink also contains collagen dissolved in acetic acid. As the acidic condition is toxic to cells, primary keratinocytes are suspended in chilled PDβ media containing NaOH, which neutralizes the acetic acid upon mixing of these two components, resulting in collagen gelation. We estimate that in the clinical setting, surgeons may take up to an hour to apply the bioprintable epidermis onto the patient's wound. Since the pre-neutralized media (PDβ + NaOH) are not optimal for cells, viability of primary keratinocytes in the high pH environment for up to an hour was checked. Results in Figure 4D showed that there was no significant

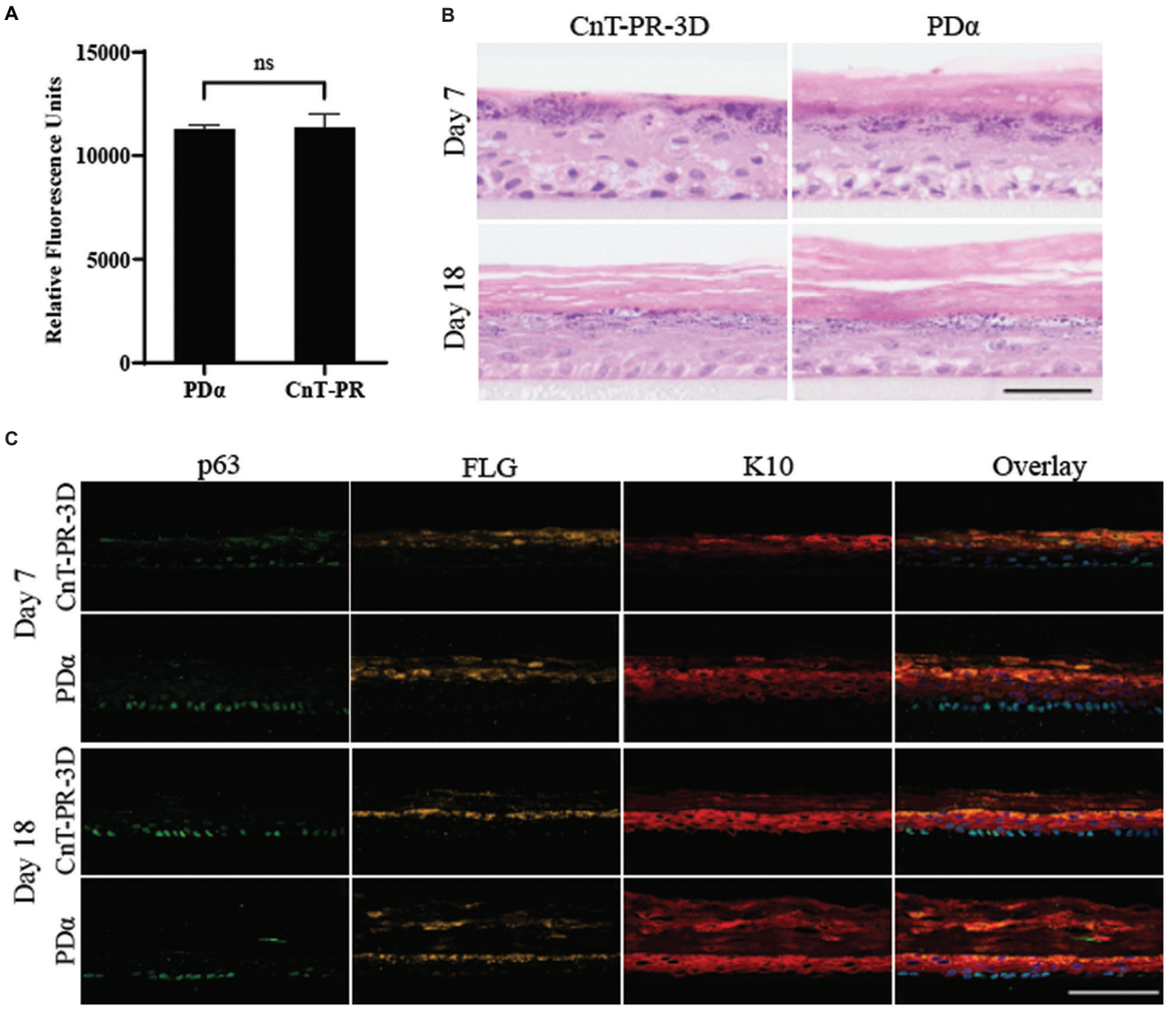

Figure 3. Characteristics of N/TERT keratinocytes cultured in PDα medium. (A) Number of viable N/TERT keratinocytes cultured in PDα was not significantly different (P = 0.8227) to cells cultured in commercial CnT-PR media after 2 days, indicating similar proliferation rates. (B) N/TERT keratinocytes cultured in PDα media were able to differentiate and form a striated epidermis that was comparable to the epidermis formed using CnT-PR-3D media. (C) Immunostaining for stem cell marker p63, keratinocyte differentiation markers filaggrin (FLG) and keratin 10 (K10) were detected in both RHE made from PDα media or CnT-PR-3D media at day 7 and day 18 post airlift. The RHEs made using the one-step PDα media were comparable to the two-step typical CnT-PR/CnT-PR-3D method. Nuclei were stained with Hoechst (Blue). Scale bar: 100 μm.

change in primary keratinocytes viability after an hour. Next, as encapsulation of primary keratinocytes in collagen could potentially affect cell viability, the cells were extracted from the collagen and cell viability determined by trypan blue staining. The results indicated that encapsulation of primary keratinocytes in the bioink did not significantly alter the viability of the cells (Figure 4E).

3.3. Development of HDF-containing dermis

The presence of HDFs has been reported to be important for the formation of a basement membrane[31], which helps anchor the epidermis onto the underlying dermis[31,32]. To simulate a dermal substrate on which to test our bioink, we generated an HDF-containing dermal template. The template contains an indent, into which keratinocytes are seeded in PDβ with or without collagen (Figure 2A and B). The cells were cultured submerged in PDβ medium initially (Figure 2A) before the removal of the media within the insert to form an air-liquid interface (airlift) for epidermis formation (Figure 2B). The template was formed with HDFs mixed with rat tail collagen and seeded into 24 mm polyester (PET) membrane inserts

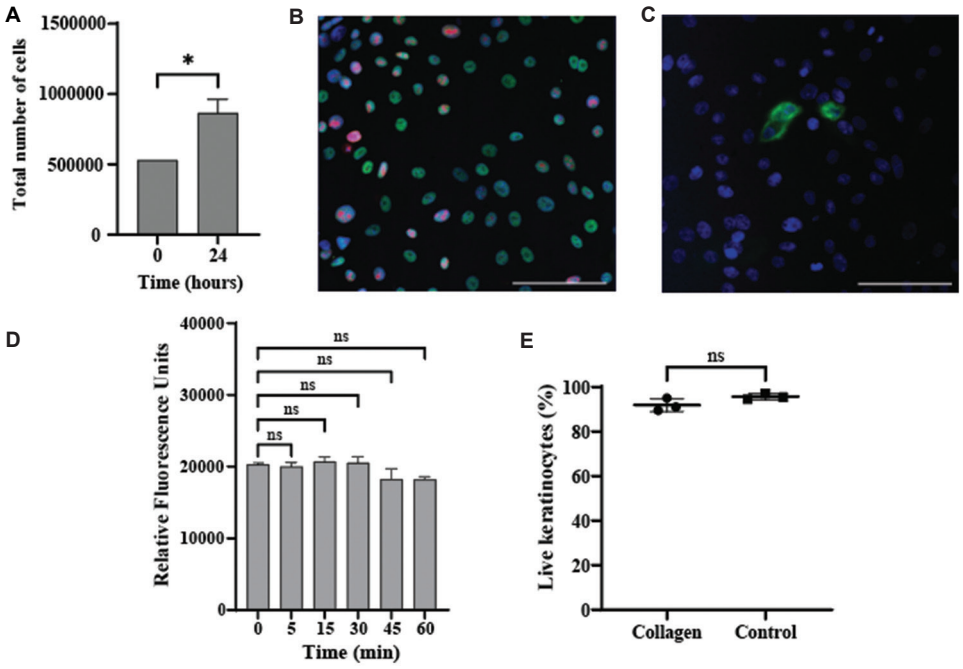

Figure 4. Characterization of primary keratinocytes cultured in PDβ bioink. (A) Primary keratinocytes were able to significantly proliferate ($P = 0.0245$) over 24 h when cultured in PDβ media. (B) Primary keratinocyte cultured in PDβ expressed stem cell marker p63 (green) and proliferative marker Ki67 (red). (C) Low levels of keratinocyte differentiative marker K10 (green) were detected while no FLG (red) (another keratinocyte differentiative marker) was detected. Thus far, the results indicate that primary keratinocytes maintained a proliferative phenotype in PDβ media. Nuclei were stained with Hoechst (Blue). Scale bar: 100 μm. (D) Prior to gelation of the bioink, primary keratinocytes were suspended in chilled PDβ media containing NaOH for up to an hour, giving surgeons sufficient time to apply the keratinocyte bioink onto the patient's wound. No significant change in primary keratinocytes viability was detected after an hour. (E) To determine if encapsulation of primary keratinocytes in bovine collagen would affect cell viability, the cells were extracted from the collagen and cell viability determined by Trypan blue stain. The results indicated that encapsulation of primary keratinocytes in the bioink did not significantly ($P = 0.0996$) alter the viability of the cells.

(Corning, USA). A silicone disk (Figure 2C), added on top of the cell-collagen solution to create an indent in the middle of the dermis layer after gelation at 37°C, serves as a basin to contain and prevent overflow of additional HDF and primary keratinocytes that will be seeded later (Figure 2D and E). The HDF-collagen construct was cultured in CnT-PR-F medium for 2 days, followed by the addition of a monolayer of HDF in the indent. This was cultured for a further 10 days with alternate days of CnT-PR-F medium changes to obtain the final dermal template. Figure 2F shows an H&E image of the dermis formed with HDF within the collagen and a monolayer of HDF covering the surface of the indent.

3.4. Primary keratinocyte HSE and c-HSE using PDβ media

To study the maturation of the keratinocytes when cultured in the PDβ media, the cells were deposited into the indent on the dermal template as described in the previous section. To arrive at our final bioink formulation, bovine collagen was added to the PDβ to create a bioprintable gel that sets quickly after deposition, and thus will not flow away from the site of deposition. Since the collagen bioink formed a gel after deposition, it was also possible to obtain visual feedback about where it was deposited. After maturation, the resulting skin organotypics with and without added collagen are referred to as c-HSE and HSE, respectively.

There were no obvious differences in the stratification of the epidermis in H&E stained sections of HSE and c-HSE (Figure 5A and B). When handling the mature c-HSE, we also noticed that the c-HSE has water-repellent properties, as evidenced when buffer is pipetted onto its surface (Videoclip S1 uploaded separately with description in Supplementary File). To further characterize and compare the neo-epidermis formed in the HSE and c-HSE, immunofluorescence was utilized. In addition to p63 (stem cell), and K10 and FLG (differentiation), we also included Ki67 and keratin 14 (K14, proliferation), and collagen type IV (ColIV) (basement membrane [BM] protein). Figure 5C–F shows the presence of p63 in both HSE and c-HSE at the base of the epidermis, while the differentiation markers are seen in Figure 5O–V. Figure 5G–N shows presence of keratinocyte proliferative markers Ki67[33] and K14[34] in both HSE and c-HSE, which identify actively

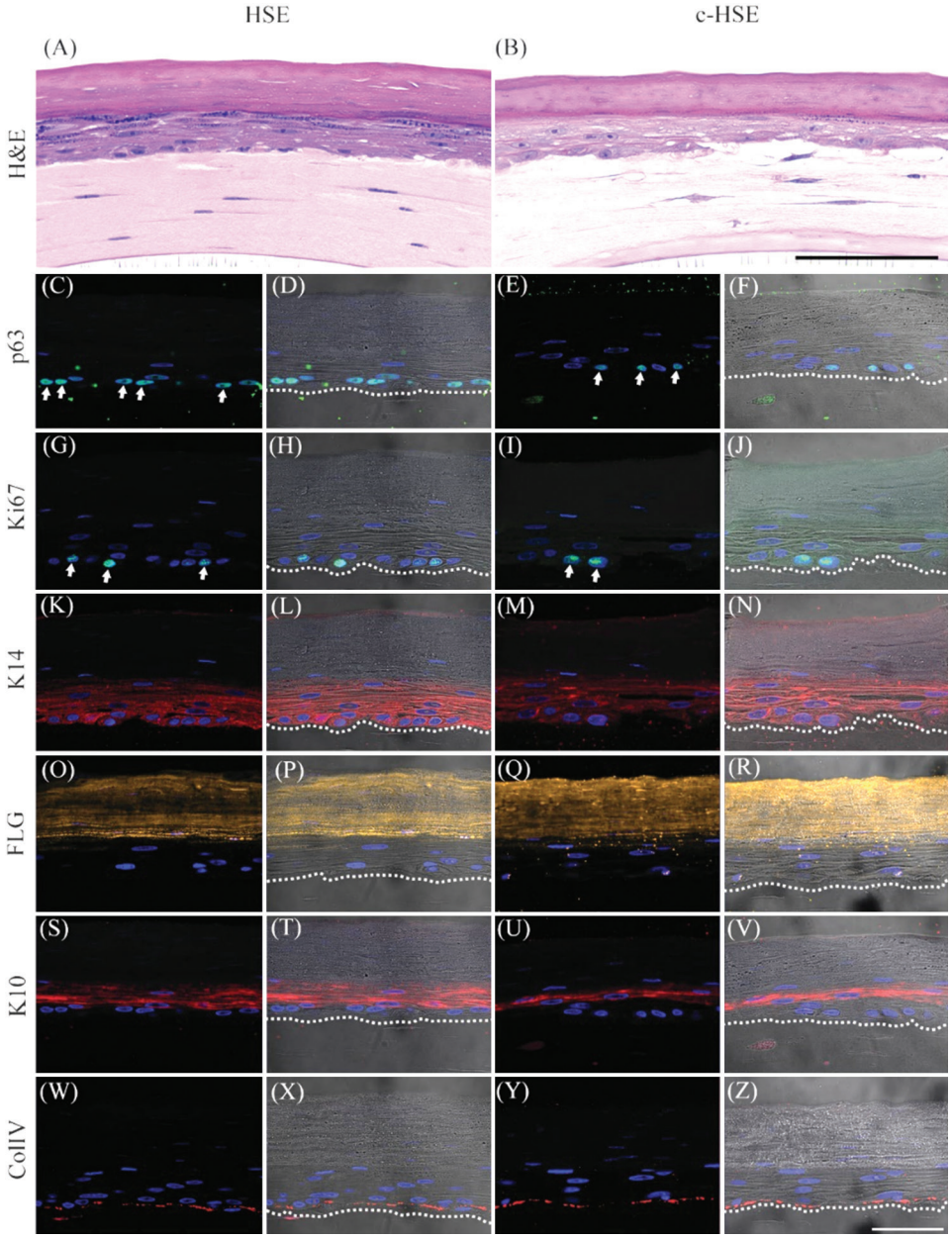

Figure 5. Characterization of the epidermis formed by HSE (PDβ media) and c-HSE (PDβ media with collagen). Stratified epidermis was observed from the H&E stained sections in both HSE (PDβ media) (A) and c-HSE constructs (B), scale bar: 100 μm. HSE and c-HSE constructs were immunostained with the following markers: Stem cell marker p63 (C–F, arrows point to p63-positive nuclei), proliferative markers Ki67 (G–J, arrows point to Ki67-positive nuclei) and keratin 14 (K14) (K–N), keratinocyte differentiation markers filaggrin (FLG) (O–R) and keratin 10 (K10) (S–V), and basement protein collagen type IV (ColIV) (W–Z). Dotted white lines indicate the interface between the epidermis and the dermis. Scale bar: 50 μm.

proliferating cells in the skin. Finally, Figure 5W–Z shows the presence of BM protein ColIV. The BM sits at the interface between the dermis and epidermis, and is crucial in maintaining the mechanical integrity of skin[35]. Its disruption results in fragile skin[6,35]. Because both keratinocytes and fibroblasts contribute to the production of the BM proteins, in the absence of fibroblasts in RHE, we did not observe any ColIV[31]. Overall, the results indicated that the neo-epidermis formed from the primary keratinocytes cultured with or without collagen were similar, and contained all the main features associated with functional skin.

4. Discussion

As the outermost layer of the human body, the epidermis serves an important role in delineating the boundary

between the body and its surroundings. It not only protects us from pathogens and harmful substances, but also prevents fluid loss to the environment. For a burn patient, restoration of this barrier function is often through the use of STSG[2], though in the most severe cases – precisely the patients most at risk – the lack of donor sites can preclude its use[3]. CEA is a relatively well-established alternative, though the absence or delayed development of BM between the epidermis and dermis contributes to its reputation for fragility, and remains a significant problem[6-8]. While "spray-on" skin has shown some encouraging results[8,9,36-38], protocols vary widely[8,38]. Furthermore, some usability considerations such as determining where the cells have been applied can be surprisingly challenging, since the low-viscosity suspension is not easily visible, and can flow away from a contoured application site[10].

In recent years, bioprinting has emerged as a promising approach to restoring tissue function. Compared with other tissues, skin is a lot more accessible, and existing protocols for forming RHEs suggest that self-organization of keratinocytes under the right conditions can yield a functional epidermis[28,39,40]. There are numerous reports that study skin bioprinting[10,15-24]. Most of these studies are concerned with *in vitro* bioprinting[17-24], where there is greater freedom to manipulate the printed constructs, which may or may not be intended for implantation. On the other hand, few recent works have identified *in situ* printing as a possible way to treat wounds, such as burns[10,15,16,26,27] (Incidentally, CEA and "spray-on" skin can be considered to be rudimentary *in vitro* and *in situ* bioprinting, respectively.)

Regardless of the type of bioprinting, the cells are delivered using a bioink, which typically consists of a suitable media or buffer, and may also contain biomaterials that can form hydrogel structures. Unlike the dermis, which has ample extracellular matrix, the epidermis is almost entirely made up of cell bodies. Consequently, epidermis bioprinting is frequently accomplished by depositing cells suspended in media, without any additional biomaterials, directly onto a dermis or dermal template[17-19,22,23]. This process is akin to the formation of RHE described in the previous paragraph, and in most cases, the keratinocytes are cultured submerged in a pro-proliferation media for a period of time, before changing to a pro-differentiation media and exposing the top surface to air, that is, airlifted. This approach is not ideal for *in situ* printing for the following reasons. First, changing of media is difficult once the bioink is printed on the patient. To address this, we developed single media formulations that can support both proliferation and differentiation (Figures 3 and 5). Second, like "spray-on" skin, the absence of any hydrogel material can result in the bioink flowing away from the site of application, which is a challenge we are trying to solve in the first place.

Different biomaterial mixtures have been explored in the literature for epidermis bioink[10,15,16,20,21,25,41]. The formulations typically contain natural polymers like fibrinogen[10,15,16,21] and collagen[16,20] which provide cell adhesion moieties necessary for cell viability. The bioink may additionally contain hyaluronic acid, pectin, gelatin, and glycerol, among others, to improve printability[10,15,20,21,41]. Previously-reported *in situ* epidermis printing utilized fibrinogen-based bioink[10,15,16]. In each report, the final concentration of biomaterial in the bioink is around 2–3% w/v, which, though not too high, may be sufficient to affect the compact organization of the epidermis[10,15,16]. Furthermore, while fibrinogen is a popular biomaterial, heterologous sources of the protein may cause severe immune reaction[41]. For our bioink formulation, we chose to use bovine collagen, because it is very well-established[27,41], and because the first U.S. Food and Drug Administration (FDA)-approved skin substitute also contains bovine collagen[27]. Perhaps because it is highly conserved cross-species, even heterologous collagen causes minimal immunological reactions[41]. To minimize the potential regulatory barrier for clinical deployment, we have also kept the composition as simple as possible, avoiding the use of other biomaterials. Since natural epidermis does not contain large amounts of collagen type I, the collagen concentration of our bioink is kept at a very low concentration of just 0.05% w/v, which was still sufficient to form a hydrogel, but is unlikely to affect epidermis formation.

In general, the previous bioprinting studies had a strong emphasis on the printing techniques. As a result, examination of the printed skin was often limited to simple H&E staining and observation of morphological structures. However, in our experience, care must be taken to analyze the molecular profile of the printed skin, since simple histology can miss out on important features. Markers were therefore chosen to confirm that the printed skin recapitulates the features of the natural tissue, namely skin self-renewal (p63), active growth (Ki67 and K14), barrier function (FLG and K10), and mechanical robustness (BM protein ColIV).

Since the epidermis is a mostly-flat structure, bioprinting can be accomplished by a "2.5D" approach – depositing a layer of bioink with appreciable thickness, and allowing self-organization to accomplish stratification into the different epidermal layers – instead of the more technically-challenging 3D approach[16]. This also simplifies the instrumentation and technique. In this work, our goal was thus to develop a bioink that we can envision being

used in a clinical setting. To that end, in addition to the obvious requirement that the bioink enables the formation of an epidermis, we identified the following key criteria: the formulation should be defined, using no complex mixtures such as serum; it should be compatible with cells before gelation, during the estimated handling time of an hour within the operating theatre (Figure 4D); and the deposition of the bioink should be controlled and visible. We systematically characterized different media formulations based on these criteria, and arrived at the serum-free, defined PDβ + collagen bioink described above, which we have demonstrated to be able to produce epidermis on an artificial dermal substrate. The airlift step, crucial in triggering stratification in typical RHE protocols[28,39,40], is achieved in the resulting epidermis as the collagen gel dries starting from the top toward the dermis. We surmise that this reduction in volume, coupled with cell proliferation, is sufficient to create cell-cell contact that can induce differentiation (Figure S3 in Supplementary File). Based on our experience with modifying the bioink formulation to favor either proliferation or differentiation (Figure S2 in Supplementary File), we believe that we will be able to achieve the goal of a bioprintable epidermis for clinical use. Compared with STSG or CEA, which typically result in visible scars and boundaries, a bioprinted epidermis can be deposited in a contiguous layer, which may yield improved cosmetic outcome. In the meantime, this protocol can also serve as a relatively easy way to obtain donor-specific skin models for testing purposes. To achieve an even more realistic model, work on incorporation of other cell types, especially melanocytes, is ongoing[18,24].

5. Conclusion

We have developed a serum-free, defined bioink formulation that can promote maturation of keratinocytes into a functional epidermis through an elegant one-step deposition process. Based on careful histological and immunofluorescence analysis, we determined that the N/TERT RHE formed with our formulation was largely indistinguishable from established protocols. Furthermore, using the general principles gleaned from the N/TERT experiments, we were able to tweak the formulation to achieve similar results with primary keratinocytes, which will be needed for clinical application. We have systematically identified and addressed the unique challenges that would affect the feasibility of the bioprinting workflow, including the handling time and the single-media requirement. While we will need to deploy this material on various animal models in the near future to verify its utility as an alternative to existing burn treatments, the current protocol can already produce donor-specific models for testing purposes.

Acknowledgments

The authors would like to acknowledge Associate Professor Hao Li, Amy for her contribution of N/TERT keratinocyte cell line.

Funding

This research is supported by Agency for Science, Technology and Research (A*STAR) under its RIE2020 Advanced Manufacturing and Engineering (AME) Programmatic Grant no. A18A8b0059 titled Additive Manufacturing for Biological Materials (AMBM).

Conflict of interest

The authors declare that they have no competing interests.

Author contributions

Conceptualization: Cyrus Weijie Beh
Formal analysis: Ruth Jinfen Chai, Wan Ling Wong
Funding acquisition: Cyrus Weijie Beh
Investigation: Ruth Jinfen Chai, Wan Ling Wong
Methodology: Cyrus Weijie Beh, Ruth Jinfen Chai, Wan Ling Wong
Writing – original draft: Cyrus Weijie Beh, Ruth Jinfen Chai, Wan Ling Wong
Writing – review & editing: Cyrus Weijie Beh

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Availability of data

Not applicable.

Further disclosure

Part of the findings has been presented in the International Conference of Additive Manufacturing for a Better World (AM Conference) 2022 and the conference abstract has been published in Materials Today: Proceedings.

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