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Handheld instrument for wound-conformal delivery of skin precursor sheets improves healing in full-thickness burns

Richard Y. Cheng^{a,*}, Gertraud Eylert^{b,*}, Jean-Michel Gariepy^c, Sijin He^c, Hasan Ahmad^c, Yizhou Gao^c, Stefania Priore^d, Navid Hakimi^c, Marc G. Jeschke^{b,d,e,¶}, Axel Günther^{a,c,¶}

^aInstitute of Biomaterials and Biomedical Engineering, University of Toronto 164 College Street, Toronto, Ontario M5S 3G9, Canada

^bInstitute of Medical Science, University of Toronto 1 King's College Circle, Room 2374, Toronto, Ontario M5S 1A8, Canada

^cDepartment of Mechanical and Industrial Engineering, University of Toronto 5 King's College Road, Toronto, Ontario M5S3G8, Canada

^dRoss Tilley Burn Centre, Sunnybrook Health Sciences Centre and Sunnybrook Research Institute, 2075 Bayview Ave, Room D704, Toronto, Ontario M4N 3M5, Canada

^eDepartment of Surgery, Department of Immunology, Division of Plastic Surgery and General Surgery, University of Toronto, 149 College Street, Toronto, Ontario M5T 1P5, Canada

Abstract

The current standard of care for patients with severe large-area burns consists of autologous skin grafting or acellular dermal substitutes. While emerging options to accelerate wound healing involve treatment with allogeneic or autologous cells, delivering cells to clinically relevant wound topologies, orientations, and sizes remains a challenge. Here, we report the one-step *in-situ* formation of cell-containing biomaterial sheets using a handheld instrument that accommodates the topography of the wound. In an approach that maintained cell viability and proliferation, we demonstrated conformal delivery to surfaces that were inclined up to 45 degrees with respect to the horizontal. In porcine pre-clinical models of full-thickness burn, we delivered mesenchymal stem/stromal cell-containing fibrin sheets directly to the wound bed, improving re-epithelialization, dermal cell repopulation, and neovascularization, indicating that this device could be introduced in a clinical setting improving dermal and epidermal regeneration.

Keywords

bioprinting; skin substitutes; regenerative medicine; microfluidics; 3D printing

Full thickness burns are categorized by the destruction of both the epidermal and dermal layers of the skin and often cover a significant portion of the total body surface area (TBSA) of a patient[1,2]. Lack of rapid and sufficient wound healing can lead to serious

¶ Corresponding authors. Dr. Marc G. Jeschke: marc.jeschke@sunnybrook.ca, Dr. Axel Günther: axel.guenther@utoronto.ca.

* Authors contributed equally to this work.

complications such as dehydration, infection, and shock, resulting in a high rate of patient mortality[3–6]. The current standard of care involves removal of damaged tissue from the wound, followed by application of an autologous skin graft with tissues obtained using a dermatome directly from the patient's own skin[7]. However, large area burns often leave insufficient quantities of healthy skin for harvesting, precluding this application in cases of severe injury. Acellular biodegradable scaffolds based on bovine collagen such as Integra® are alternatively used[8], where healing relies on cellular ingrowth from tissues surrounding the wound. Additional split thickness grafts are often necessary and imply the creation of a new wound to further increase the risk of delayed healing and pain[6,9–11].

Different cellular approaches that rely on either patient-derived autologous[12] or donor-derived allogeneic cells[13] have been proposed as alternative treatment methods to provide sustainable signaling for active recruitment of host cells and contribute directly to wound healing by continual secretion and remodeling of extracellular matrix proteins[14–16]. One such option is the formation of cell-based tissue constructs off-site by using long-term tissue culture techniques where cell sheets are generated *in vitro*[17]. While this approach provides mechanically handleable and transplantable tissue constructs from patient-derived cells, these sheets require 4.5 to 8 weeks of cell culture to generate relevant quantities necessary for burn treatment[18]. Pre-seeding acellular scaffolds with cultured cells prepared *in vitro* have also been explored as potential treatment options[19], but approaches for the efficient and homogeneous distribution of cells in the 3D matrix prior to the application onto the wound have yet to be developed. More recently, strategies have been demonstrated for the direct delivery of cells to a wound that include spraying of cells[20–22] and injection of cell-containing microgel solutions[23]. However, available approaches do not yet allow for a uniform distribution of cells and biomaterials across large, severe wounds with physiological topographies and orientations. Bioprinting approaches overcome some of the above-mentioned limitations by patterning bioinks, i.e., cell-containing biopolymer solutions, to create tissues mimicking the native architecture. Although bioprinting approaches provide flexibility in terms of cell and material use, structure, and organization of the printed tissues[24–28], they are generally designed for *in vitro* use with physical dimensions of the printer greatly exceeding the ones of the printed tissue. To accurately account for the topography of large-area wounds, additional steps such as laser-optical or ultrasonic scans need to precede the bioprinting process, and increase the required time, cost, and resources[29]. If prepared externally, bioprinted structures need to withstand the manual transfer from the printing platform to the wound bed, thus limiting material composition. The ideal *in-situ* delivery approach is compatible with different cell types and biomaterials and allows cell-laden biomaterial layers to be applied conformally to a wound bed of varying shapes. The delivered cells then secrete their own extracellular matrix, recruit host cells, and contribute significantly to overall wound healing[30]. Further, this direct delivery approach enables deposition on large and inclined surfaces, as opposed to modestly inclined wound surfaces as our group [31] and others [32] have recently shown.

Potential candidates for cell delivery include mesenchymal stromal cells (MSCs) with the multipotent capability to differentiate into cells of the mesenchymal lineage[33–35] therefore promoting wound repair and regeneration[36,37]. We selected MSCs primarily due to beneficial paracrine signaling and trophic effects[16,38,39] which promote migration of

endogenous cells towards the wound site[40]. MSCs were shown to support the growth and differentiation of local stem and progenitor cells, promote angiogenesis, reduce fibrosis, limit apoptosis induced by oxidative stress, and attenuate the immune response[41] which are all relevant in the context of skin regeneration in a burn wound model. We chose a fibrinogen-based bioink due in part to fibrinogen's role in recruiting host cells during the wound healing cascade[42,43] as well as the widespread clinical use of fibrin-based biomaterials. Furthermore, fibrin provided a transient scaffolding material with a safe degradation profile which cells readily attach to and remodel prior to secreting their own extracellular matrix (ECM)[44,45]. The inherent adhesion of fibrin-based skin precursor sheets to the wound bed and the rapid enzymatic cross-linking reaction meant that sheet formation was affected by the fibrinogen and thrombin concentrations as well as diffusive transport[46,47]. We selected a porcine full-thickness burn model to demonstrate a potential application of the handheld instrument due to its close resemblance to human skin [48].

RESULTS

Handheld instrument design and approach

We designed a handheld instrument specifically for *in-situ* deposition of skin precursor sheets in clinically relevant settings, directly onto wound surfaces of arbitrary size, shape and topography. The fibrin-based bioink is permissive for cell viability and proliferation and is cross-linked with a thrombin solution. The compact (20cm × 11cm × 15cm) and light (1.4kg) instrument is operated with one hand (Fig. 1a). Figure 1b shows a rendered image of the instrument during *in-situ* formation of skin precursor sheets (see also Supplementary Movies 1–3). The operator holds the instrument on the handle such that the soft wheel to contacts the wound bed. Upon engaging the toggle switch, the wheel rotates at velocity V , guiding the deposition process. Simultaneously, the bioink and cross-linker stored on-board in separate syringes are co-delivered at the respective flow rates Q_B and Q_C . The solutions then pass through flexible tubing to separate inlets of the microfluidic printhead that trails the wheel. Within the printhead, they are distributed through a bifurcated channel network towards a parallel array of microchannels at the exit that deposit a uniform thickness skin precursor sheet conformal to the wound bed, covered with the cross-linker. Because the widths of the printhead and deposited sheet exceed the width of the wheel, arbitrarily wide wounds can be covered by successive side-by-side sheet deposition. The deposited sheets are visually distinguishable from the wound immediately after leaving the printhead, allowing the user to adjust the printhead position to ensure neighbouring layers to be deposited without gap. Optionally, the temperature of the delivered bioink can be controlled by recirculation of a heat transfer fluid through the instrument as indicated in Fig 1b. In this work, the bioink remained at room temperature.

Deposition on physiological topographies

The components of the handheld instrument can be separated into two categories (Fig. 2a). The first set of components are single-use: the biomaterial containing syringes, the microfluidic printhead, and the silicone wheel. The second set of components are designed for repeated use and can be readily disassembled, sterilized and re-assembled: the printhead bracket with two-axis gimbal design and torsional spring (Supplementary Fig. 3) in addition

to the handheld instrument body containing the delivery mechanisms for the bioink and the cross-linker, the drive mechanism for the wheel, and the handle with switch. The microfluidic printhead is attached to a metal bracket via a snap-fit joint, allowing printheads with different widths to be selected (Supplementary Fig. 1). While the microfluidic printhead described here is designed for deposition on large area surfaces such as the posterior trunk, a modified printhead of reduced width may be used to address harder-to-reach areas or such with small curvature radii. The soft Shore OO-20 silicone wheel functions to establish controlled contact between the printhead and the wound bed, and to dissipate the forces imparted by the weight of the instrument and user. While the force applied from the handle affects the contact pressure of the wheel, the contact pressure of the printhead is operator independent and only affected by its weight and the spring force. The guiding wheel is fabricated using a silicone material to increase friction with the deposition surface. Using this system, we found that the total pressure on the substrate was less than 12kPa (Fig. 2b, Supplementary Fig. 2).

The metal bracket attached to the microfluidic printhead is connected to the instrument via a passive two-axis gimbal which enables rotation about two principle axes (Supplementary Fig. 3). The printhead is free to adjust the pitch angle, α , by rotating about the y -axis and thereby compensate for inclination angles of up to 45° (Fig. 2c, left side). The roll angle, ϕ , denotes rotations about the x -axis, and may vary between the neutral position and $\pm 25^\circ$ (Fig. 2c, right side). To initiate the deposition process, the operator engages the switch at the handle (Supplementary Fig. 4). The bioink (bottom) and cross-linker (top) solutions are consistently deposited at the exit of then printhead onto the wound bed (Fig. 2d). The deposited layer has the total height of h_T . Diffusive contact between the bioink and cross-linker initiates the enzymatic gelation of fibrin. Here, the two rotational degrees of freedom ensure consistent contact between the printhead and the wound bed and correct for operator-induced positioning changes. Without consistent contact, a portion of the printhead may locally fail to contact the wound bed and disrupt the liquid bridge, leading to inconsistent sheet formation. Even a small gap between the microfluidic printhead and the wound surface exceeding the capillary length of 2.26mm would disrupt sheet formation.

Consistency of deposition was assessed at different total flow rates, $Q = Q_B + Q_C$, and a constant nominal wheel speeds, $V_0 = 3\text{mm/s}$, and printhead width $w = 25\text{mm}$ (Fig. 2e, Supplementary Fig. 5). At high enough flow rates ($Q = 15\text{ }\mu\text{l/s}$ and $50\text{ }\mu\text{l/s}$), uniform sheets with a width comparable to the width of the printhead exit are obtained. At low flow rates ($Q = 0.1\text{ }\mu\text{l/s}$), the gap between the microfluidic printhead and the substrate failed to completely along the entire lateral direction, leading to an area loss of 45.8% (Fig. 2f, top). In addition, operator induced compression of the soft wheel impacted the actual wheel speed V , compared with the nominal one, V_0 (Fig. 2f, bottom). We found that the absolute difference between the two to be approximately the same, 0.57mm, regardless of wheel speed. The standard deviation between V and V_0 however decreased from 45% to 8% as wheel speeds were increased from 1mm/s to 5mm/s. In Fig. 2g, we report results from 30 operating conditions, with flow rates ranging from 0.1 to $50\text{ }\mu\text{l/s}$ and wheel speeds from 2 to 10mm/s. While the sheet thickness could be modulated by changing the flow rate and/or the wheel speed, very low wheel speeds ($< 2\text{mm/s}$) and flow rates ($< 0.1\text{ }\mu\text{l/s}$) lead to inconsistencies in sheet formation. Furthermore, given the predictability of our system, in Fig. 2h we describe

the anticipated time, biomaterial volume, and cells required to cover a large area for treatment of full thickness burn. For an average burn area of the human posterior trunk at 2,000cm² or about 10% TBSA, we estimate a required 13.3ml of bioink containing 1.33×10^7 cells, using the presented instrument and a deposition time of 67 min, excluding short breaks for syringe exchange. To provide evidence that the projected area coverage, biomaterial, and cells used as a function of time was accurate, we analyzed the time required to cover a wound size of 5cm $\times$ 5cm *in vivo* with 9 full-thickness burn wounds and found that for each wound, it took an average of 0.89 min, with 167 μ l of biomaterials with a total of 1.67×10^5 cells used.

Biomaterial maintains uniform coverage across non-flat surfaces

In general, clinically relevant wound surfaces are not flat, nor are they oriented horizontally. One of the most important advantages of the presented approach is that it allows for the conformal deposition of a bioink layer onto inclined surfaces (angle θ). Until the delivered layer gels, its thickness h is expected to reduce. To ensure consistent wound healing for clinically relevant inclination angles of 0–45° we require h to deviate by no more than 20%. Fig. 3a schematically shows a bioink layer right after it was deposited in the x -direction that is perpendicular to the drawing. As indicated, the thickness of the deposited layer reduces by h and due to drainage after deposition by a distance y along the inclined surface. Addition of hyaluronic acid (HA) to the fibrin-based bioink led to shear thinning behaviour with decreasing viscosity observed as the shear rate increased for all concentrations, especially relevant for bioink extrusion through a microfluidic printhead. HA addition also improved printability on inclined surfaces by increasing the viscosity and reducing drainage. The addition of 0.5% HA to the fibrin-based bioink resulted in a dynamic viscosity of 0.06 Pa's which could be increased to 1.2 Pa's by adding 1% HA or up to 3.5 Pa's by adding 2% HA (Fig. 3b, Supplementary Fig. 6a, b). By measuring the required time to reach 95% of maximum turbidity via confocal microscopy as an indicator of complete gelation, we found that adding 0.5% HA to the bioink resulted in a gelation time of 52 s, while further addition of HA significantly increased the total gelation time from 4.2 min after 1% HA addition and 23 min after 2% HA addition (Fig. 3c, Supplementary Fig. 6d). We selected a final concentration of 1% HA to incorporate into the bioink as it had the benefits of increasing viscosity (1.2 Pa's at 1/s shear rate) to improve printability on inclined surfaces balanced with an acceptable gelation time (4.2 min) in the clinical setting.

To determine the behaviour of the biomaterial sheet after deposition on an inclined surface, we tracked the biomaterial sheet immediately after extrusion via the handheld instrument at various tilted angles. Using a setup that could change the substrate angle (Supplementary Fig. 7a), biomaterial sheets were deposited using the handheld instrument and tracked via fluorescent time-lapse microscopy illuminating the FITC-labeled 1 μ m microparticles embedded within. Compared to biomaterials deposited at $\theta = 0^\circ$ where no drainage was observed, sheets deposited on 15-degree surfaces had a maximum drainage speed of 0.044 mm/s and sheets deposited on a 45° surface had a higher maximum drainage speed of 0.066 mm/s. We observed that over a tracked period of 240 s, the drainage speeds of deposited materials at both angles were reduced to zero (Fig. 3d, Supplementary Fig. 7b). While the 15° condition had a reduced magnitude of biomaterial drainage speed compared to the 45-

degree tilts condition, the rate of drainage was similar. The time required for the biomaterial to be static, here defined as when the drainage speed was less than 5% of the maximum speed, was 169 s and 194 s for deposition on 15° and 45°, whereas the percentage change in travel distance relative to the total width of the sheet was 10.5% and 19.4% for 15° and 45°. As Fig. 3e shows, the percent loss in thickness at 15° and 45° compared to a 0° deposition was 10.4% and 17.3%, respectively, with total loss of 9.4µm and 15.6µm (Supplementary Fig. 7c–d).

To determine whether the deposited bioink layer was amenable for cell viability and proliferation, we cultured them *in vitro* over 7 days. At day 0, MSCs delivered within the biomaterial sheets had rounded shapes with cytoskeletal distribution immediately surrounding the nucleus as indicated by the phalloidin and Hoechst stain. After one day of culture, the MSCs began to elongate, with the cytoskeleton extending beyond the nucleus to interact with the surrounding extracellular matrix. This continued onto day 3, where the quantity of cells increased with MSCs elongating further. The 3D distribution of cells was maintained, and by day 7 the entire surveyed area of the biomaterial sheet was populated with MSCs (Fig. 3f). We show that the cultured cells in the 3D matrix maintained over 94% viability across a 7-day culture period (Supplementary Fig. 8), with consistently increasing cell numbers. Similarly, the cell-cell distance was reduced to less than 30µm after seven days of culture from an original value of 61µm, reflecting the rapid increase of cell number within a constrained physical space. Significant elongation of the MSCs was observed, where rounded cells with an initial aspect ratio (cell width/length) of 0.9 quickly adopted a 3D morphology with increased aspect ratios of 3.5 after seven days of culture in the porous fibrin matrix (Fig. 3g, Supplementary Fig. 9).

***In-situ* delivered cell-containing biomaterials contribute to improved wound healing**

To demonstrate the clinical relevance of the *in-situ* delivery of skin precursor sheets, we treated large full-thickness burn wounds on four porcine models with MSC-containing fibrin-HA bioink compared to controls of acellular materials and burn alone with a reference to healthy skin (Supplementary Fig. 10–13 and Supplementary Tables 1–3), as well as degradable scaffolds as a proof of concept (Supplementary Fig. 14). During treatment in the operating room we measured the deposition angles of realistic physiological surfaces and found that they ranged from $\theta = 0^\circ$ (horizontal) to 45°, with most commonly found curvatures of 15°. A side profile view of the handheld instrument during the deposition process showed that only the microfluidic printhead and the silicone wheel was in direct contact with the wound substrate (Fig. 4a, left). The rotational degree of flexibility was also captured in action, where the position of the microfluidic printhead was held regardless of substrate topography such that the width of the device is continuously in contact with the wound during the entire treatment (Fig. 4a, right). As shown in the photographs immediately before and after cell and biomaterial delivery using the handheld instrument, the wounds were uniformly covered with the deposited material (Fig. 4b). After 28 days of healing, a macroscopic visual inspection of the burn wound control and the acellular materials condition indicated significant inflammation in both the wound area and surrounding regions with higher score of scarring and contracture. In contrast, the MSC-treated wounds showed a superior healing profile with a reduction in inflammation, scarring, and contraction (Fig. 4c).

Histological assessment of the same wounds revealed that the burn and acellular cases resulted in either a failure to regenerate the epidermis or the generation of partial, thin layers. These control conditions showed regions with poor cell repopulation in addition to localized areas with hyperplasia or hypoplasia. Conversely, the MSC-fibrin treated experimental conditions exhibited epidermal cell repopulation in normal ranges post-treatment (Fig. 4d), as well as a physiologically thick epidermis and a characteristic rete ridge formation indicative of normal, healthy skin. Quantitative assessments of the macroscopic wounds confirmed our observations, where we found that MSC co-delivery with fibrin-HA using the handheld instrument led to an increase in epithelialization speed, a reduction in scarring, as well as a reduction in contracture formation per wound area compared to burn only controls (Fig. 4e). Furthermore, histological sections obtained from the wound biopsies after 28 days of cell and biomaterial treatment showed a superior restoration of overall epidermal thickness and dermal collagen density comparable to healthy skin, whereas the acellular treatment group showed a delay in restoration with lower collagen formation while the burn condition failed to heal. An elevated number of CD31+ expressing endothelial cells of vessels were also found in the MSC treatment conditions, which were confirmed by the α -SMA+ assessment seen by mature counted vessels (Fig. 4f). Additionally, evaluation of cells positively expressing Type-2 macrophage marker CD163 and pan-inflammatory cell surface marker CD11b in the remodeling phase on day 28 of the wound healing process revealed lower expression levels compared to burn only controls, suggesting an attenuated immune response after cell delivery. Importantly, these histological results also showed a reduced amount of α -SMA expressing myofibroblasts in the cell-treated group compared the experimental controls, providing evidence of significant reductions in pro-fibrotic effects (Fig. 4g).

CONCLUSIONS

The use of an artificial scaffold to treat large area full-thickness burns when autologous skin is not sufficiently available has been the standard of care for over 40 years[49–51], but this approach is fundamentally limited by biomaterial composition and relies solely on the contribution of host cells to repair wounds. Direct cell delivery is an emerging option to provide a continuous source of cell signaling and matrix remodeling, but reliable delivery of engineered constructs on large physiological areas remains a significant challenge. Here, we introduce a handheld instrument which uses a microfluidic printhead to uniformly deposit skin precursor sheets comprised of cells and biomaterials directly and conformally on physiological substrates in a single step. The modular design of the handheld instrument grants the operator full control over the physical dimensions and material composition of the resulting skin precursor sheet. Shear-thinning fibrinogen-HA bioink provides printability on angled surfaces while maintaining high cell viability and increased proliferation. Furthermore, *in vivo* studies with porcine full-thickness burn models prove that the handheld instrument can safely and reliably deliver MSC-containing fibrin-based skin precursor sheets onto a burn substrate to improve wound healing outcomes. Taken together, this user-friendly and automated technology enables the next generation of safe cell and biomaterial delivery, with potential clinical applications extending beyond full-thickness burn injuries.

METHODS

Microfluidic printhead design and fabrication

Printheads were designed using software programs SolidWorks, AutoCAD, and Autodesk Inventor and additively manufactured using a 3D printer (model Projet 3500 HDMax, 3D Systems, USA) and resin Visijet M3 Crystal. 3D printing and post-processing processes were optimized to minimize the difference between expected microfluidic channel dimensions and measured channel dimensions (Supplementary Fig. 1). Representative fluidic channel structures were assessed under brightfield microscopy (model Axio Observer, Zeiss, Germany). Channel sizes were evaluated from bright-field images using ImageJ software.

Wheel characterization

Silicone wheels were designed using the SolidWorks software program and moulded in silicone (Smooth-On Ecoflex series) with shore values of OO-10, OO-20, and OO-30 (Supplementary Fig. 2). The compliant wheel of the handheld instrument was placed in contact with a stiff (PMMA plastic) or soft (Shore OO-20) substrate with only the instrument's weight and no additional forces contributing to the contact pressure and area. Bottom-up photographs allowed the extent of wheel compression to be determined based on the total contact area. Coloured dyes were added to the platform to enhance image contrast between the wheel contact area and the substrate. Image analysis of the wheel's contact area was performed using the ImageJ software. The contact pressure resulted from dividing the printer weight by the total contact area.

Biomaterial deposition characterization

Time-lapse videos and representative images of biomaterial deposition with a top-down view recorded from a charge-coupled device camera (model EXi Aqua, QImaging, Canada). Measured wheel speed across a constant length was determined by the distance the wheel traveled per second, tracked using the MTrack2 plugin in the ImageJ software. Images of the resulting deposited biomaterial sheets were assessed using ImageJ software to determine total and percent area coverage at various biomaterial flow rate and wheel speed conditions. The biomaterial flow rate versus wheel speed optimization graph was extrapolated from 8 data points characterizing different operating conditions repeated in triplicates to determine regions of uniform coverage, contraction, and non-uniform coverage.

Deposition time, biomaterial volume, and cells required for area coverage

Analytical model was generated assuming constant thickness of the combined material thickness h_T of 0.2mm and a cell concentration of 1×10^6 MSCs/ml in the bioink layer, varying wheel speed and width of the microfluidic printhead. The total covered area was determined by multiplying the speed of wheel translation, interval duration, and the width of the microfluidic printhead. The time required for syringe changes was neglected. Experimentally obtained data was generated from recorded time, biomaterials used, and cells used for 9 independent wounds during *in vivo* porcine wound depositions.

Material preparation

Extruded biomaterials were consisted of fibrinogen from bovine plasma (Sigma-Aldrich, Canada), thrombin from bovine plasma (Sigma-Aldrich, Canada), and sodium hyaluronate pharma grade 80 (Kikkoman, Japan). Fibrinogen was prepared at 20mg/ml, thrombin at 500 IU/ml, and hyaluronic acid ranging from 0.5% to 2% w/v. 2× concentration stock solutions of fibrin or thrombin were dissolved in warm saline solutions and combined with 2× concentration hyaluronic acid at a 1:1 ratio to reach the targeted viscosity.

Scanning electron microscope

Fibrin-HA biomaterial sheets were imaged using a Philips XL30 Scanning Electron Microscope (SEM) at 20kV at secondary electron mode at 25,000× magnification. Samples were fixed with 4% paraformaldehyde/1% glutaraldehyde in 0.1 M phosphate buffer pH 7.2, then washed with PBS and ddH₂O followed by alcohol dehydration. The sample was then critical point dried and coated with 15nm gold prior to imaging.

Rheology

A sample volume of 1ml fibrinogen mechanically mixed with 0.5%, 1%, or 2% HA was loaded onto the platform of a 40mm steel Peltier parallel plate rheometer with a gap of 800µm. The oscillatory shear rheological properties (storage modulus G' and loss modulus G'') during gelation was measured at 37°C with applied strain and frequency of 0.1% and 1.0 Hz, respectively, for a maximum duration of 90 minutes. Viscosity of non-gelling components was measured in Pa·s for shear rates of 1–200s⁻¹.

Turbidity measurement

Fibrinogen-based bio-inks containing 0.5%, 1%, and 2% HA cross-linked with Thrombin-HA was deposited with the handheld instrument onto a 1" × 3" glass slide placed on the stage of a confocal microscope (model A1, Nikon, Japan) with active temperature and humidity control to avoid evaporation. Time-lapse images were acquired every 1 s, for a duration of 40 min. The mean gray value of each image was plotted to determine the change in turbidity and indicate the time of gelation of the deposited biomaterial sheet (<5% change in turbidity). Control experiments consisted of fibrin-HA co-deposited with HA components only without the thrombin cross-linker component.

Material tracking

Fibrinogen solution contained 1µm FITC microparticles at 0.1% v/v (Thermo Fisher Scientific, Canada) as well as 0.5%, 1%, and 2% HA. The bioink was cross-linked with a Thrombin-HA solution was deposited with the handheld instrument onto a 1" × 3" glass slide placed on a tilted surface ranging from 0, 15, to 45 degrees. Image sequences were captured using a charge coupled device camera (model EXi Aqua, Teledyne QImaging, Canada). An optical bench that provided rotational freedom long the axis of printing was used, with the camera mounted orthogonally to the deposition surface to produce a top-down image independent of the surface inclination angle. An image exposure time of 6.1ms was selected to produce an appropriate SNR and the images were captured in a darkened room. The samples were illuminated at a low angle with a 495nm blue LED to minimize substrate

reflection. Intervals of 0.41s were chosen to estimate speed change over a maximum duration of 5min. A Macro-zoom lens with close-up filter (Navitar 7000, USA) was fitted to the camera and produced a resolution of 15 pixels/mm. A Yellow-Green cut-on filter was mounted in the optical path to filter stray blue light, autofluorescence and reflection. The speed of microparticle movement due to material drainage down an inclined slope was determined by the change in position over time. The time required for no biomaterial movement due to gelation was defined as t_s , where the tracked microparticle speed was <5% than maximum speed. The movement of the biomaterial sheet down the inclined surface was defined as y . Due to printhead travelling through the video, the data sets were captured from after the printer had left the capture frame. This creates a delay in the initial data point which corresponds to approximately 15s after t_0 . To accurately determine the t_s , or the point at which the particles are moving at <5% of initial velocity, an extrapolation using an exponential function was used to calculate the estimated speed at time t_0 . Integrating the exponential curve fit ($R^2=0.91$) with respect to time yields the estimated entire distance travelled by the particle and the average movement of the sheet.

Material thickness via confocal

Fibrinogen biomaterials deposited on a 1" × 3" glass slide located on a tilted surface with defined angles 0, 15, and 45 degrees were collected after complete gelation and imaged with a confocal microscope. Z-stack images with intervals of 1μm were obtained, then the midpoint of the sheet was visualized in 3D using ImageJ software to quantify the resulting thickness with 0-degree deposition as a point of comparison due to no drainage.

Cell culture

Mesenchymal stromal cells were extracted from the umbilical cord tissue received from the Obstetrical and Gynaecology Department at Sunnybrook Hospital. The donated umbilical cord was maximally stored for 24 hr at 4°C, prior to processing. Cells were extracted from the extracellular matrix from the Wharton's Jelly of the umbilical cord, and further cultured in Dulbecco's Modified eagle medium (Gibco™ DMEM, Thermo Fischer Scientific, Canada) enriched with 1% antibiotic-antimycotic solution (Gibco™ Antibiotic-Antimycotic, Thermo Fischer Scientific, Canada), 1% L-Glutamine (Sigma Aldrich), and 10% fetal bovine serum (FBS) (Gibco™ fetal bovine serum, Life Technologies Corporation, USA).

Flow cytometry

Flow cytometry cell sorting was performed after initial cell extraction and expansion for MSCs expressing markers (negative markers: CD34, CD45, CD11b, CD19, and HLA-DR, and positive markers: CD73, CD90 and CD105) by using a BD LSR II Flow Cytometer. Live cells were selected and gated with the negative markers CD34-/CD11b-/CD45- (FITC) (Invitrogen), CD19-/HLA-DR- (AF700, PE-Cy7) (eBioscience), and positive markers were gated for CD73+ (PE) (eBioscience), CD90+ (BV510) (eBioscience) and CD105+ (APC) (eBioscience) using a commercial software program (FACSDIVA™, BD Biosciences, Canada). Cells were trypsinized, washed and resuspended in flow buffer consisting of Hank's Balanced Salt Solution (HBSS; Wisent, Canada) and 1% bovine serum albumin (Wisent, Canada). Cells were then incubated with the conjugated antibodies (ratio 1:100) for 30min on ice in the dark, with additional washing with flow buffer, prior to

performing flow cytometry using with the laser channels mentioned above. Graphical and statistical analysis was completed in FlowJo™ software.

MSC differentiation assay

For assessment of adipogenic differentiation potential, MSCs were cultured in a low glucose DMEM supplemented with FBS, Ab/Am and 3-isobutyl-1-methylxanthine (Sigma-Aldrich, Canada), insulin (SAFC Bio-sciences, USA), indomethacin (Sigma-Aldrich, Canada), and dexamethasone (Sigma-Aldrich, Canada). After two weeks of culture, Oil Red O staining was performed to confirm adipogenic differentiation. The media was removed, and wells were rinsed with PBS. Cells were then fixed in 10% formalin for 30 min, rinsed with distilled water and stained with Oil Red O for 5 min (Sigma-Aldrich, Canada). Following multiple rinses with water, cells were stained with hematoxylin (Sigma-Aldrich, Canada). Intracytoplasmic lipid droplets appear in red and nuclei in dark blue. For assessment of chondrogenic differentiation potential, cells were cultured in a low glucose DMEM supplemented with FBS, Ab/Am, and L-ascorbic acid-2-phosphate (Sigma-Aldrich, Canada), Insulin-Transferrin-Selenium (Corning™ cellgro™ Insulin-Transferrin-Selenium, Corning Incorporated, USA), Dexamethasone (Sigma-Aldrich, Canada), sodium pyruvate (Sigma Aldrich, Canada), TGF- β 1. After 42 days of culture, Alcian Blue staining was performed to confirm chondrogenic differentiation. Cells were fixed with 4% paraformaldehyde for 30 min (Electron Microscopy Sciences, USA), rinsed with PBS and stained with Alcian Blue for 30 min (Alcian Blue 8GX, Santa Cruz Biotechnology, Canada), and washed multiple times. The cartilage extracellular matrix exhibited a strong blue stain. For assessment of osteogenic differentiation, cells were cultured in low glucose DMEM supplemented with FBS, Ab/Am, L-ascorbic acid-2-phosphate, (Sigma-Aldrich, Canada), β -glycerophosphate disodium salt hydrate (Sigma-Aldrich, Canada), and Dexamethasone (Sigma-Aldrich, Canada). After three weeks of osteogenic differentiation, the media was removed, and wells were rinsed with PBS. Cells were fixed with 4% paraformaldehyde for 30 min (Electron Microscopy Sciences, USA), rinsed with PBS and stained with Alizarin red for 45 min (Alcian Blue 8GX, Santa Cruz Biotechnology, Canada), and washed multiple times. Calcium deposits appeared in a strong red.

Viability assay and immunohistochemistry

MSCs in fibrin-HA biomaterial sheets deposited using the handheld instrument was analyzed for viability in 3D culture by staining with a Live (Calcein+)/Dead (Ethidium Homodimer+) Staining Kit (Thermofisher Scientific, Canada) at days 0, 1, 3, and 7. Cell morphology and quantity was assessed via immunohistochemistry, where MSCs in 3D culture after deposition were stained for cell nucleus (Hoechst, Thermofisher Scientific, Canada) and cytoskeleton (Phalloidin, Abcam, USA).

Burn wound infliction

One week after being acclimatized and treated with preventive antibiotics for 5 days (Ceftiofur Injection daily, intramuscular), all pigs were exposed to burns (TBSA 25%) when they reached a weight of 25kg and a length of 60cm. Prior to the treatment, animals were kept under general anesthesia and analgesia (Buprenorphine 0.05mg/kg subcutaneous, Ketamine 0.2mg/kg sc. combined with Atropine 0.5 – 1.0 mg depending on the heart rate, as

well as Isoflurane 5%/1/O₂ intubation). Additional analgesia (Tramadol 2–4mg/kg/every 8h orally) was administered regularly over the duration of the entire experiment starting 24h pre-intervention. Full-thickness burn wounds of 5cm × 5cm on the dorsal back of the pigs were created using a standardized protocol confirmed from the animal welfare committee (with a heated aluminum device (200°C) for 20 s under constant force measured using a digital force gauge of 4.0 Newton (N) (Mark-10 Corporation) (1N = 1kg·m·s⁻²)). Full-thickness excisional wounds were inflicted in all pigs, where each wound had a size of 5cm × 5cm created in two rows equidistant from the spine at a distance of 5cm from each other. Full-thickness burn was confirmed on the day of the experiment procedure, after processing and staining with Masson's Trichrome Protocol. Surgical wound excisions were performed 48–72hrs postburn, after injection of anesthesia and analgesia (Buprenorphine, Ketamine, Isoflurane), and hair removal via electrical shaving followed by chemical depilation. The operation area was disinfected with chlorhexidine, full-thickness burn biopsies were taken, skin excisions were marked, and excision of the full-thickness burn was done with a scalpel and monopolar electrocautery. Following the deposition process, the wounds were covered with wound dressing such as Jelonet® and Polysporine®, wet/dry sterile gauze, kept in place by adhesive bandages and an elastic stocking porcine suit.

***In-situ* deposition**

MSCs prepared at 1×10⁶ cells/ml were resuspended with a biomaterial solution consisting of 20mg/ml fibrinogen and 1% hyaluronic acid in a 3ml syringe, then loaded onto the handheld instrument together with the cross-linker solution consisting of 500IU/ml thrombin and 1% hyaluronic acid. The cell-containing biomaterial solution was delivered onto the wound bed at a rate of 0.3ml/min while the wheel translation speed was kept constant at 3mm/s. A 1-inch (25.4mm) wide microfluidic printhead was used for all animal experiments. The angle of the wound relative to the operating table was recorded before the deposition process began. Two side-by-side strips of width 25.4mm of biomaterial sheets were deposited for each 5cm × 5cm wound. Following the experimental procedure, the pigs were intraoperatively monitored, with 500–1000 ml NaCl was administered intravenous (iv.). Daily antibiotic injections were continued between 3–5 days post-surgery. No wound infection occurred. After surgery and bandaging, pigs could recover in a warm, well-ventilated recovery pen with essential amounts of oxygen and under observation.

Wound inspection

Photographs of the wounds were taken at each dressing change (every 2–3 days in general anaesthesia) using the ruler of a scalpel for scale on each photo. Images were then analyzed using ImageJ software. Terminal end-point photos were taken on day 28 to assess for wound healing progress. To determine the extent of macroscopic wound regeneration, the re-epithelialized area was compared with the initial burn wound excised. Contracture assessments were made by quantifying the scar and contraction area per wound region. For scar assessments, the Vancouver Scar Scale[52] was used to rate vascularity, pigmentation, pliability, and height. Macroscopic wound evaluation was done from two external blinded researchers in plastic surgery research.

Histological evaluation

Skin histology was assessed after biopsies were taken on day 28 of all wounds in all experiments. The tissue was (immediately) fixed in 10% formalin, dehydrated in 70% ethanol (EtOH), embedded in paraffin, and cut into 5µm sections. For Masson's Trichrome Stains, paraffin-embedded slides were deparaffinized through CitriSolv and rehydrated through grades of ethanol for staining (Electron Microscopy Sciences). Samples were kept overnight in Bouin's solution at room-temperature, washed and stained with Weigert's iron hematoxylin working solution (Sigma-Aldrich) (Nuclei Stain), Biebrich scarlet-acid fuchsin solution (Plasma Stain, Solution A), phosphomolybdic-phospho-acid solution (Solution B), aniline blue solution (Fibre Stain, Solution C), multiple times washed and refreshed in acid, until fixed via rehydration and citrosol. For each biopsy, images were obtained using a Leica light microscope (LEICADM 2000 LED) from consistent locations and depth, then individually analyzed with two blinded controls. For immunohistochemistry, slides were dewaxed and rehydrated for staining. First, antigen decloaking was performed with a pressure cooker at 110°C for 4 min. for antigen retrieval. Next, samples were blocked with 3% H₂O₂ for 10 min. before antibody incubation over-night. The tested antibodies were CD11b (Abcam ERP1344), CD163 (Abcam 87099), CD31 (Abcam 32457), α-SMA (Abcam 7781) and HLA-ABC-CI.1 (Abcam 70328). Antibodies were visualized using an HRP probe and polymer detection kits (Biocare) for either mouse or rabbit, as well as a betazoid DAB chromogen kit (DAKO, Biocare). Finally, slides were counterstained with hematoxylin, dehydrated, and mounted with xylene-based mounting medium. Stained slides were then scanned, and positively stained cells were quantified in a dermal layer from three different locations. Immunohistochemistry assessments were made for inflammation, stem cell presence and vascularization.

Ethics approval

This study was approved and performed in accordance with the guidelines and regulations of the Research Ethics Board (REB), Sunnybrook Health Science Centre (REB #017–2011). All procedures were executed accordingly in agreement with the Animal Policy and Welfare Committee of the University of Toronto. Animal procedures were reviewed and approved by Sunnybrook Research Institute and Sunnybrook Health Sciences Centre animal care and use committee (AUP #: 16–600). All controlled drug exemptions were obtained from Health Canada (44219.11.17, 44220.11.17) under the Controlled Drug and Substances Act.

Statistical evaluation

Data are expressed as the mean ± s.d., as well as median and IQR of at least three independent experiments. The number of independent experiments (*n*) is indicated in the figure legends. Wilcoxon-Mann-Whitney tests using two-independent samples with the data points (median IQR) for comparison of the data and differences were considered with significance at *P* < 0.05.

Data availability

The datasets generated and analyzed in this work are available from the corresponding authors upon reasonable request.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. In-situ formation of precursor skin tissue using an intraoperative approach. **a**, Schematic illustration of handheld approach for delivering cell-laden biomaterial sheets conformal to full-thickness burn wound of arbitrary size and topology. **b**, Rendered image of handheld instrument for controllable delivery of bioink consisting of mesenchymal stem/stromal cells (MSCs) in fibrin-based bioink (green color) supplied at flow rate Q_B with cross-linker (clear) supplied at flow rate Q_C through microfluidic printhead, while driven by soft wheel along wound surface at velocity V .

Figure 2. Conformal deposition of biomaterial layers onto physiologically relevant topologies. **a**, 3D rendering, exploded view of handheld instrument and disposable bioink syringes, microfluidic printhead, and silicone wheel. **b**, Left: Side view of printhead illustrating conformal sheet deposition by printhead onto wound substrate being unaffected by wheel deformation. Right: contact pressures measured for wheels of different hardness on stiff and soft substrates. Dotted line corresponds to stiffness of wound tissue. Data expressed as mean $\pm$ s.d., $n = 5$ independent experiments. **c**, Left: rendered image of printhead rotating about y -axis with pitch angle α , compensating for inclinations of up to 45° . Right: printhead rotation.

about x -axis with roll angle ϕ , accounting for $\pm 25^\circ$ variation in instrument position with respect to the normal direction of the deposition surface. **d**, Left: side view photograph of bioink extrusion. Q_C and Q_B indicate perfusion of cross-linker and bioink through printhead (Supplementary Table 1; right column). Wheel rotates clockwise to advance instrument at nominal speed V_0 in deposition direction. Middle: schematic cross-sectional view showing bioink and cross-linker exiting printhead to form biomaterial sheets on deposition surface with height h_T . Right: photograph of fibrin sheet formation. Scale bar: 2mm. **e**, Micrographs of deposited sheets for different values of V_0 and total flow rate Q and expected sheet width w for conditions of uniform coverage (top), sheet contraction (middle), and non-uniform coverage (bottom). Scale bar: 10mm. **f**, Top: percentage area covered for $V_0 = 3\text{mm/s}$. Bottom: measured actual wheel speed, V , versus V_0 . Data expressed as mean $\pm$ s.d., $n = 3$ independent experiments; $*p < 0.05$, t -test. **g**, Graphical representation of operating conditions to generate biomaterial sheets of varying thicknesses depending on total flow rate (Q) and wheel speed (V_0) for expected sheet width (w). **h**, Projected time, biomaterial volume, crosslinker volume, and cell number required for area coverage for $h_T = 0.2\text{mm}$, $c = 1 \times 10^6$ cells/ml, and $w = 25\text{mm}$ or 50mm . Blue dots: experimental conditions used *in vivo*, $n = 9$ independent wounds.

Fig. 3. Fibrin-HA biomaterials maintain uniform coverage over tilted surfaces following microfluidic extrusion to provide a homogenous environment for 3D cell growth.

a. Schematic of biomaterial sheet behavior after deposition from microfluidic printhead (Supplementary Table 1; right side) onto surface with inclination angle θ . Selected values represent inclination angles of burn wounds *in vivo*. Biomaterial sheet of initial width w undergoes drainage-induced reduction in thickness, h , and lateral translation, y . **b.** Viscosity measured at different shear rates and hyaluronic acid content in fibrinogen-based bioink. **c.** Time t_g associated with reaching 95% change in turbidity as obtained through

confocal microscopy. Data expressed as mean $\pm$ s.d., $n = 3$ independent experiments. **d**, Drainage velocity as determined by tracking 1% HA-containing biomaterial sheets containing 1 μm microspheres on surface with tilt angle θ . Data expressed as mean $\pm$ s.d., $n = 5$ individually tracked particles. Black lines indicate best fit, $R^2 > 0.91$. **e**, Left: time t_s until drainage ended due to gelation-induced viscosity increase. Center: Relative lateral drainage distance y , at time t_s . Right: percentage change in biomaterial thickness h due to drainage at time t_s determined from confocal microscopy. **f**, Representative time snaps of 1×10^5 MSC/ml fibrin-HA biomaterials in 3D culture for 0 to 7 days after sheet deposition, stained for cell nucleus (Hoechst, blue) and α -Actin (Phalloidin, green). Scale bar: 100 μm . **g**, Quantification of cell viability, cell number, distance to nearest neighbour, and cell aspect ratio of MSCs in 3D culture after microfluidic extrusion from days 0 to 7. Data expressed as mean $\pm$ s.d., $n = 3$ independent experiments.

Fig. 4. MSC-containing fibrin-HA biomaterials deposited homogeneously on a porcine full thickness burn surface using the handheld device contribute to improved wound healing.

a. Left: photograph showing the side view of the handheld device in the process of depositing MSC-containing fibrin-HA biomaterials onto a porcine full-thickness burn model with only the microfluidic printhead and silicone wheel in contact with the wound substrate. Right: isometric view of the handheld instrument during the deposition process, with the body of the instrument positioned at a different angle relative to the microfluidic printhead, which is held parallel and in direct contact with the wound substrate underneath. Scale bar:

2.5cm **b**, Top down photographs showing the 5cm × 5cm burned wound prior to (left) and immediately after (right) *in-situ* deposition. Scale bar: 2cm **c**, Top down photographs indicating macroscopic wound healing after 28 days of recovery comparing acellular biomaterial treatment only (left), and MSC-containing biomaterials (right). Scale bar: 2cm. **d**, Masson`s Trichrome stained tissue sections of healthy skin and wounds treated with burn alone, acellular biomaterials only, and MSC-containing biomaterials after 28 days post-biomaterial deposition. Scale bar: 200µm. **e**, Quantification of epithelialization speed, scar quality, and contracture per wound from macroscopic assessment. **f**, Quantification of epidermal thickness, collagen density, and CD31+ vessels from histology. **g**, Quantification of CD163+ expression, CD11b+ expression, and α-SMA+ density from histology. Data expressed as median IQR, $n = 4$ separate pig models with >3 replicates per condition per animal, * $p < 0.05$, Wilcoxon–Mann–Whitney test.