Light-Activated Decellularized Extracellular Matrix-Based Bioinks for Volumetric Tissue Analogs at the Centimeter Scale

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Tissue engineering requires not only tissue-specific functionality but also a realistic scale. Decellularized extracellular matrix (dECM) is presently applied to the extrusion-based 3D printing technology. It has demonstrated excellent efficiency as bioscaffolds that allow engineering of living constructs with elaborate microarchitectures as well as the tissue-specific biochemical milieu of target tissues and organs. However, dECM bioinks have poor printability and physical properties, resulting in limited shape fidelity and scalability. In this study, new light-activated dECM bioinks with ruthenium/sodium persulfate (dERS) are introduced. The materials can be polymerized via a ditirosine-based cross-linking system with rapid reaction kinetics and improved mechanical properties. Complicated constructs with high aspect ratios can be fabricated similar to the geometry of the desired constructs with increased shape fidelity and excellent printing versatility using dERS. Furthermore, living tissue constructs can be safely fabricated with excellent tissue regenerative capacity identical to that of pure dECM. dERS may serve as a platform for a wider biofabrication window through building complex and centimeter-scale living constructs as well as supporting tissue-specific performances to encapsulated cells. This capability of dERS opens new avenues for upscaling the production of hydrogel-based constructs without additional materials and processes, applicable in tissue engineering and regenerative medicine.

1. Introduction
The tissue engineering and regenerative medicine (TERM) field has a tremendous opportunity to build transplantable functional tissue constructs to overcome organs shortage. It can also be applied to build in vitro tissue models to study disease mechanism, high-throughput drug screening, drug repositioning, and drug toxicity studies.[1–5] However, traditional approaches mainly rely on functional biomaterials seeded with cells, supplemented with bioactive factors, or in situ recruitment of cells after transplantation.[6–9] These methods are insufficient to make a finely tuned complex milieu for providing multiscale 3D architectures, such as curved cornea with crisscross internal collagenous pattern or a chamber-shaped helical cardiac chambers.

3D bioprinting technology enables precise control over the placement of cells and materials with the desired structures at the designated locations. This technology allows the versatile emulation of tissue-specific cellular arrangements and structural features with extensive process flexibility, which has huge potential to increase the functions by advancing the bioinks (printable biomaterials mixed with cells). Yet, there is a tradeoff between printing architectural fidelity and biocompatibility within bioinks,[10–12] limiting the multi-scalar control to produce clinically relevant human-scaled designs. Moreover, the recapitulation of the tissues with the organ-specific microenvironments is necessary to provide intrinsic biochemical cues for guiding various cellular behaviors (e.g., proliferation, migration, differentiation, and maturation).[1,13] As a result, there remains a high

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Decellularized extracellular matrix (dECM) has recently been considered as the desired biomaterial to emulate the intricacy in the native microenvironment because of its similar component and composition of various biomolecules to the native tissues and organs. In contrast to conventional bioinks (e.g., collagen, silk fibroin, and gelatin) that contain one main natural component, dECM can provide tissue-specific complex networks contributing to the cell specification and tissue morphogenesis. We have demonstrated the effectiveness of heart, blood vessels, pancreas, and cornea tissue-derived dECM-based bioinks on engineering tissue- and organ-specific microenvironment to promote the maturation of the tissue constructs. In particular, dECM-based bioinks provide a great advantage to be used as temperature-sensitive hydrogels due to physical cross-linking of their abundance of collagenous proteins through intermolecular interaction. In addition, the unique spatial distribution of biochemical milieu in dECM-based bioinks facilitates the regulation of various cellular behaviors, and supports tissue regeneration as well as matrix remodeling at the injured site.

Despite such promising advances, the present dECM-based bioinks have insufficient mechanical stability and printability, which risks hampering the practical applications for printing the desired 3D constructs. Thus, to fabricate clinically relevant large-volume tissue constructs, various physical strategies (e.g., support baths, sacrificial hydrogels, nanofiber-reinforced dECM) have been attempted to increase the shape fidelity and stability during bioprinting processes. Although these approaches enable the fabrication of free-standing complex and volumetric constructs, they also often require intensive postprocessing including the removal of the sacrificial/supporting polymer with a risk of collapsing the engineered construct. Alternative chemical strategies have also been attempted, such as the application of cross-linker (e.g., genipin-dECM hydrogel), blending of dECM-based bioinks with other rapid cross-linking systems (e.g., dECM/alginate, dECM/GelMA), or the photo-crosslinking of dECM-based bioinks through the conjugation of the methacryloyl functional groups to dECM-based bioinks (e.g., methacrylated dECM/Irgacure 2959). However, these methods still limit in that such chemical modification inevitably diminish the native bioactivity of pristine dECM.

Light-activated cross-linking method has been directly employed in dECM-based bioinks to induce formation of covalent cross-links between the matrix molecules or proteins within dECM (e.g., dECM/riboflavin). Although the photosensitizer, riboflavin is clinically approved and applied as a convenient treatment of cornea tear, the reaction is initiated by UVA (365 nm), which has been previously shown to be cytotoxic and mutagenic, resulting in damage to the anterior stroma. Moreover, the riboflavin and UVA photo-crosslinking system also limited in cross-linking efficiency and cross-linking speed, which are major drawbacks in fabrication of large-volume tissue constructs. To overcome this limitation, visible light-induced cross-linking method using the photoinitiator of ruthenium (Ru)/sodium persulfate (SPS) has come into greater prominence owing to three advantages. First, the absorptivity in the visible light range (400–450 nm) prevents UV-induced DNA damage within encapsulated cells. Second, the high molar extinction coefficient allows efficient curing reaction at a relatively low initiator concentration. Third, the high penetration depth is effective to overall the curing of the large constructs homogeneously. Ru-based photoinitiators have already been applied to the cross-linking of fibrin-, collagen-, as well as silk-based natural biomaterials. In the presence of visible light and an electron acceptor (SPS), Ru undergoes photolysis into Ru³⁺. The presence of Ru³⁺can in turn oxidize aromatic residues, including tyrosine. The oxidized tyrosine groups are further converted into tyrosyl free radicals, where quenched by forming covalent di-tirosine bonds with nearby tyrosine moieties. In addition, because of the high absorbance of Ru in the visible light chemical stability at the excited state, Ru/SPS cross-linking system has been proven to be highly efficient and rapid. Given that dECM bioinks contain abundant tyrosine-carrying proteins, we hypothesize that the Ru/SPS system is able to facilitate cross-linking of dECM bioinks in a rapid and cytocompatible manner. Furthermore, we believe that these light-activated dECM biomaterials have significant potential as bioinks for 3D bioprinting, where dECM-based constructs with high shape fidelity and complex architecture can be fabricated.

2. Results and Discussion

Herein, we developed a novel cross-linking system for dECM-based bioinks through application of Ru/SPS photoinitiator. We investigated the functional mechanism of this system on the rapid cross-linking of dECM-based bioinks and observed its versatility by controlling the ratio of dityrosine synthesis in the dECM-based bioinks. In particular, we applied this cross-linking system to cornea-derived and heart-derived dECM bioinks for the fabrication of 3D functional tissue analogs with hierarchical multiscale. These two different types of tissue-derived dECMs came from different body systems were considered because of their two representative opposing characteristics related to the vascular system as follows: 1) Cornea is an avascular transparent tissue, and 2) heart is one of the highly vascularized vital organs. The materials were prepared through intermixing of each dECM bioinks with Ru/SPS initiator, named as dERS. The dERS compound samples were cross-linked via visible light-activated oxidation mechanism. This mechanism is initiated via the production of tyrosyl free radicals by irradiation of visible light (400–450 nm), which will then form covalent dityrosine cross-links with nearby tyrosine moieties (Figure 1A). Tyrosine, one of the abundant amino acids, exists as a versatile residue in regulating the structural conformation transitions of proteins. The most abundant protein in both dECMs is collagen, which was previously reported to contain tyrosine residues. Further examinations using liquid chromatography with tandem mass spectrometry (LC–MS/MS) analysis successfully identified and quantified the presence of tyrosine within dECMs. After light irradiation, the amount of tyrosine residues significantly reduced within the groups containing photoinitiators, indirectly indicating that these tyrosine groups were converted into covalent cross-links (Figure 1B). In order to confirm the formation of dityrosine cross-link bonds in the light-activated dERS, we further measured the autofluorescence intensity of the samples when exposed to UV light at 320 nm. It has been previously
reported that stable dityrosine bonding that contains phenolic groups emits fluorescence in response to the absorption light at 280 and 320 nm, respectively. The fluorescence images indicated the presence of dityrosine groups in the light-activated dERS. (Figure 1C; Figure S1, Supporting Information). Furthermore, an increased amount of dityrosine and trityrosine groups was observed within all the photo-crosslinking process conditions (Figure 1D; Figure S2, Supporting Information). Interestingly, 2/20 \times 10^{-3} \text{m} \text{Ru/SPS} photoinitiator compound revealed the most amount of dityrosine group compared with that in the 0.5/5 \times 10^{-3} and 1/10 \times 10^{-3} \text{m} samples. These observations may be due to the presence of more complicated cross-linking mechanisms, such as formation of tri-tyrosine bonding within the high concentration of photoinitiator. Thus, it would be interesting to conduct further experiments exploring the mechanism of dERS cross-linking reactions.

Nevertheless, the dityrosine bonding was confirmed as one of the main dominant driving forces for the dERS cross-linking reaction to occur. We then conducted sol fraction and swelling analyses of both dERS samples to evaluate the physico-chemical properties. The sol fraction values, which are measures of the polymers that are not covalently bound to the network after cross-linking, were observed inversely related to the concentration of Ru/SPS initiator, as shown in Figure 1E (more detailed data are presented in Figure S3, Supporting Information). These results approve the previous observation with the fluorescence measurements (Figure 1D), where a higher concentration of Ru/SPS increases the free radical dimerization cross-linking reaction rate to form covalent cross-link bonds. The swelling ratios were also found inversely related to the concentration of Ru/SPS, implying that the lower sol fraction corresponds to a lower swelling ratio due to a tighter network (Figure 1F; Figure S3, Supporting Information).

Bioinks must provide non-Newtonian shear-thinning behavior to alleviate direct shear stress from bioinks into the

Figure 1. Tyrosine-based light-activated cross-linking reaction in dECM bioink. A) A schematic of visible-light active dityrosine synthesis. B) LC–MS/MS analyses of tyrosine group with or without photo-crosslinking (*\text{$p$*} < 0.01). C–G) Effects of dityrosine cross-linking initiated by Ru/SPS: C) an autofluorescence of dECM by dityrosine, D) fluorescence intensity of the dECM and dERS bioink (**\text{$p$*} < 0.0001), E) sol fraction (**\text{$p$*} < 0.1), F) swelling ratio (*\text{$p$*} < 0.1, **\text{$p$*} < 0.01), G) cross-linking time in light-activated and thermally cross-linked dECM hydrogels. H, I) Rheological properties of pre- and post-printed bioinks using 2% hECM and 2% Co-dECM: H) viscosities of preprinted dECM and dERS at 4 °C (ready-to-print condition), I) increased storage and loss moduli after light exposure for 3 min (light source: visible light (30 mW cm$^{-2}$ postprinting condition). J) Improved mechanical properties of compressive moduli by dityrosine synthesis after light-activated and thermal cross-linking processes (**\text{$p$*} < 0.1, ***\text{$p$*} < 0.01).
encapsulated cells upon the fabrication process. Because the previously developed dECM bioinks showed proper shear-thinning behavior for 3D bioprinting, we investigated the effects of Ru/SPS concentration on the viscosity of dERS. The rheological profiles exhibited few changes regardless of the concentration of Ru/SPS from 0.5/5 × 10⁻³ to 2/20 × 10⁻³ m in 1–1000 s⁻¹ range of the shear rate (Figure 1H). On the other hand, a significant improvement in the mechanical properties was observed in the postprinted dERS under the visible light, which was primarily due to the dityrosine cross-links formed within the dERS structure. The experiment was performed at 37 °C, which is the temperature physiologically relevant to sol–gel transition as well as identical to that of the printing bed in the bioprinting process. In comparison with hdECM, the hdERS material at its highest ratio exhibited 12.8 times enhanced complex moduli after exposure to visible light for less than 3 min (Figure 1I; Figure S5, Supporting Information). In general, collagenous proteins usually require ≈30 min at 37 °C to induce the thermal cross-linking reaction. Therefore, it can be noted that the additional light-activated cross-linking system increases the complex moduli depending on the concentration of the cross-linker.

Furthermore, we investigated other types of mechanical characteristics using a universal testing machine (UTM). In nature, dityrosine is found in the structural connective tissues of dragonflies, human aorta, and bovine skin with high elasticity, strong mechanical properties, and energy-storage properties. In our study, dityrosine cross-links were introduced within the dERS that were both chemically and thermally cross-linked using a combination of light and Ru/SPS, leading to enhanced mechanical properties. Previous studies show the compressive moduli of other dECM hydrogels in the range of 0.18 to ≈3.0 kPa. However, the dERS product showed a highly improved value for the compressive modulus up to 86.4 kPa (1/10 × 10⁻³ m Co-dERS). Moreover, a sample of 2/20 × 10⁻³ m Ru/SPS mixed Co-dERS showed 2.55, 3.79, and 20.04 times increases in compressive, elastic, and resilient moduli, respectively, compared with those of thermally cross-linked Co-dECM without Ru/SPS (Figure 1J; Figures S6 and S7, Supporting Information). Thus, it can be noted that the formation of dityrosine cross-links improves such mechanical and rheological properties that can increase the threshold limit for tolerance against various forms of stresses. These improvements provide mechanical properties larger than those of the native tissues as well as ultimately result in better products for both printability and shape fidelity characters.

We further fabricated geometrically complex and large structures via extrusion-based 3D bioprinting technology (Figure 2A). For light exposure during the extrusion-based printing process, an LED module was mounted at the bottom side of the extrusion head as a light source adjusted to an intensity of 30 mW cm⁻² (Figure S8, Supporting Information). The entire extrusion-based printing process was carried out under the irradiation of visible light (400–450 nm of wavelength) (Figure 2A). To investigate the shape fidelity of dERS, we designed a cylindrical tubular structure with a height of 5.5 mm and diameter of 8.0 mm and compared it with the postprinted constructs fabricated using hdERS and with the desired dimensions. As a result, only the light-activated dERS group (dERS + VIS) irradiated with visible light could reproduce up to 96.87% of the size of the desired construct in height, whereas other groups (dECM, dECM + VIS, and dERS) showed a height 22% far shorter than the desired height (Figure 2C,D). In addition, the dERS + VIS group displayed the narrowest line width, which is a similar dimension to the diameter of the used nozzle, enabling the fabrication of precise and elaborate structures. Such outstanding dimensional fidelity was not achieved in other groups in which the printed struts showed wider line widths and formed narrow areas compared with the dERS + VIS group (Figure 2E; Figure S9, Supporting Information).

We also successfully fabricated geometrically complex and sloped structures, such as auricular constructs, hollow pyramids, and grid structures (Figure 2F; Figure S10, Supporting Information). Interestingly, the constructs could be folded, bended, and even twisted without any deformation or disruption due to their high elasticity and resilience (Figure 2F; Figure S11, Supporting Information). Taken together all these observations, it can be noted that dERS bioinks cured with the visible light offer a versatile fabrication capacity in all x, y, and z directions. Apart from the extrusion-based printing, we also showed proof-of-concept data where dERS can also be used for DLP photopatterning (Figure 2B). Using hdERS as bioresin in a DLP bioprinting system (Cellink Lumen XTM), we were able to fabricate constructs including complex structures with high resolution (Figure 2G; Figure S12, Supporting Information), where the minimal strand diameter was 100 μm.

To further demonstrate the cytocompatibility of the printing process, we used a bioresin containing human bone marrow-derived mesenchymal stem cells and hdERS to fabricate a wheel-shape structure. After the printing and additional 24 h culture, we obtained the printed construct with over 82% cell viability, indicating an excellent cytocompatibility (Figure 2G). These results also demonstrate potential application of dERS for DLP printing, which further showcases the versatility of dERS in the biofabrication field.

We then demonstrated that these living tissue constructs fabricated via visible light-activated cross-linking system have excellent tissue regeneration and remodeling capability similar to pure dECM. Cornea-derived ECM (Co-dECM) has been used to provide cornea-specific biological/biochemical cues to the encapsulated or in vivo surrounded cells. Moreover, the rheological characteristics indicate the potential of Co-dECM working as a bioink. However, the Co-dECM bioink showed inferior shape stability due to its long gelation time, causing issues to remain the corneal curved structure integrity during the thermal gelation process. The introduction of the visible light-activated cross-linking system to Co-dECM through intermixing with Ru/SPS initiator compound (Co-dERS) resulted in rapid gelation of Co-dECM, making biofabrication of curved corneal structures possible (Figure 3A). These curved corneal designs were based on the 3D anatomical images of the eyeball (from NIH 3D Print Exchange) by a few modifications of the design to enhance the stability of the printed structure. The printed corneas provided the desired dimension of curvature without any supporting materials. In addition, because the transparency of the printed cornea is highly related to the degree of swelling and color-removal of the yellowish tone from the Ru component, we conducted experiments to determine
the degree of swelling of the printed corneas as well as their light transmittance at different time points until the cornea became fully transparent. The cornea only transmitted the yellowish light (550 nm of wavelength) at ≈55% immediately after printing. However, the transparency gradually recovered to a transmittance over 94% after immersing the printed tissue in normal saline for 30 min (Figure 3B,C).

Furthermore, to evaluate the cellular performances, human turbinate-derived mesenchymal stem cells (hTMSCs) were differentiated into keratocytes by 3-day culturing in the keratocyte differentiation medium. The predifferentiated keratocytes encapsulated in the corneal construct exhibited an increase in the metabolic activity with high cell viability when the cells were printed at 37 °C (Figure 3D; Figures S13 and S14, Supporting Information). Interestingly, the amount of hydroxyproline also increased, indicating the promotion of a newly synthesized collagen by the printed cells (Figure 3E). Recent studies have demonstrated that the matrix stiffness affects the performances of keratocytes, including phenotype and cellular interaction.[53–55]

The higher concentrations of Ru/SPS leads to an increasing trend of the tensile modulus which is identical to the stiffness (Figure S7, Supporting Information). For assessment...
of the cellular performances, we prepared corneal structures at different concentrations of Ru/SPS. Cornea-specific genes (keratocan, KERA; aldehyde dehydrogenase, ALDH) were significantly upregulated over time in the cells printed with Co-dECM (Figure 3F). The results obtained from the experiments with Co-dERS indicated that the stiffer matrix has a positive effect on the upregulation of the cornea-specific genes. Furthermore, 0.5/5 × 10⁻³ and 1/10 × 10⁻³ M of Ru/SPS-mixed groups showed little enhancement in the mRNA expression of KERA and ALDH over time. Conversely, 2/20 × 10⁻³ M of Ru/SPS-mixed group showed highly improved mRNA expression of corneal genes; whereas, suppressing ACTA 2, a gene representing myofibroblasts. The mRNA expression levels of all Co-dERS groups were observed larger than that of Co-dECM group, demonstrating that a soft matrix but with stiffer properties promotes the encapsulated cells to upregulate mRNA expression of the cornea-specific genes. Large amounts of collagen type I were also detected in 1/10 × 10⁻³ M of Co-dERS group, a trend similar to that of the human cornea (Figure 3G).

Similarly, we observed that heart-derived dECM (hdECM) with visible light-activated cross-linking system could retain a stable 3D cardiac shape while supporting cellular growth. The rendering image of cardiac tissue was obtained from NIH 3D Print Exchange and was simplified into the 3D anatomical images including the left and right ventricles (Figure 3H). The printed ventricular constructs using hdECM intermixed with Ru/SPS (hdERS) could stably sustain their hollow shape. The hTMSCs and human-induced pluripotent stem cell-derived cardiomyocytes (hiPSC-CMs) were encapsulated using 1/10 × 10⁻³ M Ru/SPS and their viability characters were examined using live/dead cell assay. It was observed that the hTMSCs laden construct remained greater than 90% viable even after 48 h postprinting process; whereas, the hiPSC-CMs laden construct showed ≈60–80% viability for 7 days (Figure 3I; Figure S15, Supporting Information). Because 0.2/2 × 10⁻³ M of Ru/SPS showed greater viability of above 80% for hiPSC-CMs (Figure 3I, Figure S15, Supporting Information), this composition was selected for the 3D printing process of the cardiac tissue analog.

Figure 3. Tissue printing process with dERS: Corneal stroma mimicking constructs providing cornea-specific biochemical environment. A) 3D printed curved cornea based on anatomical image of the eyeball (scale bar: 5 mm). B) Dissolve of Ru/SPS photoinitiator in printed cornea over time and C) their transmittance values (**p < 0.001, ****p < 0.0001). Activated cellular performances observed by: D) metabolic activity (***p < 0.001) and E) collagen synthesis (**p < 0.001). F) Values of Log₂ fold changes normalized to GAPDH: Upregulation of keratocyte-positive genes while downregulation of myofibroblast-positive genes in dERS (**p < 0.1, ***p < 0.01, ****p < 0.001, *****p < 0.0001). G) Immunofluorescence images of tissue formation-related protein (ColI) in printed cornea. H) Heart mimicking constructs providing cardiac-specific biochemical environment and 3D printed heart based on anatomical image of the heart (scale bar: 5 mm). I) Cell viability of hiPSC-CMs encapsulated in hdECM construct at 1, 3, and 7 days. J) Images of live/dead cell assay at day 2 of hiPSC-CMs encapsulated in hdECM construct (scale bars: 200 μm). K) Immunofluorescence images at day 14 (scale bars: 20 μm). K) Calcium imaging frequency response at day 14 of hiPSC-CMs encapsulated in hdERS (scale bar: 500 μm).
The quantitative analyses of the expressions of cardiac-specific gene markers were performed to evaluate cellular performances within hdERS (Figure S16, Supporting Information). Both cardiac troponin T (cTnT) and myosin heavy chain 6 (MYH6) within hdERS showed similar expressions to that of hdECM group after 14 days. Calcium-handling genes, such as ATPase sarcoplasmic/endoplasmic reticulum Ca\(^{2+}\) transporting 2 (ATP2A2) and calcium voltage-gated channel subunit alpha1 C (CACNA1C), were also similarly expressed within the two groups of hdERS and hdECM. With these results, it can be noted that hdERS provided the heart muscle cells with cardiосpecific biological/biochemical cues as hdECM did.

To study the formation of cardiac-specific proteins, Cardiac troponin T (cTnT) and connexin 43 (Cx43)—a gap junction cardiac surface protein—were qualitatively characterized at day 14 to demonstrate the regulation of muscular contraction and intercellular communication. The cardiac tissues using hdERS exhibited an organized sarcomeric structure, which approximates to that of the hdECM group. In addition, the immunofluorescence of Cx43 protein was highly expressed in both of the two groups of hdERS and hdECM. With these results, it can be noted that hdERS provided the heart muscle cells with cardiосpecific biological/biochemical cues as hdECM did.

Furthermore, we carried out a Ca\(^{2+}\) wave propagation analysis to assess the Ca\(^{2+}\) handling capacity by the printed cardiac muscle tissue. Both of the hdECM and hdERS groups displayed a spontaneous beating motion and Ca\(^{2+}\) sparks, which is time-consistent (Figure 3K; Figure S18, Supporting Information). These results clearly indicated that hdERS could support cardiac maturation and functionality in the optimized conditions. The application of hdERS-based bioinks may be given extended consideration to direct printing of a hollow chambered pulsating constructs. Taken together, it was recognized that dERS imparts both tissue-specific environments to encapsulated cells and 3D fabrication versatility in every axis.

4. Experimental Section

Preparation of hdECM Powder: The porcine heart for decellularization was purchased from a slaughterhouse. The left ventricle was separated from the whole porcine heart and decellularized following previously reported protocols with minor adjustments.[14,16,49] Briefly, the heart tissues were sliced into 1 mm thickness and stirred in tap water for 1 h to remove blood. Then, the tissues were decellularized with a solution of 1% sodium dodecyl sulfate (SDS; BioShop, Canada) anionic surfactant for 60 h and treated with 1% Triton X-100 (Biosesang, Korea) nonionic surfactant solution in phosphate buffered saline (PBS; T&A, Korea) for 1 h. The decellularized tissues were then stirred in isopropyl alcohol (Samchun, Korea) for 2 h. The treated tissues were immediately cleaned with 10× PBS (T and I, Korea) for 72 h to ensure that residual detergent was removed, followed by sterilization with 0.1% peracetic acid (32 wt% in dilute acetic acid, Sigma-Aldrich, USA) in 4% ethyl alcohol for 3 h. Finally, the sterilized tissues were rinsed with 1× PBS (Hyclone, USA) for 6 h. After lyophilization and pulverization, the obtained powder of hdECM should be stored at −80°C until further use.

Preparation of Co-dECM Powder: The preparation of Co-dECM was also described in previous article.[18] In brief, the whole corneas were dissected from bovine eyeballs, washed using PBS with penicillin (100 units mL\(^{-1}\)) and streptomycin (0.1 mg mL\(^{-1}\)). Stromal layers were separated from corneas and stirred in 20× 10\(^{-3}\) m ammonium hydroxide (NH\(_4\)OH; 4.98 N solution in water, Sigma-Aldrich, USA) containing 0.5% Triton X-100 for 4 h. Then, the tissues were treated in hypotonic Tris hydrochloride (Tris-HCl; pH 7.4, Biosesang, Korea) buffer solution for 24 h and 10× 10\(^{-3}\) m Tris-HCl solution with 1% (v/v) Triton X-100 for 24 h at 37°C, resulting in Co-dECM tissues. The Co-dECM tissues were sterilized using 1% peracetic acid (32 wt% in dilute acetic acid, Sigma-Aldrich, USA) in 50% ethyl alcohol for 10 h. After the decellularization process, the Co-dECM was lyophilized overnight and cryogenically crushed into fine powder using liquid nitrogen and a milling machine and stored at −80°C.

Preparation of dECM Bioinks: Each Co-dECM and hdECM powder sample was dissolved in 0.5 M acetic acid solution (Merck Millipore, USA) with pepsin (Sigma-Aldrich, USA) at room temperature for 72 h. The digested solution was filtered through a 40-μm pore mesh (Falcon, USA) and stored at 4°C. Prior to experiment, each Co-dECM and hdECM solution was neutralized (pH 7.4) using 10 n NaOH solution (Biosesang, Korea). Additionally, 10× PBS and autoclaved water were added to the neutral hdECM solution.

Measurement of dECM Autofluorescence from Dityrosine: For visible light cross-linking process, 2 wt% of dECM was mixed with different amounts of Ru/SPS (0.5/5 × 10\(^{-3}\), 1/10 × 10\(^{-3}\), and 2/20 × 10\(^{-3}\) m). The mixed solution was deposited into a premade silicon module (diameter 5 cm, height 2 mm) and placed under a visible light source (30 mW cm\(^{-2}\)) for 3 min. For thermal cross-linking process, 2 wt% of dECM was dropped into the silicon module and placed at 37°C for 1 h until gelled. After the gelation, the hydrogel constructs were placed under the UV light (3UV-38 UV Lamp, UVP, Cambridge, UK) with a wavelength of 302 nm. Pictures were taken using an iPhone 11 Pro. The images were further converted into black and white color using GNU Image Manipulation Program (GIMP 2.10.22).

Sol Fraction and Swelling Ratio: The sol fraction and swelling ratio values were determined following the previous study procedure.[18] Hydrogel samples were fabricated as explained above. After the photo-crosslinking or thermal cross-linking, all the samples were weighed and...
recorded as $M_{wp}$. Three samples from each condition were lyophilized ($M_{d}$) directly. Another three samples from each condition were soaked in PBS at 37 °C overnight. The swollen samples were weighed as $M_{s}$ and weighed again after lyophilized as $M_{d}$.

The actual macromer weight fractions were calculated using Equation (1)

$$m\% = \frac{M_{d} - M_{wp}}{M_{d}} \times 100\%$$

(1)

The sol fraction ($M_{sol\, fraction}$) and swelling ratio ($q$) were calculated using Equations (2) and (3)

$$M_{sol\, fraction} = \frac{100 \times (M_{wp} \times m\%) - M_{s}}{M_{wp} \times m\%}$$

(2)

$$q = \frac{M_{s}}{M_{wp}}$$

(3)

**Liquid Chromatography Tandem Mass Spectrometry (LC–MS/MS):** The LC–MS/MS examinations were performed based on the protocol previously reported.[10] Briefly, all gels used for LC–MS/MS analyses were prepared as mentioned in the auto-fluoresce experiment. The fabricated hydrogel constructs were hydrolyzed using 4 M methanesulfonic acid containing 1 wt% phenol under the nitrogen environment for 18 h. Solid phase extraction (SPE) using Strata C18-E (55 µm, 70 Å) cartridges was performed to the hydrolyse process to remove the acid and was eluted with 80% (v/v) methanol. Eluents were further dried and reconstituted in 0.1% (v/v) formic acid for analysis. A 2.6 µm Kinetex C18 100 Å column (150 × 2.1 mm) and an Agilent 1290 binary pump (200 µL min⁻¹) were used to perform the reverse-phase HPLC–MS/MS experiments. The gradient started with 2% aqueous acetonitrile with 0.1% (v/v) formic acid for 4 min, increasing to 80% acetonitrile over 3 min, maintaining this over 3 min then equilibrated with the starting eluent. The analytes were delivered into a QTRAP 6500 mass spectrometer (Sciex) and detected in multiple reaction monitoring mode using positive ion mode. The ion spray was set to 5.5 kV and the temperature was set to 600 °C. Nitrogen was used as the collision gas and the collision energy was set at 25%. The peaks were calculated using Analyst Software v 1.6.2 (Sciex).

**Rheological Examinations:** The rheological characteristics of pure dECM and dERS mixed with 0.5/5 × 10⁻³, 1/10 × 10⁻³, and 2/20 × 10⁻³ M Ru/SPS were examined with Advanced Rheometric Expansion System (TA Instruments, USA) using 25-mm-diameter plate geometry. A steady shear sweep analysis of each bioink was performed at 4 °C to evaluate its viscosity. A time sweep analysis was used to measure the complex moduli of postprinted bioinks at 37 °C. Before performing the time sweep examinations, all bioinks were exposed to visible light for 3 min. All the measurements were conducted in triplicate.

**Mechanical Properties Examinations:** Tensile testing was conducted using a universal test machine (Nano-UTM, MTS) with a maximum load and load resolution of 500 × 10⁻³ and 50 × 10⁻⁶ N, respectively. The constructs, made of pure dECM and dERS mixed with 0.5/5 × 10⁻³, 1/10 × 10⁻³, and 2/20 × 10⁻³ M Ru/SPS, were washed twice with PBS for 5 min. The constructs were then fixed between two clamps with papers as geometrical constraints. The connection of the paper was removed, and the samples werepreload to remove slack and elongated at a set strain rate of 0.005 mm s⁻¹ until failure. Elastic and resilient moduli were respectively calculated from the slope and area of the linear region of the stress–strain curves. Specifically, the resilient moduli ($U_0$) were calculated using Equation (4)

$$U_0 = \frac{\sigma_0 \epsilon_0}{2}$$

(4)

where $\sigma_0$ is the yield stress and $\epsilon_0$ is the yield strain. Compressive testing was performed based on a previous study.[33] Compressive moduli were determined from the linear region of the stress–strain curves (10–15%) using an MTS Criterion 42 mechanical testing machine with a 5 N loading cell. Samples were prepared based on the autofluorescence experiment, further incubated in PBS at 37 °C overnight prior to the examinations. During the mechanical testing experiments, samples were placed parallel to their long axis and compressed at a constant crosshead speed of 0.01 mm s⁻¹. The experiments were performed at ambient temperature with a preload setting at 0.1 N.

**Extrusion-Based Printing:** Visible-light activated bioinks were printed using an in-house 3D bioprinting system. A LED module was installed at the bottom of a dispenser head for simultaneous extrusion and photopolymerization. An amount of 2% hDECM or 2% Co-dECM bioink neutralized with 10 m sodium hydroxide solution and containing 2/20 × 10⁻³ M Ru/SPS was loaded into a syringe that was set to 4 °C. For the extrusion process, two different sizes of nozzles of 22 and 25 G were used. The applied pneumatic pressure ranged from 20 to 50 kPa. Both extruded dERSs were deposited onto a printing bed kept above the gelation temperature of 40 °C and were cross-linked under visible-light irradiated from the LED module. To evaluate the printability, the diffusion degree was calculated using Equation (5)

$$\theta = \frac{S - S_0}{S_0}$$

(5)

where $S$ is the actual area and $S_0$ is the theoretic desired area.[56]

**DLP Photopatterning:** DLP photopatterning experiments were conducted using CELLINK Lumen X printer. A solution of 1% hDECM was neutralized with 10 m sodium hydroxide solution. An amount of 1 mL of hDECM supplemented with 1/10 × 10⁻³ M Ru/SPS was placed on the petri dish, which later was placed onto the print station. For the 3D bioprinting process, 5 million cells were also included. The printing process was performed using the software included with the Lumen X printer. The printing parameters were set as: light intensity 50%, exposure time 30 s, and penetration depth 100 µm. After conducting the printing process, the constructs were washed with PBS at 4 °C to remove the un-crosslinked bioresin. Printed constructs were further photographed under a microscope. The images were further converted into black and white color in CMIP for improvement of their contrasts.

**Optical Transparency Characterization:** Corneal transparency was examined through measurement of light transmittance using a microplate reader. Postprinted corneal constructs were prepared with 200 µm thickness, placed into wells of a 48-well plate. The excess liquid covering the surface of the samples was removed using a blotting paper. After setting the 48-well plate in the microplate reader (WALLAC 1420 VICTOR3, PerkinElmer, USA), light absorption values under the wavelength of 550 nm were determined. Each sample was assessed in triplicate and all the transmittance results were corrected by measuring the absorbance of a blank well as a reference.

**Metabolic Assay:** As the corneal constructs were cultured, proliferation of encapsulated cells was measured with a Cell Counting Kit-8 (CCK-8) assay (Dojindo, Japan). After culturing for 1 and 7 days, the constructs were moved to a new well and 5 mg mL⁻¹ CCK-8 agent was added to each new well. After 3 h, aliquots (100 µL) were pipetted into the wells of a 96-well plate and measured using the microplate reader (WALLAC 1420 VICTOR3, PerkinElmer, USA) at an absorbance of 450-nm wavelength.

**Hydroxyproline Assay:** The contents of total collagen were determined using hydroxyproline assay. For quantitative analysis of collagen, the absorbance at a wavelength of 540 nm was measured using the microplate reader (WALLAC 1420 VICTOR3, PerkinElmer USA) by referring to a standard curve made from hydroxyproline (Sigma-Aldrich, USA). All samples were assessed in triplicate.

**Cell Culture:** hTMSCs were obtained from the Catholic University of Korea, St. Mary’s Hospital after approval from the Institutional Biosafety Committee of POSTECH (PIBC-035) and cultivated in Dulbecco’s modified Eagle’s medium (Hyclone, USA) supplemented with 10% fetal bovine serum (Hyclone, USA). To encapsulate in Co-dECM bioink, the normal medium was replaced with differentiation medium containing 10 ng mL⁻¹ KGF/EGF for one day to obtain the differentiated keratocytes. hiPSC-CMs (Cardiosight-S; NEXEL, Korea) were seeded onto 1.6 mm Matrigel (Corning, USA):DMEM/F-12 (Gibco, USA)) Matrigel-coated plates with RPMI 1640 medium (Gibco, USA) containing 10% fetal bovine serum (Hyclone, USA), 1× B-27 Supplement Minus Vitamin A (Gibco, USA), and 5 × 10⁻⁶ M Y-27632 (Tocris, UK) (Approval no. PIBC-035). Y-27632 including medium was changed for fresh medium without ROCK inhibitor.
1 day after seeding. To prevent bacterial contamination, 1% penicillin/streptomycin (HyClone, USA) was added to each culture medium.

Gene Expression Analysis: Total RNA was extracted from the postprinted constructs at day 14 of culture using the TRIzol reagent (Takara Bio, Japan). The constructs were rinsed with 1x PBS and lysed in TRIzol reagent. Subsequently, 200 µL of chloroform (Samchun, Korea) was added per 1 mL of TRIzol reagent to separate the phases. The total RNA in the upper aqueous phase was recovered with isopropyl alcohol (Samchun, Korea) precipitation process. The precipitate was then washed with 75% ethanol alcohol followed by a dissolution in DEPC-treated water (Ambion, USA). Concentration and purity of RNA were determined in a NanoDrop (Thermo Fisher Scientific, USA) spectrophotometer. Additionally, potential DNA or protein contamination was assessed through measurement of the A260/A280 ratio. The extracted RNA underwent reverse transcription to synthesize cDNA using a SuperScript IV First-Strand Synthesis System (Thermo Fisher Scientific, USA). Each cornea- and cardiac-specific primers were designed based on the published literature. Quantitative real-time PCR (qPCR) experiments were performed with StepOnePlus Real-Time PCR system (Applied Biosystems, USA) and PowerSYBR Green PCR Master Mix (Applied Biosystems, USA). Relative gene expression levels were normalized to GAPDH and calculated using 2^−ΔΔCt method.

Immunofluorescence Analysis: Immunofluorescence staining examinations of the corneal and cardiac constructs were performed after culturing the samples for 14 days. The cultured corneal structures were fixed with paraformaldehyde solution in PBS (4% (w/v)); permeabilized with Triton X-100 (0.1%); treated with bovine serum albumin (Affimetrix, USA) in PBS (3%) for 1 h to block the nonspecific binding; washed three times with PBS for 15 min; incubated with Anti-Collagen I antibody (Abcam, UK) overnight at 4 °C; washed with PBS; exposed to Alexa Fluor 488 goat anti-mouse antibody (Invitrogen Life Technologies, USA) for 1 h at 37 °C; counterstained with 4,6-diamidino-2-phenylindole (DAPI). The cultured cardiac constructs were rinsed with 1x PBS (HyClone, USA); fixed in 4% paraformaldehyde (Chembio, USA) overnight at 4 °C; permeabilized with 0.2% TritonX-100 for 15 min; blocked with 5% normal goat serum for 1 h; stained with anti-cardiac troponin T (cTnT) antibody (Invitrogen, USA) and anti-connexin 43 (Cx43) antibody (Abcam, UK) overnight at 4 °C; exposed to Alexa Fluor 488 goat anti-mouse antibody and Alexa Fluor 594 goat anti-Rabbit Secondary Antibody (Invitrogen, USA) for 2 h at 4 °C in the dark condition; counterstained with VECTASHIELD antifade mounting medium with DAPI (Vector Laboratories, USA). Images of all stained samples were obtained using a FV1000 Olympus confocal microscope (Olympus, Tokyo, Japan).

Cell Viability Assay: The live/dead assay protocol was conducted with 1 × 10^6 mL^−1 hTMSCs and 2 × 10^6 mL^−1 iPSC-CMs for each tissue to determine the cell viability of the visible-light cross-linked constructs after culturing for 2 days. The samples were stained with Live/Dead viability/cytotoxicity kit (Thermo Fisher Scientific, USA) following the manufacturer’s instruction. The fluorescence images of live/dead cells were captured using a Nikon Eclipse Ti inverted microscope (Nikon Instruments, USA).

Ca^2+ Wave Propagation Analysis: To validate the contraction in the cardiac tissues, spontaneous Ca^2+ signaling was measured. In brief, fluorescent Ca^2+ indicator solution was made using Fluo-4 AM ester (Thermo Fisher Scientific, USA) dissolved in DMSO. The cardiac tissues were incubated in 10 × 10^−6 m Fluo-4 AM diluted with the maintaining medium for 1 h at 37 °C. For de-esterification of dye, the stained tissues were rinsed with Tyrode’s solution and incubated for 20 min at 37 °C. Tyrode’s solution consists of 140 × 10^−3 m NaCl (Samchun, Korea), 5 × 10^−3 m KCl (Sigma-Aldrich, USA), 5 × 10^−3 m HEPES buffer (Sigma-Aldrich, USA), 1.8 × 10^−3 m NaH2PO4 (Samchun, Korea), 1.1 × 10^−3 m MgCl2 (Junsei, Japan), 1.8 × 10^−3 m CaCl2 (Kanto, Japan), and 10 × 10^−3 m glucose (Sigma-Aldrich, USA). The Ca^2+ signaling data were recorded using a Nikon Eclipse Ti inverted microscope (Nikon Instruments, USA).

Statistical Analysis: Quantitative results were analyzed with GraphPad Prism 8 (GraphPad Software). One-way analysis of variance (ANOVA) and student’s t-test were applied to mean comparisons. Statistically significant differences were considered when p-value was smaller than 0.05.

Supporting Information

Supporting Information is available from the Wiley Online Library or from the author.

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Conflict of Interest

The authors declare no conflict of interest.

Author Contributions

J.J. and K.S.L. designed and directed this study. All authors designed and performed research. H.K., B.K., and S.-H.L. conducted the experiments using the extrusion-based printing, and X.C. performed the DLP photopatterning experiments. W.H. and K.L. contributed to use the Nano UTM and its analysis of mechanical tensile testing. H.K., B.K., X.C., J.J., and K.S.L. analyzed data, and wrote and revised the manuscript. D.-W.C. and T.B.F.W. intellectually contributed to this work.

Data Availability Statement

Research data are not shared.

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3D bioprinting technology, decellularized extracellular matrix, hydrogel, photopolymerization, scalable tissue manufacturing, tissue engineering

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