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A Photo-crosslinkable Kidney ECM-derived Bioink Accelerates Renal Tissue Formation

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Abstract

3D bioprinting strategies in tissue engineering aim to fabricate clinically applicable tissue constructs that can replace the damaged or diseased tissues and organs. One of the main prerequisites in 3D bioprinting is finding an appropriate bioink that provides a tissue-specific microenvironment supporting the cellular growth and maturation. In this respect, decellularized

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Conflict of Interest

The authors declare no conflict of interest.

extracellular matrix (dECM)-derived hydrogels have been considered as bioinks for the cell-based bioprinting due to their capability to inherit the intrinsic cues from native ECM. In this study, we developed a photo-crosslinkable kidney ECM-derived bioink (KdECMMA) that could provide a kidney-specific microenvironment for renal tissue bioprinting. Porcine whole kidneys were decellularized through a perfusion method, dissolved in an acid solution, and chemically modified by methacrylation. A KdECMMA-based bioink was formulated and evaluated for rheological properties and printability for the printing process. The results showed that the bioprinted human kidney cells in the KdECMMA bioink were highly viable and matured with time. Moreover, the bioprinted renal constructs exhibited the structural and functional characteristics of the native renal tissue. We demonstrated the potential of the tissue-specific ECM-derived bioink for cell-based bioprinting that could enhance the cellular maturation and eventually tissue formation.

Keywords

Bioprinting; kidney; bioink; extracellular matrix (ECM); decellularization; tissue engineering

1. Introduction

Current therapeutic options for end-stage renal disease (ESRD) include dialysis and kidney transplants; however, problems such as limited donor supply, graft failure, and other complications remain a concern. Bioengineering of living organ-like constructs can be an alternative to address these limitations.¹ Recently, three-dimensional (3D) bioprinting has evolved as an innovative technology capable of bioengineering of clinically applicable tissue constructs with high precision and resolution by imitating structural, anatomical, and functional features of native tissues or organs.²⁻⁴ The promising evolution in 3D bioprinting can be attributed to its ability to bioprint multiple cell types and biomaterials simultaneously, as it is designed to fabricate complex biological constructs.⁵

The hydrogel-based “bioink” is one of the major components for cell bioprinting that should provide the printability, structural integrity, and biological properties.⁶ The bioinks have been made from synthetic and naturally derived hydrogels that have their own characteristic merits and limitations. Synthetic hydrogels have the advantageous ability to allow for control of the composition and mechanical properties, while natural hydrogels can provide biological microenvironment mimicking that of the native extracellular matrix (ECM), which facilitates cell attachment, growth, and maturation.⁷ Recently, tissue-specific ECM-derived bioinks have been introduced for 3D bioprinting.⁸ These ECM materials can either be obtained from cell-derived matrices that are secreted during *in vitro* culture, or derived directly from native tissues through a decellularization process during which all the cellular components removed to avoid adverse immunological response.⁹⁻¹⁴ To utilize the tissue-derived ECM materials as bioinks, the ECM-rich materials can be solubilized to reformulate as a gel type.^{15, 16}

Importantly, the ECM provides a structural architecture that contains adhesion sites for cell surface receptors¹⁷ and preserves normal tissue function by its tissue-specific mechanical and biochemical properties.¹⁸ The interaction between cells and the surrounding ECM

regulates a variety of physiological cellular processes, including motility, migration, invasion, and proliferation.^{19, 20} Moreover, the ECM regulates signal transduction pathways by binding to integrins or by modulating the activity of signaling molecules.²¹ The ECM hydrogels are composed of the structural and functional molecules that characterize the native tissue ECM such as collagen, laminin, fibronectin, growth factors, glycosaminoglycans, glycoproteins, and proteoglycans.²² Thus, bioinks derived from decellularized tissue-specific ECM can provide these same functions as naturally occurring ECM.^{23–25}

In this study, we hypothesized that kidney ECM materials could provide renal-specific molecules and structural and biomechanical signals to regulate the renal cell behavior and, eventually, tissue maturation and formation. First, we aimed to develop a novel photo-crosslinkable kidney ECM-derived bioink formulation for 3D bioprinting of functional renal constructs without any polymeric material support. Second, we investigated whether this kidney-specific bioink containing human renal cells could provide improved renal cell functions and accelerated tissue formation. We examined the cell viability, proliferation, and renal cell functions after bioprinting of human renal cells.

2. Results and Discussion

2.1. Decellularization of porcine kidneys

Decellularization aims to remove the cellular components in tissues or organs via mechanical and/or chemical manipulation to produce ECM-rich materials. These ECM materials have been used as tissue-specific bioinks for 3D bioprinting applications.^{15, 16} Thus, a decellularization protocol is needed that is efficient, effective, and is able to maintain tissue-specific ECM components. The development of this protocol will contribute to biological integrity and facilitate the production of an ideal tissue-specific bioink material.

In this study, the porcine kidneys were treated by serial perfusion of sodium dodecyl sulfate (SDS, anionic detergent) and Triton-X 100 (non-ionic detergent) followed by nuclease enzyme to remove the cellular components (Figure 1A). The decellularized kidneys were clear in appearance while retaining the overall structural features of the native kidney. Decellularization and ECM preservation of kidneys were confirmed by histomorphological analysis (Figure 1B). In comparison with the native kidney, the cellular components were mostly removed by the decellularization process, while the inner structures, including glomerular and tubular structures, were preserved. Masson's Trichrome staining indicated the preservation of collagenous matrices in the decellularized kidney. The glycosaminoglycan (GAG) presented in the renal papillary interstitium was detected by Alcian Blue staining. The Sirius Red staining in conjunction with the Alcian Blue staining identified the collagenous bundles in the kidney. Alcian Blue/Sirius Red staining indicated the preservation of glycogen and GAG in the decellularized kidney. DNA contents after decellularization were measured to confirm the removal of cellular components in the decellularized kidney. The DNA content of the decellularized kidney was significantly lower than that of the native kidney (Figure 1C). A study indicated that less than 50 ng DNA per mg tissue dry weight can suffice to satisfy the intent of decellularization.²⁶ According to our previous work in the decellularization of whole kidneys,²⁷ we were able to obtain kidney-

derived ECM materials with complete removal of cellular components, as well as intact kidney-specific ECM.

2.2. Kidney ECM-derived hydrogel (KdECM) and KdECM methacrylate (KdECMMA)

Decellularized ECMs have been widely used as scaffold materials for tissue engineering applications. The structural and compositional features of each ECM are different, which can provide unique biochemical cues and microenvironments. Preparation of a photo-crosslinkable ECM hydrogel involves three key steps. Figure 2A shows the illustration of the entire process, including decellularization, solubilization, and methacrylation of a kidney ECM-derived hydrogel. The most prevalent method used to solubilize ECM materials is through pepsin-mediated solubilization. It is well known that pepsin can cleave the telopeptide bonds of collagen triple helix structure to unravel collagen fibril aggregates. In this study, the decellularized ECM material was lyophilized, pulverized, and dissolved in 0.5 M acetic acid solution containing 0.1 mg/mL pepsin, resulting in a kidney ECM-derived hydrogel (KdECM). The solubilized KdECM was concentrated and dialyzed to form a neutralized KdECM.

Although ECM-derived hydrogels could support the cell adhesion, proliferation, and tissue specificity, these hydrogels lack tunable biochemical properties. To overcome this limitation, we chemically modified the KdECM as a photo-crosslinkable hydrogel by methacrylate reaction, which allowed spatiotemporal control of biomechanical properties of KdECM. After methacrylate, the degree of substitution of amino groups in KdECM before and after methacrylate estimated by TNBS assay and NMR analysis indicated $79.3 \pm 3\%$ and $73.5 \pm 2\%$, respectively, of methacrylate percentage in KdECM methacrylate (KdECMMA). Methacrylate percentage can be controlled by adjusting methacrylic anhydride amounts (data not shown). In order to form a gel type, the KdECMMA was reacted with Irgacure 2959 by UV light (Figure 2B).

2.3. KdECMMA-based bioink formulation

The bioinks for the cell-based microextrusion printing should fulfill the following basic requirements: (i) relatively higher viscosity to provide homogenous cell suspension and initial structural integrity, (ii) strong shear-thinning behavior to prevent the cell damage from shear stress during the printing process, and (iii) rapid crosslinking process after printing.^{6, 28} Based on our previous studies,^{3, 29, 30} the KdECMMA-based bioink formulation was developed by combining with various hydrogel materials, including gelatin, hyaluronic acid (HA), and glycerol. Gelatin was used due to its thermo-sensitive properties: it is a liquid form above 37°C and become a solid form below 25°C. HA functions to enhance dispensing uniformity, while glycerol prevents nozzle clogging. Moreover, the photo-crosslinkable KdECMMA could provide structural stability to the printed construct and the kidney-specific microenvironment conducive to cell adhesion, proliferation, and tissue formation. After cross-linking of KdECMMA, the uncross-linked components (gelatin, HA, and glycerol) can be gradually washed out under the culture condition.

It was established that storage modulus (G') and loss modulus (G'') have independent effects on the bioink extrudability.⁶ Increasing the concentration of both KdECM and KdECMMA

in the formulations resulted in an increase in the storage and loss moduli (Figure 2C). This suggests that these bioink formulations are viscous rather than elastic. After UV crosslinking process, the gel stiffness of the KdECMMA-based bioink formulations with different concentrations was measured. The result indicates that the stiffness of the KdECMMA-based bioink formulations increased with an increase of KdECMMA concentrations (Figure 2D). The highest modulus expressed by 3% KdECMMA-based bioink formulation was 4405 ± 277 Pa. However, the gel stiffness of the KdECM-based bioink formulations could not be measured because of weak structural stability.

The printability precision values of the KdECMMA bioinks extruded at different air pressure showed that 3% KdECMMA extruded at lowest pressure with the highest precision compared to 1% and 2% KdECMMA (Figure 3A,B). The printability value calculated from a square pattern printed in the 2D plane using 2% (40 kPa) and 3% KdECMMA (40 kPa) showed *Pr* values of 0.96 and 1.05, respectively, where the ideal printability value (*Pr*=1). However, 1% (30 kPa) KdECMMA showed a less desirable printability value of 1.58. Based on this outcome, we optimized the printing parameters for the KdECMMA bioinks. Based on this experiment, we were able to print KdECMMA-based constructs (Figure 3C).

The structural stability of the printed KdECMMA constructs was analyzed for up to 14 days in the culture medium at 37°C. The 1% KdECMMA construct lost its structure as early as 7-day incubation, whereas the 2% KdECMMA maintained its structure but deformed after a 7-day incubation. The 3% KdECMMA was stable in retaining the shape without structural deformity at 14-day incubation. In addition, the 3% KdECMMA constructs showed the minimum weight loss compared with 1% and 2% KdECMMA constructs. Figure 3D shows the volume change (length, width, and thickness) of the printed constructs with 3% KdECM and 3% KdECMMA. Even though the KdECM (without methacrylate) was successfully printed with the supporting components (gelatin, HA, and glycerol), the printed KdECM-based constructs did not maintain the structural integrity at room temperature because the gelatin became a liquid form.

2.4. *In vitro* biological evaluation of KdECMMA

It has been reported that the ECM obtained from decellularized tissues influences the cellular behavior through bioactive soluble molecules and intracellular signaling activated by cell adhesion molecules.^{31, 32} Furthermore, the ECM consists of a number of tissue-specific bioactive molecules following decellularization process, including growth factors, cytokines, and a variety of proteins.^{31, 33} In this study, we examined the biological properties of the KdECM and KdECMMA using human primary kidney cells that it is a clinically relevant cell source. These cells are a heterogeneous population of cells, including proximal and distal tubular epithelial cells, podocytes, and so on.³⁴ In order to determine whether the ECM-derived hydrogel could support the cell viability, adhesion, and proliferation, the KdECM and KdECMMA were coated on the non-treated culture plates, respectively. The human kidney cells cultured on the ECM-coated plates were analyzed at 1, 3, and 5 days in culture. Additionally, gelatin methacrylate (GelMA) was used as a control, as it is the most commonly used hydrogel as a bioink in 3D bioprinting. The live/dead images indicated the cell viability on the KdECM-, KdECMMA-, and GelMA-coated plates, and over 95% cell

viability with all groups was observed (Figure 4A). The proliferation of human kidney cells on the KdECM and KdECMMA at different concentrations showed the increase of the cells with time, while the cells on the non-treated plate were decreased with time (Figure 4B). In alignment with our hypothesis, the proliferation of the cells on the KdECMMA-coated plates was significantly increased when compared with GelMA and non-treated groups ($*P<0.05$), indicating that kidney-derived ECM could provide an adequate substrate for the cell adhesion and proliferation. Moreover, the methacrylate of KdECM seemed to be more stable while maintaining the biological properties of kidney-derived ECM.

Based on the aforementioned experiments, we selected 30 mg/mL of KdECMMA for 3D bioprinting of renal constructs due to its printability, structural stability, and biological properties. For 3D bioprinting, the human primary kidney cells (Figure 5A) were mixed with the KdECMMA-based bioink formulation, and a renal construct was fabricated using our integrated tissue-organ printing (ITOP) system (Figure 3C). After printing, the bioprinted renal constructs were crosslinked by UV light and cultured in the growth medium in a CO₂ incubator for the further analyses. The live/dead staining of the bioprinted constructs with KdECMMA and GelMA was performed at 1, 5, and 14 days in culture (Figure 5B). The result indicated over 90% cell viability with both GelMA and KdECMMA bioinks (Figure 5C). The cell proliferation in the bioprinted renal constructs was determined by CCK-8 assay. The KdECMMA-based bioinks showed a significant increase in the cell proliferation when compared with GelMA-based bioinks ($***P<0.05$, Figure 5D).

2.5. *In vitro* functional evaluation of bioprinted renal constructs

To investigate whether the microenvironment provided by the kidney-specific ECM hydrogel could improve the cellular functions and functionality, we examined the bioprinted renal constructs by measuring electrolyte reabsorption and amino acid transportation activity. Sodium ion (Na⁺) is the main cation in extracellular fluid (ECF) that contributes to maintaining the normal fluid osmolarity and fluid volume. Particularly, the sodium/hydrogen exchanger is the major sodium transporter in the proximal tubular kidney cells. To evaluate the electrolyte reabsorption capability of the human primary kidney cells in the bioprinted construct, sodium uptake was detected at 2 weeks in culture by a sodium fluorescent dye. Fluorescent imaging of sodium green indicated that the bioprinted kidney cells showed a significant amount of sodium uptake (Figure 6A). Quantitatively, the percentage of sodium-positive cells in the KdECMMA constructs was significantly higher than in the GelMA constructs ($*P<0.05$, Figure 6B). The negative control (not treated with Sodium Green) showed no fluorescent intensity. The result indicated that the sodium uptake capability of the human kidney cells was improved in the renal constructs bioprinted by KdECMMA-based bioink.

Additionally, amino acid transportation into the kidney cells by hydrolase enzymes, gamma glutamyl transpeptidase (GGT) and leucine aminopeptidase (LAP), was analyzed in the bioprinted renal constructs. Hydrolases on the proximal tubular kidney cells play significant roles in the amino acid transfer.³⁵ To determine the hydrolase enzyme activity, the bioprinted renal constructs were incubated with L-glutamic acid γ -(4-nitroanilide) and L-leucine-*p*-nitroanilide. The results indicated that the human kidney cells in the KdECMMA constructs

showed significant higher hydrolase activity when compared with those in the GelMA constructs (* $P < 0.05$, Figure 6C,D). This finding suggests the importance of tissue-specific ECM bioinks that can support the functions of cells in the bioprinted tissue constructs.

2.6. Histological and immunohistochemical examination of bioprinted renal constructs

To examine whether the kidney-derived ECM could support the human primary kidney cells to form the tubular or glomerular-like structures in the bioprinted renal constructs, the histomorphological examination was performed by H&E staining and immunohistochemistry. The results indicated the cellular organization into tubular and glomerular-like structures in the bioprinted renal constructs at 2 weeks of culture (Figure 7). The cells in the bioprinted renal constructs revealed a change in the cellular organization during the culture. Moreover, the tissue formation in the bioprinted KdECMMA constructs showed better the cell growth and organization when compared with the GelMA constructs. Immunohistochemical analysis performed with tubular marker, AQP1, and glomerular marker, NPHS2, showed that the bioprinted human kidney cells maintained kidney-specific phenotype and formed tubular and glomerular-like structures in the construct. The results indicated that the KdECMMA could provide an appropriate microenvironment to support the self-assembly of the kidney cells, resulting in the formation of tubular and glomerular-like structures in the bioprinted renal constructs.

3. Conclusions

We successfully developed a photo-crosslinkable kidney-specific ECM bioink formulation for renal tissue bioprinting. This bioink formulation composed of gelatin, HA, glycerol, and KdECMMA provided the desired printability and structural integrity. More importantly, this KdECMMA-based bioink formulation offered the kidney-specific microenvironment that could support the human kidney cell maturation and tissue formation. Therefore, the bioprinted renal tissue constructs showed high cell viability and proliferation, and also exhibited the structural and functional characteristics of the native renal tissue. 3D bioprinting strategy with kidney-specific ECM bioink has great potential to bioengineer a functional renal tissue construct for use in future regenerative medicine applications. Further *in vivo* studies using KdECMMA are currently being performed in an animal model to investigate the clinical feasibility, and, additionally, the interaction between the bioprinted renal construct and host tissue.

4. Experimental Section

Decellularization of porcine kidneys:

Whole kidney decellularization was performed with minor modifications from a previous work.²⁷ Kidneys obtained from adult Yorkshire pig were collected with an intact renal artery and vein for cannulation in accordance with Wake Forest University Animal Care and Use Committee (ACUC) guidelines. Kidneys were connected to a custom-made high-throughput decellularization perfusion system with peristaltic pumps supplied with 10 USP units/mL sodium heparin (Hospira Inc., Lake Forest, IL) in phosphate buffered saline (PBS) for 15 min at 0.75 L/h (this flow rate was used for all steps). The heparin treatment was followed

sequentially by detergent solutions 0.5% sodium dodecyl sulfate (SDS) in deionized water and 1% Triton X-100/0.1% ammonium hydroxide at a flow rate of 5 mL/min for 36 h and 24 h, respectively. Kidneys were then perfused with normal saline for 72 h to remove detergent residuals and again perfused with 500 mL of DNase solution [0.0025% (w/w) DNase and 10 mM magnesium chloride in 0.1 M PBS]. The decellularized kidneys were given a final rinse with PBS for 1 h to remove residual chemicals.

To validate the decellularization process, tissue samples were fixed in 10% neutral-buffered formalin (NBF) for 48 h and dehydrated in a graded series of alcohol. The dehydrated samples were immersed in xylene and embedded in paraffin blocks. Thin tissue sections of 5 μ m thickness were cut using a microtome (Leica Microsystems, Inc., Buffalo Grove, IL), and the sections were stained for hematoxylin and eosin (H&E), Alcian Blue/Sirius Red, and Masson's Trichrome. Stained sections were observed under a light microscope Leica Microsystems DM4000B (Leica Microsystems, Inc.), and images were captured (Olympus cell sense dimension 1.16 software, Tokyo, Japan). For DNA quantification, the decellularized samples were freeze-dried using a lyophilizer (Labconco, Kansas City, MO). DNA was extracted from the dried samples using Qiagen DNeasy Blood and Tissue Kit (Qiagen Inc., Valencia, CA) and quantified by Quant-iT PicoGreen dsDNA Assay Kit (Invitrogen Corp., Carlsbad, CA) according to manufacturer's instruction. The fluorescence was measured using a spectrophotometer (SpectraMax M5 Microplate Reader, Molecular Devices, San Jose, CA) at 525 nm (excitation 490 nm). Native kidney served as a control. All chemical reagents were obtained from Sigma-Aldrich Co. (St. Louis, MO) unless stated otherwise.

Preparation of KdECM and KdECMMA:

For solubilizing the decellularized kidney matrix, the decellularized samples were chopped into approximately 5×5 mm² pieces and freeze-dried using the lyophilizer. Subsequently, the freeze-dried samples were pulverized by Cryo-Mill (SPEX Certiprep™ Cryogenic Mill 6870, Thermo Fischer Scientific, Waltham, MA) under -198°C temperature. After pulverizing, the samples were digested using 1 mg/mL pepsin in 0.5 M acetic acid solution at a ratio of 10 mg sample per mL of pepsin solution. The solution was continuously stirred for 48 h at room temperature. The solubilized kidney ECM (KdECM) was precipitated by adding sodium chloride at a final concentration of 5% in the solution. The precipitated KdECM was separated by centrifuging at 10000 rpm for 15 min. The KdECM was collected and dialyzed in deionized water (2 changes per day) at 4°C for 2 days using a dialysis tubing (molecular cutoff of 3,500 kDa). The neutralized KdECM was freeze-dried and stored at -20°C until the use.

Methacrylation of KdECM was performed for the structural integrity after bioprinting. Briefly, 1 g of KdECM was dissolved in 0.5 M acetic acid (100 mL) and adjusted to pH to 8–9 by adding 1M NaOH. Under continuous stirring, the methacrylic anhydride was added at a ratio of 2.5 mL per 1 g of protein. The solution was stirred for 2 days to allow chemical modification of KdECM methacrylate (KdECMMA), and the solution was dialyzed and freeze-dried.

The degree of methacrylate was confirmed using a 2,4,6-Trinitrobenzene Sulfonic Acid (TNBS) assay kit (Thermo Fischer Scientific) and $^1\text{H-NMR}$ analysis. For TNBS assay, KdECMMA was dissolved in 0.1 M sodium bicarbonate solution (pH=8.5) to a concentration of 200 $\mu\text{g/mL}$.³⁶ Following dissolution, 250 μL TNBS was added in 0.5 mL of the sample solution, and the solution was incubated at 37°C for 2 h. The reaction was stopped by adding 250 μL 10% SDS and 125 μL of 1N hydrochloric acid (HCl). The optical density was obtained using a spectrophotometer at 335 nm. The KdECM before methacrylation served as a control. The extent of functionalization was calculated by the difference of remaining free amino groups in KdECMMA and KdECM using the below equation:

$$\text{Degree of methacrylation} = \left(1 - \frac{\text{OD of KdECMMA}}{\text{OD of KdECM}}\right) \times 100$$

Where OD represents the optical density.

The degree of methacrylate was also determined using $^1\text{H-NMR}$ according to previously described protocol.³⁷ $^1\text{H-NMR}$ spectra were collected at 35°C in deuterium oxide at a frequency of 400 MHz using an NMR spectrometer (Bruker, Billerica, MA) with a single axis gradient inverse probe.

Formulation of KdECMMA-based bioinks:

For the bioprinting process, a KdECMMA-based bioink was formulated with gelatin, hyaluronic acid (HA), and glycerol based on the previous study⁵. The KdECMMA-based bioinks consisted of 30 mg/ml gelatin, 3 mg/ml HA, 10% (v/v) glycerol, and 10–30 mg/mL KdECMMA in serum-free Dulbecco's Modified Eagle's Medium/Nutrient Mixture F-12 (DMEM/F-12). The photoinitiator 2-hydroxy-1-(4-(hydroxy ethoxy)phenyl)-2-methyl-1-propanone (Irgacure 2959, CIBA Chemicals, Tarrytown, NY) was added at a final concentration of 0.5% to the bioink solution. A formulation containing gelatin methacrylate (GelMA) instead of KdECMMA served as a control.

Rheological properties and gel stiffness of KdECMMA-based bioink formulations:

The mechanical properties of KdECMMA-based bioink formulations were analyzed using Discovery Hybrid Rheometer DHR-2 (TA Instruments, New Castle, DE). The rheological testing (storage and loss modulus) was measured from a strain sweep at a frequency of 1 Hz and within a range of 0.02 to 1.0% strain. The viscoelastic properties of the bioink formulations were analyzed by measuring the storage and loss moduli without crosslinking. Additionally, KdECMMA-based bioink formations were cast and crosslinked using a mold to obtain discs of 1 cm in diameter and 2 mm in height. Stiffness was measured using a parallel plate contact mode at 18°C using 8 mm geometry.

Printability of KdECMMA-based Bioink:

The printability of KdECMMA-based bioinks with different concentration of KdECMMA (10, 20, and 30 mg/mL) was analyzed using our integrated tissue-organ printing (ITOP) system that contains an X, Y, Z-axis stage/controller and multiple dispensing modules.⁵ A

three-axis stage system having 200×200×100 mm³ travel and controller was used to provide motions for the printing process. The dispensing modules had a precision pneumatic pressure controller, a customized metal syringe, and micro-scale nozzle. Line patterns in two-dimensional (2D) plane with fixed printing speed (150 mm/min), nozzle diameter (460 μm), environment temperature (18°C) and different pneumatic pressure were created and imaged. The minimum pressure required to initiate extrusion from the nozzle tip was considered as the starting point and two consecutive higher pressure at 10 kPa interval were used. The images were analyzed using Image J software for measuring the printing precision using the equation $P = DN/DP$,³⁸ where DN is the diameter of the nozzle and DP represents the width of the printed lines. Printability in the 2D plane was also analyzed semiquantitatively with closed square line pattern using the following equations, derived from previously reported protocols.³⁹

$$C = \frac{4\pi A}{L^2} \quad \text{equation 1}$$

$$Pr = \frac{\pi}{4C} \quad \text{equation 2}$$

In the above equations, C represents the circularity of the enclosed space within the square pattern, L represents the perimeter, and A represents the area. The printability (*Pr*) shown in equation 2 was calculated from the circularity obtained by printing with KdECMMA at 1, 2, and 3% w/v at different air pressures (40, 50, and 60 kPa). The printability is based on the circularity formed at the center of the square design. The settings with the highest printability will give a *Pr* value of 1. Based on the printability values, a square solid construct having (6×6×1.2 mm³) was printed using KdECMMA at different concentrations (1%, 2%, and 3% w/v).

For the structural stability, we measured the volume changes of the printed constructs. We calculated the change in volume at each time point by comparing the total volume (length, width, and thickness) of the samples under the culture condition.

Human primary kidney cell culture:

The biological evaluation of KdECMMA as a bioink was performed using human primary kidney cells. The cells were isolated as previously described³⁴ and were maintained in growth medium containing 1:1 mixture of Keratinocyte Serum-Free Medium supplemented with 2.5% fetal bovine serum (FBS), 1% penicillin-streptomycin (P-S), 0.4% insulin-transferrin-selenium (ITS), 0.2% epidermal growth factor (EGF), and 0.2% bovine pituitary extract (BPE) and DMEM supplemented with 10% FBS, 1% P-S at 37°C with 5% CO₂. Cell culture medium was replaced every 3 days. All reagents for cell culture were purchased from Life Technologies (Grand Island, NY) unless stated otherwise.

In vitro biological evaluation of KdECMMA:

For biological evaluation, KdECM, KdECMMA, and GelMA were coated on Falcon® 48-well Clear Flat Bottom Not Treated Multiwell Cell Culture Plate (Corning Inc., Corning, NY). KdECM and KdECMMA were dissolved in DMEM and prepared at concentrations of 10, 20, and 30 mg/mL, respectively. GelMA and nonadherent plate without coating were used as controls. After overnight incubation at 37°C, KdECMMA, and GelMA were exposed by UV light (365 nm). Human primary kidney cells at a concentration of 1×10^5 cells per well were seeded on the plates. After 1, 3, and 5 days in culture, the cell viability and proliferation were examined by live/dead staining kit containing 2 mM calcein AM, 4 mM ethidium bromide (Thermo Fisher) and Cell Counting Kit-8 (CCK-8, Sigma-Aldrich), respectively. Live/dead cells were imaged using a fluorescence microscope (Zeiss Axiovert 200 M, Carl Zeiss, Oberkochen, Germany). For the cell proliferation, the optical density (OD) was measured at 460 nm using a microplate reader (Spectra Max M5).

To evaluate the cell viability and proliferation in the bioprinted constructs, KdECMMA and GelMA bioinks containing human kidney cells at a concentration of 10^7 /mL were printed using the ITOP system as previously described. The concentration of KdECMMA and GelMA was 30 mg/mL in the bioink formulation. The bioprinted renal constructs of $6 \times 6 \times 1.2$ mm³ were crosslinked by UV exposure for 120 s using Omnicure UV curing system. The constructs were placed in the growth medium and incubated in a CO₂ incubator for 5 days. The cell viability and proliferation were analyzed at 1 and 5 days after bioprinting, as previously described.

In vitro functional evaluation of bioprinted renal constructs:

Electrolyte reabsorption capability was determined by sodium uptake into the kidney cells in the bioprinted constructs using a Sodium Green (Life Technologies) after 2 weeks of culture.⁴⁰ Briefly, the constructs were incubated with 50 μ M ouabain for 1 h at 37°C to inhibit Na⁺/K⁺ ATPase and washed with PBS thoroughly. The samples were treated by 200 μ L of Sodium Green solution in loading buffer (90 mM NaCl, 60 mM N-methyl-d-glucamine, 2 mM NaH₂PO₄, 5 mM KCl, 1 mM CaCl₂, 1.2 mM MgSO₄, 32 mM HEPES, and 10 mM glucose) at pH 7.4. After 1-hr treatment, the samples were washed and fixed in 4% paraformaldehyde for analysis. Images were taken using a fluorescent microscope (Zeiss Axiovert 200M).

Amino acid transportation activity in the bioprinted renal constructs was measured using proximal tubule associated hydrolases gamma glutamyl transpeptidase (GGT) and leucine aminopeptidase (LAP). For GGT activity assay, the bioprinted constructs were minced into small pieces, then incubated with 200 μ L of substrate reagent containing [2.5 mM L-glutamic acid γ -(4-nitroanilide) in Tris-HCl (pH =8.5), 150 mM NaCl and 50 mM glycylglycine] for 1 h at room temperature. Next, the mixture was centrifuged at 1500 rpm for 5 min, finally, 100 μ L from the supernatant was added to 96-well plate and the absorbance was measured at 405 nm using the spectrophotometer. For LAP activity, the bioprinted constructs were minced and incubated with 200 μ L of substrate reagent contain (3 mM L-leucine-*p*-nitroanilide in PBS) for 1 h at room temperature then centrifuged at 1500 rpm for 5 min. the

supernatant (100 μ L) was added to the 96-well plate and the absorbance was measured at 405 nm. All samples are measured in triplicate.

Histological and immunohistochemical examination of bioprinted renal constructs:

The bioprinted renal constructs were collected, fixed overnight in 4% paraformaldehyde, paraffin-embedded. Cell organization and tissue formation in the construct at 1 and 2 weeks in culture were determined by H&E staining. Moreover, the bioprinted constructs were characterized by immunohistochemistry for the renal-specific marker, including anti-aquaporin 1 (Abcam, Cambridge, UK) and anti-NPHS2 (Abcam). Following deparaffinization, antigen retrieval was performed by boiling sections in citrate buffer 10 mM, pH 6, and the endogenous peroxidase was blocked using a Dako Endogenous Enzyme Block (Agilent Technologies, Santa Clara, CA). Additionally, nonspecific site binding was blocked using Dako Protein Block solution for 20 min. The sections were incubated with primary antibodies at dilution (1:500) for 1 h at room temperature and then treated with biotinylated anti-rabbit IgG (1:300) for 30 min at room temperature. The color was generated by addition of DAB chromogen (Vector Laboratories Inc., Burlingame, CA). Sections were counterstained with hematoxylin, dehydrated, mounted with a coverslip, and observed by a light microscopy (Leica Microsystems DM4000B, Leica Microsystems, Inc.).

Statistical analysis:

Data were analyzed with Student's *t*-test or one-way ANOVA using GraphPad Prism software version 3.0a (GraphPad Software, Inc., La Jolla, CA). $P < 0.05$ was considered statistically significant.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

Decellularization of the porcine kidney. (A) Gross images of decellularization process: (a) normal kidney, (b) SDS treatment for 36 h, (c) Triton X-100 treatment for 24 h, and (d) washing in saline for 72 h. (B) Histomorphogenic examination of native and decellularized porcine kidney tissues as confirmed by H&E, Masson's Trichrome, and Alcian Blue/Sirius Red staining. Scale bar = 200 μ m. (C) DNA contents before and after decellularization ($n=3$, $*P<0.05$).

Figure 2. Preparation of KdECM and KdECMMA-based bioink formulations. (A) Schematic illustration of a photo-crosslinkable kidney-specific ECM hydrogel. (B) Photography of KdECMMA before and after UV crosslinking. (C) Rheological properties (storage and loss moduli) of KdECM and KdECMMA-based bioink formulations. (D) The stiffness of the KdECMMA-based bioink formulations with different concentrations of KdECMMA after UV crosslinking ($n=3$, $*P<0.05$).

Figure 3. Printability of KdECMMA-based bioinks with different concentrations. (A) Printability testing of KdECMMA bioinks through the fabrication of heterogeneous structures in accordance with different pneumatic pressures. (B) The printing precision of KdECMMA bioinks extruded with different pneumatic pressures ($n=5$). (C) Printing code and gross images of the printed KdECMMA-based constructs. (D) The structural stability of the KdECM and KdECMMA-based printed constructs.

Figure 4.

In vitro biological evaluation of KdECM- and KdECMMA-coated plates at 1, 3, and 5 days in culture. (A) Live/dead images of human primary kidney cells (green- live, red – dead). (B) CCK-8 cell proliferation assay ($n=3$, $*P<0.05$). GelMA-coated and non-treated plates served as controls.

Figure 5. Cell viability and proliferation of human primary kidney cells in bioprinted KdECMMA constructs at 1 and 5 days in culture. (A) Human primary kidney cells, expressing renal specific markers. (B) Live/dead images of bioprinted renal constructs (green- live, red – dead). Scale bar = 50 μ m. (C) Percentage of cell viability in bioprinted renal constructs ($n=3$). (D) Cell proliferation by CCK-8 assay ($n=3$, ***, $P<0.05$).

Figure 6.

In vitro functional evaluation of bioprinted renal constructs. (A) Fluorescent images of Sodium Green expression in bioprinted constructs at 2 weeks in culture. Scale bar = 100 μ m. (B) Percentage of Sodium Green (+) cells in bioprinted renal constructs ($n=3$, $*P<0.05$). Hydrolase activity of (C) gamma glutamyl transpeptidase (GGT) and (D) leucine aminopeptidase (LAP) of bioprinted renal constructs at 2 weeks in culture ($n=4$, $*P<0.05$).

Figure 7. Histological and immunohistochemical examination. H&E images revealed the organization of human primary kidney cells in the bioprinted renal constructs into glomerular and tubular-like structures after 2 weeks in culture. Scale bar = 50 μ m. Immunohistochemical staining for AQP1 and NPHS23 showed maintenance of kidney-specific phenotype in the bioprinted renal constructs. Scale bar = 50 μ m.

Table 1.

Growth factor and cytokine compositions of kidney-derived hydrogels (*indicates value above the limit of detection)

(pg/mL)	Decellularized kidney	KdECM	KdECMMA
Amphiregulin	4.8	69.3	228.5*
BDNF	0.3	1.8	1.5
bFGF	22.3	17.4	24.6
BMP-4	0.0	16.9	0.0
BMP-5	3,407.3	2,812.0	0.0
BMP-7	237.6	1,123.5*	1,497.8*
Beta NGF	0.1	0.0	0.0
EGF	0.2	0.0	0.2
EGF receptor	1.3*	2.7*	3.0*
Prokineticin-1	4.5*	0.0	0.0
FGF-4	132.3	0.0	36.9
FGF-7	51.2*	20.7	10.2
GDF-15	0.6*	0.0	0.3
GDNF	7.3*	0.0	0.0
Somatotropin	15.4	39.7*	38.1*
HB-EGF	5.0	5.7	1.7
HGF	0.0	0.0	0.0
IGFBP-1	0.8	3.2	10.8
IGFBP-2	14.2	8.7	28.6
IGFBP-3	0.0	0.0	1,238.3*
IGFBP-4	371.9	528.9	3,670.3*
IGFBP-6	0.0	125.5	99.4
IGF-1	0.0	0.0	107.3*
Insulin	0.0	0.0	0.0
MCSF receptor	0.0	0.0	0.0
NGF receptor	2.0	6.6	9.3*
NT-3	10.3	0.0	0.0
NT-4	6.6	3.2	6.8
Osteoprotegerin	0.0	0.0	22.1*
PDGF-AA	14.6*	0.0	0.0
PIGF	0.0	0.0	0.0
SCF	0.1	0.0	4.1
SCF receptor	3.5	0.0	0.0
TGF alpha	0.0	0.0	0.0
TGF beta-1	289.0	778.8	2,586.4*
TGF beta-3	0.0	0.0	0.0

(pg/mL)	Decellularized kidney	KdECM	KdECMMA
VEGF	9.4*	0.0	3.1
VEGF receptor 2	0.0	13.3	11.6
VEGF receptor 3	0.0	0.0	0.9
VEGF-D	0.0	0.0	0.0

BMP: bone morphogenic protein, NGF: neural growth factor, HB: heparin binding, EGF: epidermal growth factor, FGF: fibroblast growth factor, GDF: growth differentiation factor, GDNF: glial cell-derived neurotrophic factor, IGFBP: insulin-like growth factor binding protein, IGF: insulin-like growth factor, MCSF: macrophage colony-stimulating factor 1, NT: neurotrophin, PDGF: platelet-derived growth factor, PIGF: placenta growth factor, SCF: stem cell growth factor, TGF: transforming growth factor, VEGF: vascular endothelial growth factor