Chapter 6

Decellularization of Rat Kidneys to Produce Extracellular Matrix Scaffolds

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Abstract

The extracellular matrix (ECM) retains three-dimensional structures for the stimulation of cell growth, with components of the ECM relatively conserved between species. Interest in the use of decellularized scaffold-based strategies for organ regeneration is increasing rapidly. Decellularized scaffolds derived from animal organs are a promising material for organ engineering, with a number of prominent advances having been reported in the past few years.

In this article we describe a simple and robust methodology for generating decellularized rat kidneys. To obtain these scaffolds, we perfuse rat kidneys with detergents through the abdominal aorta. After decellularization, kidney scaffolds are harvested for evaluation of vascular structure and histology. Qualitative evaluation involves vascular corrosion casting, transmission electron microscopy, and several different histological and immunofluorescent methods. SDS residue levels are assessed by ultraviolet-visible spectrophotometer (UV-VIS).

Key words Kidney, Decellularized scaffolds, Tissue/organ engineering, Regeneration, Extracellular matrix

1 Introduction

Chronic kidney disease is an increasing public health issue, affecting between 8 and 16 % of the global adult population [1]. Although end stage renal disease (ESRD) can be managed with dialysis, transplantation remains the only available curative treatment. However, there is a long-term requirement for immune suppression. Furthermore, the supply of donor kidneys is inadequate to meet demand, with less than 20 % of US patients [2, 3] and only 1 % of patients in China [1] with ESRD expected to receive a kidney transplant. Tissue-engineered kidney substitutes generated in vitro or in vivo could therefore offer new strategies for ESRD. Possibilities include implantation of a bioactive kidney scaffold with a three-dimensional structure for the stimulation of cell growth, with and without implanting cells. The former needs a

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bioreactor for the cultivation of cells under monitored and controlled environmental and operational conditions [4], while the latter will use organisms as an autologous bioreactor. Both concepts require a kidney scaffold with a three-dimensional (3D) structure to mimic the real kidney and stimulate cell growth.

Meezan et al. [5] first described a method for the isolation of extracellular matrix from tissues in 1975. This has allowed the exploration of decellularization. With the development of tissue engineering, decellularized (DC) scaffolds have become a sensational basic material, as it can potentially retain the 3D architecture, including the microvasculature, of the original tissue. Notable advances in the development of tissue engineering have been made in multiple organs including the kidney [6], heart [7], trachea [8, 9], liver [10], and lung [11]. Since the first report by Nakayama et al. [12] of a decellularized rhesus monkey kidney, decellularized kidneys have been obtained from a number of species including the pig [13–15], rodent [16, 17], and human [6]. In addition, based on our previous experience on transplanting kidney DC scaffolds [16], DC kidney scaffolds are able to act as inductive template for functional organ recovery, allowing the damaged area to recellularize with autologous stem cells or differentiated cells. Given the importance of maintaining renal function, the decellularization of kidney tissue has received much attention.

We have recently developed a reliable protocol to generate DC rat kidney scaffolds, which is described here. These scaffolds retain intact vascular trees and overall architecture, such as a continuous Bowman's capsule, the basement membrane of the glomerular capillaries, and mesangial matrix, but lose all cellular components. Importantly, the detergent residue is controlled within nontoxic levels.

2 Materials

2.1 Preparation of Decellularized (DC) Kidney Scaffolds

1. Deionized water (dH_2O).
2. 0.01 M phosphate-buffered saline, pH 7.4 (PBS).
3. 5 % chloral hydrate.
4. 24-gauge cannula.
5. Peristaltic pump.
6. Antibiotics: 100 U/mL penicillin and 100 ng/mL streptomycin.
7. 50 U/mL heparin in 0.01 M PBS.
8. 0.8 % sodium dodecyl sulfate (SDS) in dH_2O (w/v) (*see* **Notes 1, 2**).
9. 0.1 % Triton X-100 in dH_2O (v/v).
10. Sprague-Dawley (SD) rats, approximately 2 months of age and 200–250 g in weight.

2.2 Assessing SDS Residue Levels in DC Kidney Scaffold

1. SDS standards: Prepare eight different concentrations of SDS ranging between 0 and 200 mg/mL in dH_2O.
2. 100 mg/mL proteinase K (Biomiga, San Diego, CA, USA) in protein lysis buffer (Biomiga).
3. Ultraviolet-visible spectrophotometer.
4. High-speed centrifuge.

2.3 Vascular Corrosion Casting of DC Kidney Scaffolds

1. Acetone.
2. ABS Sudan solvent mixture: 0.5 % w/v Sudan red dye in 10 % solution of acrylonitrile butadiene styrene (ABS) in acetone (w/v).
3. ABS blue pigment mixture: 0.1 % w/v blue pigment in 10 % solution of acrylonitrile butadiene styrene (ABS) in acetone (w/v).
4. 50 % hydrochloric acid in dH_2O (v/v).
5. Stereomicroscope (with camera).

2.4 Histology and Immunofluore scence Analysis

1. Dimethylbenzene.
2. Ethanol: 100, 90, 80, 70, and 50 % v/v solutions of ethanol in dH_2O.
3. Harris hematoxylin.
4. 1 % hydrochloric acid in ethanol (v/v).
5. 1 % ammonia in dH_2O (v/v).
6. 1 % eosin in dH_2O (w/v).
7. Neutral balsam.
8. Periodic acid.
9. Schiff reagent.
10. 0.5 % sodium metabisulfite: Combine 10 mL of a 10 % aqueous sodium metabisulfite stock, 10 mL 1 N hydrochloric acid, and 180 mL dH_2O.
11. Sulfuric acid.
12. Ponceau acid magenta.
13. 1 % phosphomolybdic acid in dH_2O (v/v).
14. Aniline blue.
15. 3 % solution of hydrogen peroxide in dH_2O (v/v).
16. Sodium citrate buffer: 10 mM sodium citrate, 0.05 % Tween 20 (v/v), pH 6.0.
17. Pressure cooker.
18. 2 % acetic acid in dH_2O (v/v).
19. Block: 5 % bovine serum albumin (BSA) in PBS.

20. Primary antibodies diluted in 1 % BSA in PBS: rabbit anti-collagen IV antibody (1:200, Sigma-Aldrich, St. Louis, MO, USA), rabbit anti-laminin antibody (1:200, Sigma-Aldrich), and rabbit anti-fibronectin antibody (1:200, Sigma-Aldrich).
21. Secondary antibody diluted in 1 % BSA in PBS: goat anti-rabbit IgG-FITC antibody (1:250, Sigma-Aldrich).
22. 4′,6-Diamidino-2-phenylindole (DAPI).
23. Antifade mounting medium.
24. Bright-field microscope.
25. Fluorescent microscope with filter sets for DAPI and FITC.

2.5 Transmission Electron Microscope Observation

1. 2.5 % glutaraldehyde in 0.1 M phosphate buffer: Combine 10 mL of 25 % stock glutaraldehyde and 50 mL 0.2 M phosphate buffer, pH 7.4. Make up to 100 mL with dH_2O.
2. 1 % osmium tetroxide in dH_2O (w/v).
3. 3 % uranyl acetate in dH_2O (w/v).
4. Acetone.
5. Embedding media: Epon 812.
6. Ultramicrotome.
7. EM grids.
8. Lead citrate.
9. Electron microscope.

3 Methods

3.1 Preparation of DC Kidney Scaffolds

1. Anesthetize rats with 5 % chloral hydrate (0.6 ml/100 g) via intraperitoneal injection.
2. Open abdominal cavity by a ventral midline incision, extending from the pubis to the xiphoid process.
3. Ligate suprarenal abdominal aorta, suprarenal inferior vena cava, lumbar arteries, testicular arteries of male rats, and ovarian arteries of female rats (*see* **Note 3**).
4. Insert a 24 G cannula into the infrarenal abdominal aorta.
5. Remove bilateral kidneys with blood vessels connected.
6. Connect the cannula with the peristaltic pump to allow continuous rinsing with various detergents (*see* **Note 2**). Perfuse at approximately 8 mL/min (*see* **Note 4**) in the following order (*see* **Note 5**):
   (a) 50 U/mL heparin in 0.01 M PBS for 30 min (*see* **Note 6**)
   (b) 0.1 % Triton X-100 for 3 h (*see* **Note 7**)

(c) dH_2O for 30 min

(d) 0.8 % SDS in dH_2O (v/v) for 3 h (*see* **Notes 1** and **7**)

(e) dH_2O containing 100 U/mL penicillin and 100 mg/mL streptomycin for 24 h (*see* **Note 5**)

7. Once perfusion is complete, DC kidney scaffolds can be stored in 50 mL of dH_2O containing penicillin and streptomycin at 4 °C until use (*see* **Note 9**).

3.2 Assessing SDS Residue Levels in DC Kidney Scaffold

Residual SDS can be assessed using an ultraviolet-visible spectrophotometer (UV-VIS).

A standard curve for SDS is generated from the absorbance of eight different known concentrations of SDS. This curve is then used to calculate SDS residue in samples.

1. Prepare standard solutions of SDS in dH_2O (0–200 mg/mL).
2. Read absorbance by UV-VIS at 499 nm.
3. Plot to create a standard curve and use statistical software to calculate a regression line and equation.
4. Sponge up water inside the DC kidney scaffolds, and digest scaffolds with 100 mg/mL proteinase K (*see* **Note 10**) in protein lysis buffer at 50 °C overnight.
5. Centrifuge at 14,000 × *g* for 5 min to remove the precipitate.
6. Measure light absorption of the supernatant at 499 nm.
7. Calculate content of SDS in the supernatant by reference to the calibrated standard curve prepared above.

3.3 Vascular Corrosion Casting of DC Kidney Scaffolds

To confirm the integrity of microvasculature in the DC kidney scaffolds, we perform vascular corrosion casting at the end of perfusion in a representative normal and DC kidney.

1. At the end of perfusion, catheterize the inferior vena cava and abdominal aorta.
2. Inject 1–2 ml of acetone into DC kidney scaffolds through the inferior vena cava.
3. Pass 5 ml of 10 % acrylonitrile butadiene styrene (ABS) Sudan solvent mixture (*see* **Note 11**) through the abdominal aorta.
4. Simultaneously perfuse 10 mL of 10 % ABS blue pigment mixture via the inferior vena.
5. Harvest bilateral kidneys.
6. Cool samples in running water and corrode in 50 % hydrochloric acid in dH_2O for 1–3 days.
7. The morphology and distribution of vasculature can be observed under stereomicroscope and recorded by imaging.

3.4 Histology and Immunofluorescence Analysis

Samples are prepared for histological and immunofluorescence analyses by following standard protocols for paraffin embedding and sectioning (*see* **Note 12**).

3.4.1 Hematoxylin-Eosin Staining

1. Dry kidney sections at 60 °C, 8 h.
2. Dewax with dimethylbenzene for 30 min × 2.
3. Rehydrate with 100, 100, 90, 80, 70, and 50 % ethanol consecutively over 5 min.
4. Wash in dH_2O, 5 min.
5. Immerse in hematoxylin, 2 min.
6. Rinse under running water, 5–10 s.
7. Color separate with 1 % hydrochloric acid in ethanol for 3 s.
8. Rinse under running water, 1–2 s.
9. Promote blue with 1 % ammonia, 5–10 s.
10. Rinse under running water, 1–2 s.
11. Immerse in 1 % Eosin in dH_2O, 10 s.
12. Rinse under running water, 1–2 s.
13. Dehydrate with 80 %, 90 %, and 100 % alcohol for 2 min each.
14. Immerse in dimethylbenzene for 5 min × 2.
15. Mount with neutral balsam.
16. Examine and image slides with a bright-field microscopy.

3.4.2 Periodic Acid-Schiff Staining

1. Dry kidney sections at 60 °C, 8 h.
2. Dewax with dimethylbenzene for 30 min × 3.
3. Rehydrate with 100, 100, 90, 80, 70, and 50 % v/v ethanol in distilled water for a total of 5 min.
4. Wash in distilled water, 5 min.
5. Immerse in 0.5 % periodic acid solution, 5 min.
6. Rinse under 70 % alcohol, 5–10 s.
7. Immerse in Schiff reagent, 20 min.
8. Immerse in 0.5 % sodium metabisulfite solution, 2 min.
9. Rinse under running water, 10 min.
10. Immerse in hematoxylin, 2 min.
11. Promote blue with 1 % ammonia, 5–10 s.
12. Rinse under running water, 30 s.
13. Sequentially dehydrate with 80 %, 90 %, and 100 % v/v ethanol in distilled water for 5 min.
14. Immerse in dimethylbenzene for 10 min × 2.
15. Mount with neutral balsam.
16. Observe slides and capture images using a bright field.

3.4.3 Masson's Staining

1. Dry kidney sections at 60 °C, 8 h.
2. Dewax with dimethylbenzene for 30 min twice.
3. Rehydrate with 100, 100, 90, 80, 70, and 50 % v/v ethanol in dH_2O sequentially for a total of 5 min.
4. Wash in dH_2O, 5 min.
5. Immerse in hematoxylin, 5 min.
6. Wash in dH_2O, 10 min.
7. Immerse in Ponceau acid magenta, 5 min.
8. Immerse in 2 % acetic acid, 30 s.
9. Immerse in 1 % phosphomolybdic acid, 3 min.
10. Dye with aniline blue, 5 min.
11. Immerse in 0.2 % acetic acid, 30 s.
12. Dehydrate with 90 % and 100 % alcohol for 3 min.
13. Immerse in dimethylbenzene for 5 min × 2.
14. Mount with neutral balsam.
15. Observe slides with a bright-field microscope and capture images.

3.4.4 Immunofluorescent Staining

1. Dry kidney sections at 60 °C, 8 h.
2. Dewax with dimethylbenzene for 30 min × 2.
3. Rehydrate with 100, 100, 90, 80, 70, and 50 % ethanol sequentially for 5 min each.
4. Wash in PBS, 3 × 5 min.
5. High-pressure antigen retrieval: Heat slides to 90 kpa in citrate buffer antigen retrieval solution for 2 min, and then naturally cool to room temperature.
6. Quench with 0.3 % hydrogen peroxide for 10 min to eliminate endogenous peroxidase activity.
7. Wash in PBS, 3 × 5 min.
8. Incubate in block (5 % BSA in PBS) for 1 h.
9. Drain and wipe off excess serum and incubate in primary antibody overnight at 4 °C.
10. Wash in PBS, 3 × 5 min.
11. Incubate in secondary antibody at room temperature for 2 h.
12. Wash in PBS, 3 × 5 min.
13. Counterstain nuclei in DAPI for 10 min.
14. Wash in PBS, 3 × 5 min.
15. Mount with antifade mounting medium.
16. Observe and image slides with a fluorescent microscope using filter sets appropriate for DAPI and FITC.

3.5 Transmission Electron Microscope Observation

Transmission electron microscopy is used to characterize the microstructure of the DC kidney scaffolds, with very high spatial resolution.

1. Fix samples with 2.5 % glutaraldehyde in 0.1 M phosphate buffer overnight at 4 °C.
2. Wash in PBS, 3 × 15 min.
3. Postfix with 1 % osmium tetroxide for 1 h at 37 °C.
4. Wash in PBS, 2 × 15 min.
5. Stain with 2 % uranyl acetate, 1 h.
6. Dehydrate with 70, 80, 90, 100, and 100 % ethanol in 0.1 M PBS for 15 min each.
7. Infiltrate with a 1:1 mixture of acetone-embedding fluid, 1 h at 37 °C.
8. Infiltrate with acetone-embedding (1:4) fluid overnight at 37 °C.
9. Infiltrate with embedding fluid, 1 h at 45 °C.
10. Solidify 3 h at 45 °C and 48 h at 65 °C.
11. Use an ultramicrotome to cut ultrathin sections (80 nm) and mount on grids.
12. Double stain with a 3 % aqueous solution of uranyl acetate and lead citrate for 2 min each, with a wash in dH_2O in-between.
13. Examine sections using an electron microscope at 70 kV, and image by high-resolution CCD digital camera.

3.6 Analysis and Interpretation

After decellularization with continuous detergent perfusion, DC kidney scaffolds have a somewhat transparent appearance (Fig. 1a). Vascular corrosion casting shows that the vascular tree in the DC kidney scaffolds is well maintained compared with intact kidney (Fig. 1b, c). Standard H&E staining reveals that blue-stained nuclei are not observed, but pink-stained components are present in the DC kidney scaffolds. Control tissue is shown in Fig. 1d, e. As pink-stained components include both cytoplasm and extracellular matrices, this may reflect the morphological difference in H&E staining between intact kidney and DC kidney scaffolds (Fig. 1d, e). In addition, PAS and Masson's staining can be used to examine the ECM (e.g., scaffolds lose renal cells but keep normal vascular tree and continuous extracellular matrix). Immunofluorescence analysis shows that protein components (notably collagen IV, laminin, and fibronectin) of DC kidney scaffolds remain intact, while DAPI staining was negative in our scaffolds (Fig. 2). Our previous electron microscopy studies have shown that DC kidney scaffolds maintain an intact Bowman's capsule, glomerular capillary basement membrane, and mesangial matrix (Fig. 1h).

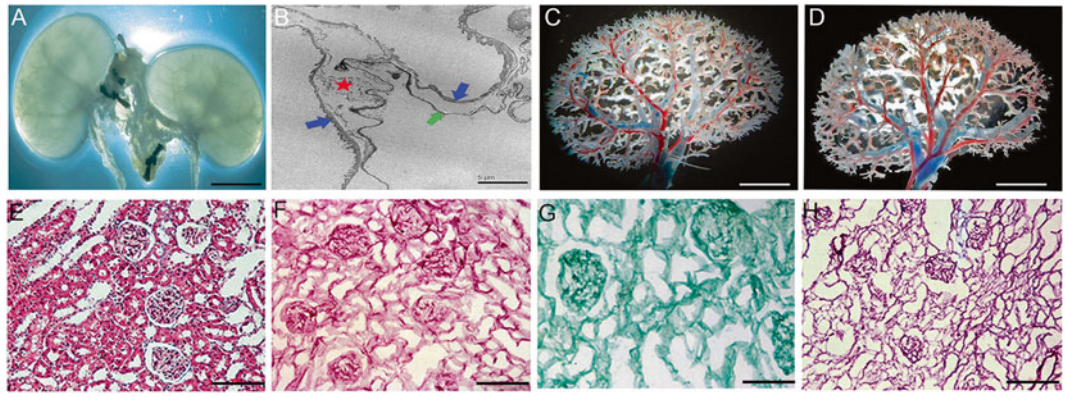

Fig. 1 Characterization of the DC kidney scaffolds. (**a**) Gross appearance of harvested DC kidney scaffolds. (**b**) Electron microscopy observation shows intact extracellular matrix in DC kidney scaffold. *Blue arrows* indicate the membrane of Bowman's capsule, a *green arrow* indicates the basement membrane of the glomerular capillaries, and a *red star* points to the mesangial matrix. (**c** and **d**) Vascular corrosion casting shows a normal vascular tree of DC kidney scaffold (**d**) compared with intact kidney (**c**). (**e** and **f**) H&E staining shows the existence of *blue*-stained nuclei in intact kidney (**e**) but not DC kidney scaffold (**f**). (**g**) Masson's staining shows that green-stained collagenous fibers in DC kidney scaffold. (**h**) PAS staining shows the presence of the ECM (e.g., basement membranes) in DC kidney scaffold. Note that the capillary loops of the glomeruli are clearly displayed. Scale bars = 5 mm (**a**, **c**, and **d**), 100 μm (**e**–**h**) and 5 μm (**b**) (Reproduced from YL Yu et al. Biomaterials 2014; 35: 6822–6828 with permission of Elsevier)

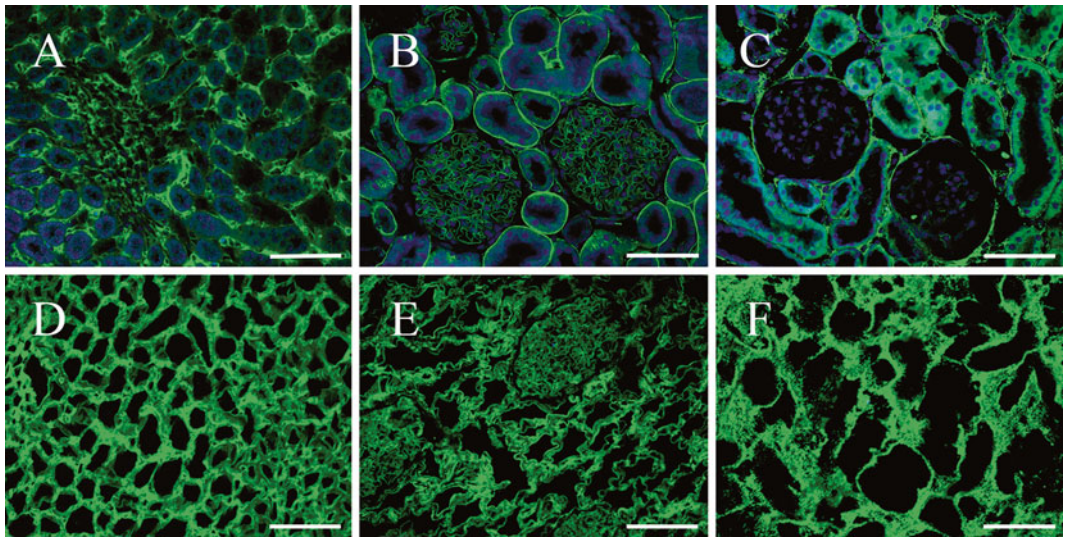

Fig. 2 Immunofluorescence of collagen IV, laminin (LN), and fibronectin (FN). (**a**–**c**) Native kidney. (**d**–**f**) Decellularized kidney scaffolds. (**a** and **d**) Scale bars = 100 μm. (**b**, **c**, **e**, and **f**) Scale bars = 50 μm. In all panels, cell nuclei stain was *blue* with DAPI, while fluorescent immunohistochemical staining for specific markers appears *green*. These indicate the intact kidney architecture and matrix proteins are retained and undisturbed, while cells and nuclear material are removed compared with the native

Given that SDS is toxic to cells, it is important to confirm the absence of SDS residues in the scaffolds. In our studies, the concentration of SDS residues in DC kidney scaffolds is typically 50.0 ± 1.7 μg/g, which is well below the toxic level of 133.3 μg/g.

4 Notes

1. In the decellularization process, it is very important that the detergent used is sodium dodecyl sulfate, not sodium dodecyl sulfonate.
2. For decellularization, we don't use trypsin, as it will damage the structural proteins severely.
3. Before cannulation, make sure that ligated vessels include the suprarenal vena cava, suprarenal abdominal aorta, lumbar arteries, testicular arteries of male, and ovarian arteries of female animals.
4. A higher or lower flow velocity is not suitable.
5. All of the fluids for decellularization should be preheated to 25–30 °C.
6. After abdominal aorta cannulation, perfuse with heparin in PBS for 30 min to prevent blood clotting.
7. The concentration of SDS and Triton X-100 must be checked, as elevated SDS and Triton X-100 will damage the extracellular matrix microstructure. Conversely lower detergent concentrations do not work well.
8. Given that SDS is toxic to cells, it is necessary to ensure that there is sufficient perfusion of deionized water to remove all traces of SDS.
9. The prepared DC scaffolds can be preserved for 1 week at 4 °C and 2 months at −80 °C.
10. To detect SDS residue, the samples must be digested completely.
11. Avoid over injection of pigments into the scaffolds when preparing vascular corrosion casts.
12. Sections for histology and immunofluorescent analysis should be approximately 5 μm in thickness.

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