参赛队员姓名：朱昀天

中学：合肥一中美国高中

省份：安徽

国家/地区：中国

指导教师姓名：丁卫平

指导教师单位：中国科学技术大学

论文题目：Construction of Fully Structured Scaffold-Free Renal Tubule

Contents

Construction of Fully Structured Scaffold-Free Renal Tubule

Yuntian Zhu

Sino-US Curriculum Program of Hefei No.1 High School, Hefei, Anhui, China

Abstract

The renal tubule is an indispensable part of the human kidney, and it performs multiple functions in human physiological activities. Renal tubules created *in vitro* can offer a significant implement for drug nephrotoxicity testing. However, the constructed renal tubules that were previously reported in the literature were still relatively simple and could hardly elaborate the unique structure with thick proximal and distal tubules and thin medullary loop. Herein, in this work, we constructed a fully structured scaffold-free renal tubule (FSSRT) that *in vitro* replicated the segmented structure of the human renal tubule *in vivo*. First, an alginate hollow tube that is thin in the middle and thick at the ends was fabricated using the coaxial coextrusion technology with well-programmed flow parameters. Then, renal epithelial cells (RECs) were cultured on the outer surface of the printed tubule. Next, the cell-laden tube was embedded in collagen in a petri dish, and the cell culture was continued for 24 hours. Finally, the alginate tube was degraded with ethylene diamine tetraacetic acid (EDTA) and the fully structured scaffold-free renal tubule was then formed preliminarily. In the process, the alginate hollow tube was characterized with experiments and the viability of the cultured cells was also studied preliminarily. Our experimental results suggested that the FSSRT fabricated by us has the potential to model and reveal the features of the fully structured renal tubule. This study provides insights into the development of the biomimetic kidney and is promising for future application in tubule-related diseases.

Keywords: Renal tubule, Scaffold free, Coaxial coextrusion technology, Alginate hollow tube

1. Introduction

The kidney is a vital organ consisted of millions of nephrons. The renal tubule is an important part of the nephrons and is mainly responsible for the reabsorption of various molecules and the regulation of physiological state including fluidic balance or blood pressure [1]. The renal tubule is segmented into the proximal tubule, the loop of Henle and the distal tubule (**Figure 1**). In terms of diameters, the proximal and distal tubules are thick while the loop of Henle is thin [1]. Studies show that most kidney diseases resulted from renal tubule dysfunction [2]. To understand the function of the renal tubule, many efforts have been made using *in vitro* models of the renal tubule.

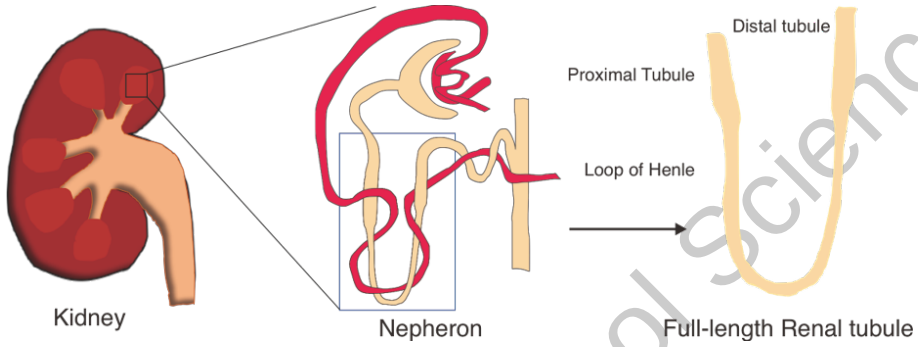

Figure 1. Schematic of human's kidney, nephron and renal tubule

In the past two decades, various renal tubule chips have been developed. However, these chips usually adopted porous membranes as the supporting scaffold for cells [3]. Recently, the bio-printing technology has been developed rapidly to fabricate *in vitro* renal tubule models. For example, Gao et al. produced tubular structures using sodium alginate based on the coaxial technology [4], Homan et al. presented the application of bio-printed renal proximal tubule models [5], Lin et al printed a proximal tubule model with a blood vessel [3], and Singh et al. produced renal tubule with monolayer and bilayer cells [6]. However, most of these reported bio-printed renal tubules needed some biomaterials as a scaffold, and thus they were not cell self-assembly tubular structures. Moreover, the current *in vitro* models usually only focused on a specific section of the renal tubule [7], rather than the complete renal tubule. Because of the structure and cell specificity of the renal tubule, it is difficult to construct a cell-assembly tubular structure simultaneously containing the proximal tubule, the loop of Henle, and the distal tubule.

In this work, we constructed a biomimetic renal tubule with a full structure based on the coaxial coextrusion technology. The fully structured scaffold-free renal tubule (FSSRT) was developed using collagen, with sodium alginate as sacrificial material and human renal epithelial cells (RECs) cultured inside. Firstly, an alginate hollow tube that is thin in the middle segment and thick at both ends was fabricated using the rate-tunable coaxial coextrusion technology. Secondly, RECs were seeded and cultured on the outer surface of the hollow tube and then the cell-laden hollow tube was embedded

into collagen gel in a pattern of U shape (the *in vivo* morphology of renal tubule). Finally, the alginate tube was degraded by ethylene diamine tetraacetic acid (EDTA) pumped through the lumen of the hollow tube, and the FSSRT with human renal tubule structure was developed after cell assembly.

2. Materials and Methods

2.1 Printing of Alginate Hollow Tube

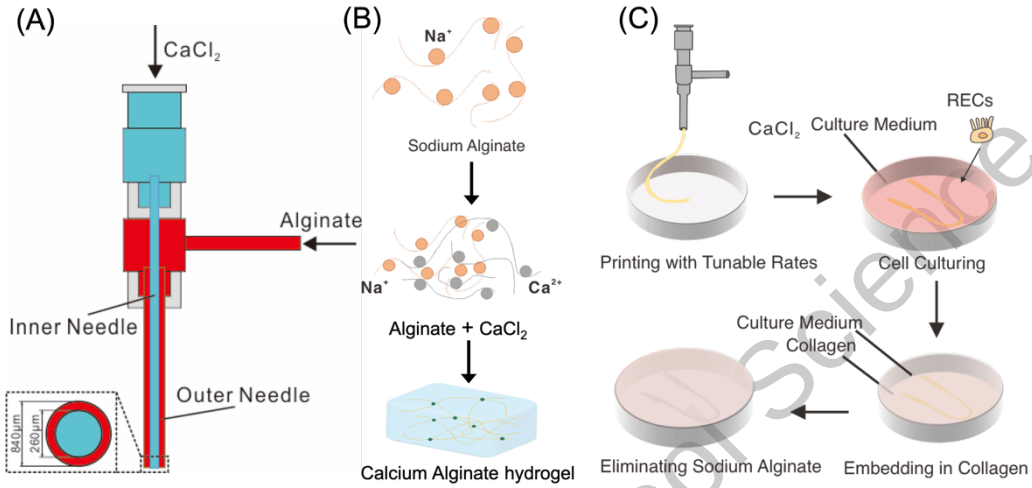

Figure 2. Schematic of the construction of the FSSRT: (**A**) schematic of the coaxial device, (**B**) formation of calcium alginate hydrogel, and (**C**) main experimental procedure.

The alginate hollow tube in this study was printed using the coaxial coextrusion technology. The coaxial nozzle is the crucial device (inner diameter of outer needle: 840 μm; inner diameter of inner needle: 260 μm) (Hefei Sipin Technology Co., Ltd., Hefei, China) **(Figure 2A)**. Since sodium alginate can crosslink with calcium ion (Ca^{2+}) and form alginate calcium hydrogel (**Figure 2B**) [8], in this study, the outer needle of the coaxial device was perfused with sodium alginate solution, and the inner needle was perfused with $CaCl_2$ solution. Here, sodium alginate solution and $CaCl_2$ solution both have a concentration of 3% (w/v). The specific bio-printing procedure is described as follows: first, sodium alginate powder was exposed to ultraviolet (UV) light for 4 hours and then mixed with sterilized deionized water, and $CaCl_2$ solution was sterilized at 121 °C; then, the alginate solution and $CaCl_2$ solution were loaded into two syringes, respectively, and the syringes are connected with the coaxial device through flexible pipes; finally, the alginate solution and $CaCl_2$ solution were simultaneously pumped using two syringe pumps (New Era Pump Systems Inc., NY, USA), and the printed alginate hollow tube was immersed in $CaCl_2$ solution in a petri dish for sufficient crosslinking (**Figure 2C**). It should be noted that to simplify the experiments, the flow rates of alginate solution and $CaCl_2$ solution were set to be within the ranges suggested by the literature [4].

2.2 Cell Culture

In this study, RECs stored in the lab were used. RECs were cultured in Dulbecco's modified Eagle's medium (DMEM; Gibco Inc., NY, USA) supplemented with 10% fetal bovine serum (Life Science Products & Services, Australia) and 1% penicillin-streptomycin (Sangon Biotech Co., Ltd., Shanghai, China) using a petri dish (Corning, NY, USA) 100 mm in diameter, and maintained in a 37 °C and 5% CO_2 humid cell incubator (Thermo Fisher Scientific, MA, USA). Before being used, cells were trypsinized using 0.25% trypsin-EDTA solution (Gibco Inc., NY, USA) for 1.5 minutes, and centrifuged at 200 g for 5 minutes. After centrifugation, the supernatant was discarded and cells were re-suspended in fresh DMEM for usage.

2.3 Analysis of Cell Viability

Calcein-AM (Shanghai Aladdin Biochemical Technology Co., Ltd., Shanghai, China) and propidium iodide (PI; Sangon Biotech Co., Ltd., Shanghai, China) staining was used to show the viability of cells. Briefly, firstly, the cell-laden alginate hollow tube was washed with phosphate-buffered saline (PBS) for three times; then, RECs on the outer surface of the alginate hollow tube were stained with Calcein-AM and PI dyes for about 20 minutes at 37 °C in darkness; finally, the tube was washed with PBS again before being imaged using an inverted fluorescence microscope.

2.4 Analysis of Cell Structure and Function

Cytoskeleton and ATPase were analyzed by cell staining to show the structure and function of cells. For the ATPase staining, the procedure is as follows: firstly, the alginate tube was washed with PBS for three times, and RECs on the alginate tube were fixed using 4% paraformaldehyde; secondly, RECs were washed with PBST (0.05% Tween in PBS), permeabilized with 0.2% Triton-100 for almost 10 minutes; then, cells were treated with 2% bovine serum albumin (BSA) solution for about 2 hours after washing with PBST; subsequently, cells were incubated with ATPase mouse antibody (Proteintech Group Inc, Wuhan, China) overnight; finally, cells were stained with Alexa Fluor 594 secondary antibody (Proteintech Group Inc, Wuhan, China) for about 40 minutes at room temperature. For cytoskeleton staining, cells were fixed, washed, and permeabilized the similar steps as above, and then labeled with phalloidin-FITC (Shanghai Maokang Biotechnology Ltd., Shanghai, China) and Hoechst 33342 (Sangon Biotech Co., Ltd., Shanghai, China) dyes for almost 20 minutes at 37 °C. Images were taken using the inverted fluorescence microscope after cells were washed with PBST.

2.5 FSSRT Construction

Rat-tail collagen (Type I; Solarbio Technology Co., Ltd., Beijing, China) was employed to construct the FSSRT. The collagen gel was prepared as follows: firstly, 2 mL of collagen solution (5 mg/mL) was well mixed with 1.2 mL of PBS solution; secondly, the above mixture was well mixed with 800

μL of 5 × PBS solution that contained phenol red; thirdly, the collagen mixture was transferred to a precooled centrifugal tube that contained 50 μL of 0.1 M NaOH solution to adjust pH; finally, the collagen mixture was quickly added to a petri dish (100 mm in diameter) for the cell-laden tube. The experimental process should be operated on ice.

In the process of embedding the alginate hollow tube into the collagen-coated petri dish, firstly, the collagen mixture was directly dripped on the tube; then, the tube was submerged in the collagen mixture; next, the petri dish was placed in a 37 °C cell incubator for 15 minutes to form the collagen gel, and finally, fresh DMEM was added into the dish for culture. It should be noted that the alginate tube needed to be completely submerged in the collagen mixture, except the small segments at both ends (the ports at two ends of the tube were used to load EDTA solution)

In the process of degrading alginate tube and forming the FSSRT, firstly, 2 mL of EDTA solution (25 mM) was loaded into a syringe; then, a port at two ends of the alginate tube was chosen as the inlet of EDTA solution, and the syringe was gently inserted into the port; finally, EDTA solution was slowly injected into the lumen of the alginate tube to degrade alginate gel. In the experiments, it took about 10 minutes for the alginate hollow tube to degrade sufficiently. After the alginate tube was degraded, fresh DMEM was added into the petri dish and the dish continued to be cultured for several days to allow cell growth and assembly to the FSSRT.

3. Results and Discussion

3.1 Alginate Hollow Tube Fabricated

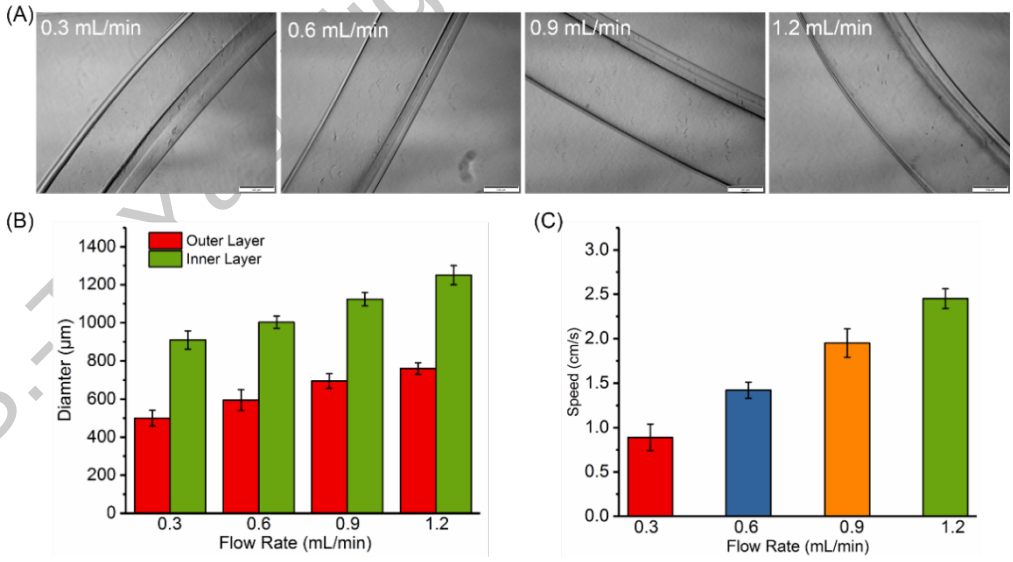

Figure 3. Characterization of alginate hollow tubes: (**A**) bright-field images of the tube fabricated at different flow rates (scale bar is 500 μm), (**B**) relation between flow rate and tube diameter, and (**C**) Relation between flow rate and printing speed.

In order to choose the suitable flow rates specifically for the three segments in a renal tubule (*i.e.*, the proximal tubule, the loop of Henle, and the distal tubule), we printed alginate tubes at various flow rates (**Figure 3A**). It could be seen that the alginate tube was hollow and the tube diameter varied with the flow rate. The inner and outer diameters of the printed tubes were detected under different flow rates (**Figure 3B**). The statistical results showed that the faster the flow rate is, the larger the tube diameter is, which is in accordance with the literature [3]. When the flow rates of sodium alginate solution and $CaCl_2$ solution were both 0.6 milliliters per minute (mL/min), the outer and inner diameters of the alginate hollow tube were approximately 1000 μm and 600 μm, respectively; when the flow rates were both 1.2 mL/min, the outer and inner diameters of the alginate hollow tube were approximately 1300 μm and 800 μm respectively. In addition, the speed of the bio-printing increased with the flow rate (**Figure 3C**). In the experiments, it was found that when the flow rate was too low, the morphology of alginate tubes was unstable and the printing speed was slow. Based on these experimental results, we, in the following experiments, empirically set the flow rate for printing the two thick segments (*i.e.*, the proximal tubule and the distal tubule) of alginate tubes as 1.2 mL/min, and chose the flow rate for the thin middle segment (*i.e.*, the loop of Henle) as 0.6 mL/min.

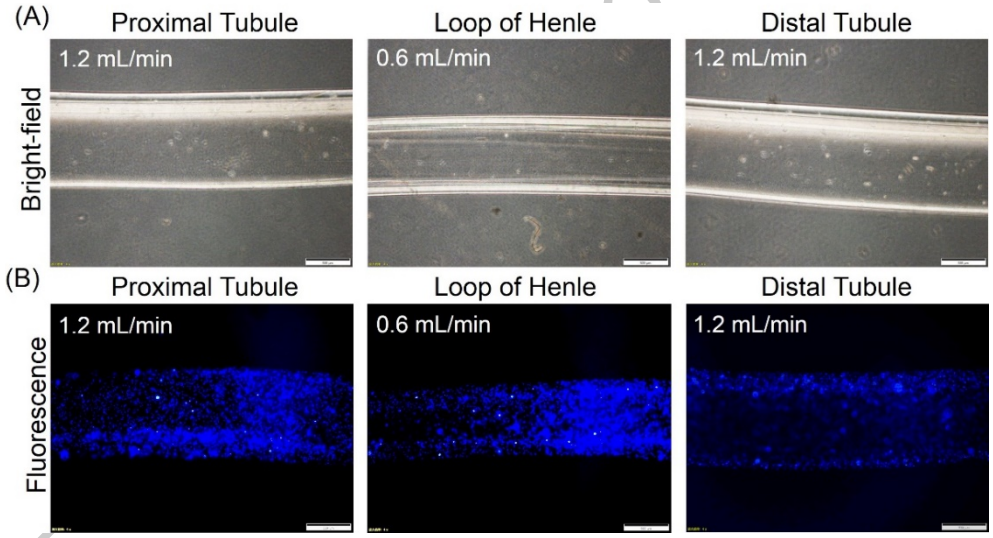

Figure 4. Alginate hollow tube fabricated at 0.6 mL/min and 1.2 mL/min, respectively, for proximal/distal tubules and loop of Henle: (**A**) bright-field images of the alginate tubes, and (**B**) fluorescence images of alginate hollow tubes (scale bar is 500 μm).

In this study, the alginate hollow tube with thin middle segment and thick segments was printed at 1.2 mL/min for 4 seconds, 0.6 mL/min for 8 seconds and 1.2 mL/min for 4 seconds, and the obtained tube has a length of about 15cm. The thin middle segment was about 9 cm long. The thick segments of the alginate tube for the proximal and distal tubules, and the thin segment for the loop of Henle are presented in **Figure 4A**. Additionally, blue fluorescent microbeads were added into sodium alginate to demonstrate the tube (**Figure 4B**). The clear contour lines of the tubes can be seen from the fluorescence images. The integrated results suggested that the alginate tube with a thin middle

segment and two thick ends was successfully obtained by changing the flow rates of the bio-printing. Meanwhile, it should be noted that the printing experiments of alginate tube need to be performed under sterile environment to avoid contamination.

3.2 Cell Seeding/Coating

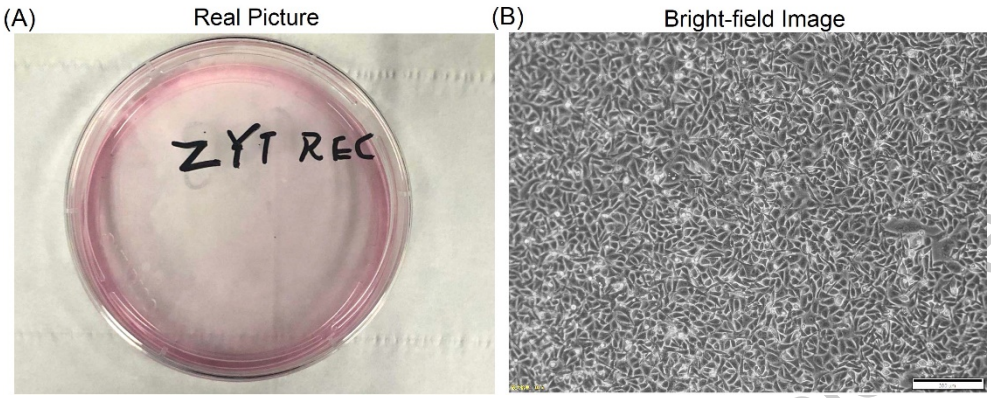

Figure 5. RECs used in this study: (**A**) real picture and (**B**) bright-field image of RECs (scale bar is 200 μm). The results showed that the RECs grew well and were appropriate for the subsequent application.

To fabricate the FSSRT, we firstly need to seed and culture cells on the printed alginate hollow tube. RECs cultured in the petri dish (**Figure 5**) were trypsinized and re-suspended in DMEM at a concentration of 5×10^6 cells/mL. Before cell seeding/coating, the printed alginate hollow tube was washed with PBS for 3 minutes. To make RECs adhere to the outer surface of the alginate tube, we immersed the tube in gelatin methacrylate (GelMA), which is a kind of modified gelatin and friendly to cell growth [9]. The tube was then exposed to UV light for 36 seconds to make GelMA crosslink. Thereafter, we directly dripped RECs on the outer surface of the alginate hollow tube.

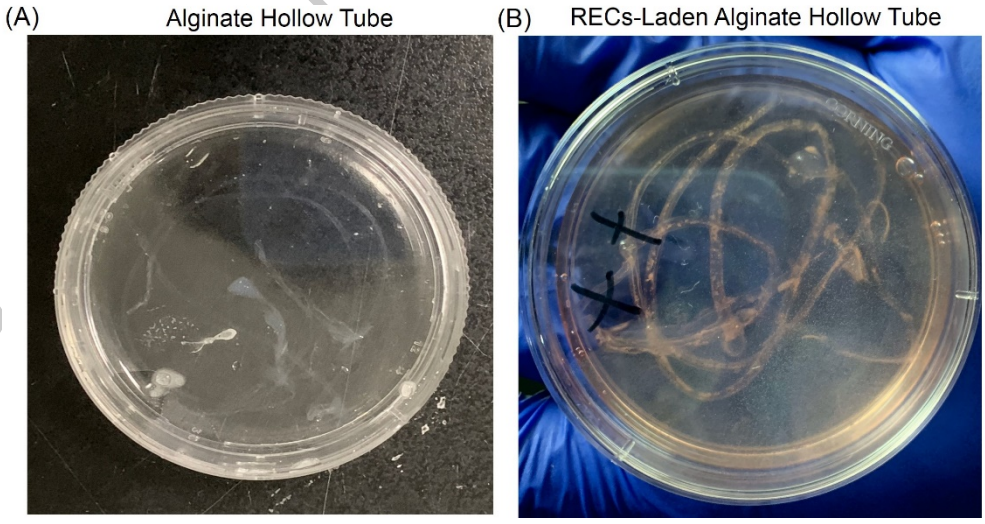

Figure 6. Real pictures of alginate hollow tube (**A**) before and (**B**) after seeding RECs.

After being cultured for two hours in an incubator, RECs outside the printed tube attached to the tube; then fresh DMEM was added into the petri dish. The pictures of the tubes without cells (**Figure 6A**)

and with RECs (**Figure 6B**) were recorded and compared. The cell-laden alginate tube was cultured for 5 days, and pictures of the tube were taken on day 3 and day 5, respectively (**Figure 7A**). The results showed that RECs rapidly proliferated. The fluorescent images of the RECs stained with DiI (*i.e.*, the cell membrane probe) in the thick and thin middle segments of the tube were also photographed (**Figure 7B**). The experimental consequence showed that it was suitable for the alginate tube to make RECs locate on its outer surface.

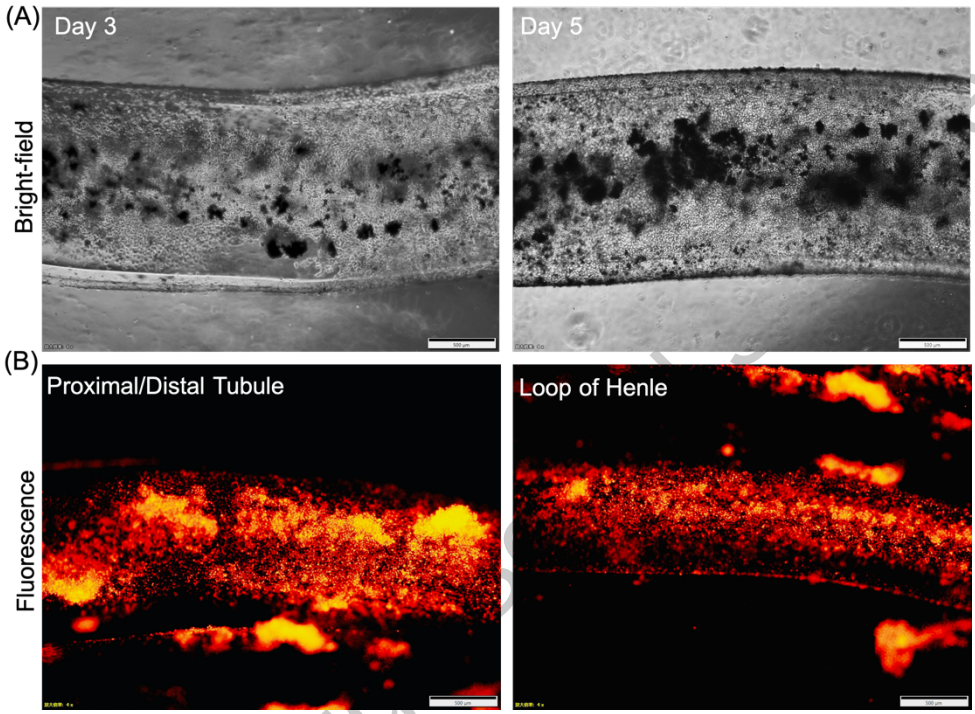

Figure 7. Cell-laden alginate hollow tubes: (**A**) growth of RECs cultured on the alginate tube, and (**B**) images of RECs labeled with DiI dye (scale bar is 500 μm).

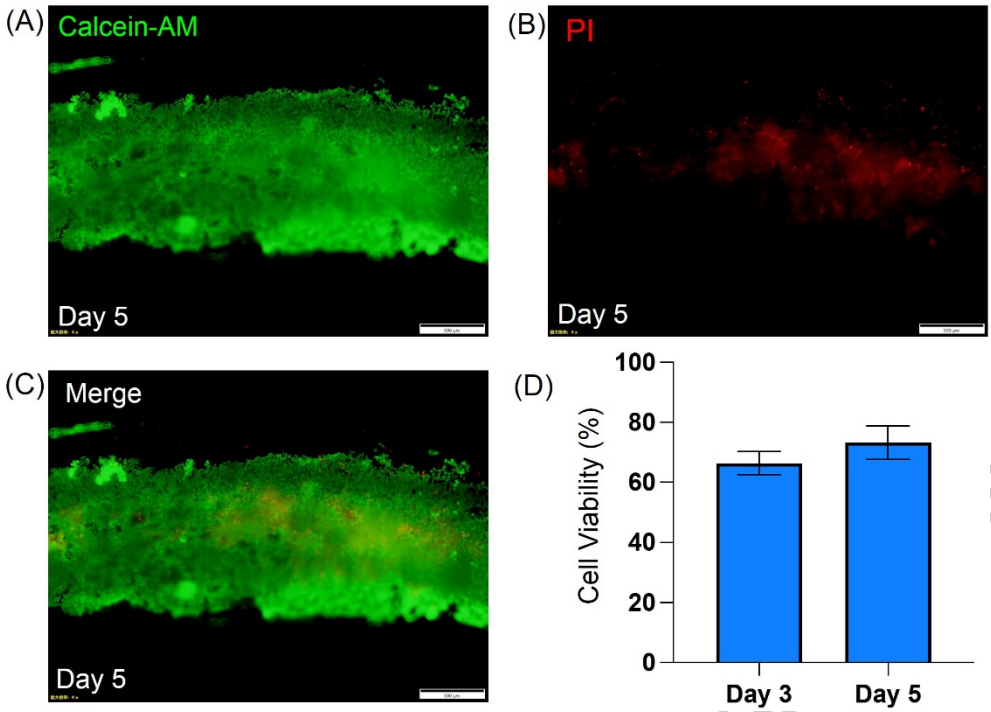

Figure 8. Cell viability of RECs on the alginate hollow tube. Images of Cells stained with (**A**) Calcein-AM and (**B**) PI, and (**C**) merged image of Calcein-AM and PI stained cells (scale bar is 500 μm). (**D**) Cell viability.

3.3 Viability of Seeded Cells

To demonstrate the growth of RECs, we analyzed the viability of cells. In the imaging results (**Figure 8**), the living cells stained with Calcein-AM appeared green while the dead ones stained with PI appeared red. It can be seen that most of the cells were green (**Figure 8A**) and only a few of them were red (**Figure 8B**), indicating that most of the cells on the alginate tube were living after being cultured for 5 days (**Figure 8C**). In addition, the cell viability reached about 75% on day 5 (**Figure 8D**). These results suggested that RECs cultured on the surface of the alginate tube maintained a relatively high cell viability during the culturing.

3.4 Cell Structure and Function

To present the structure and function of RECs cultured on the alginate hollow tube, cytoskeleton and ATPase were characterized using cell staining, respectively. The fluorescent images of F-actin (green), Hoechst (blue) and ATPase (red) staining are shown in **Figure 9**.

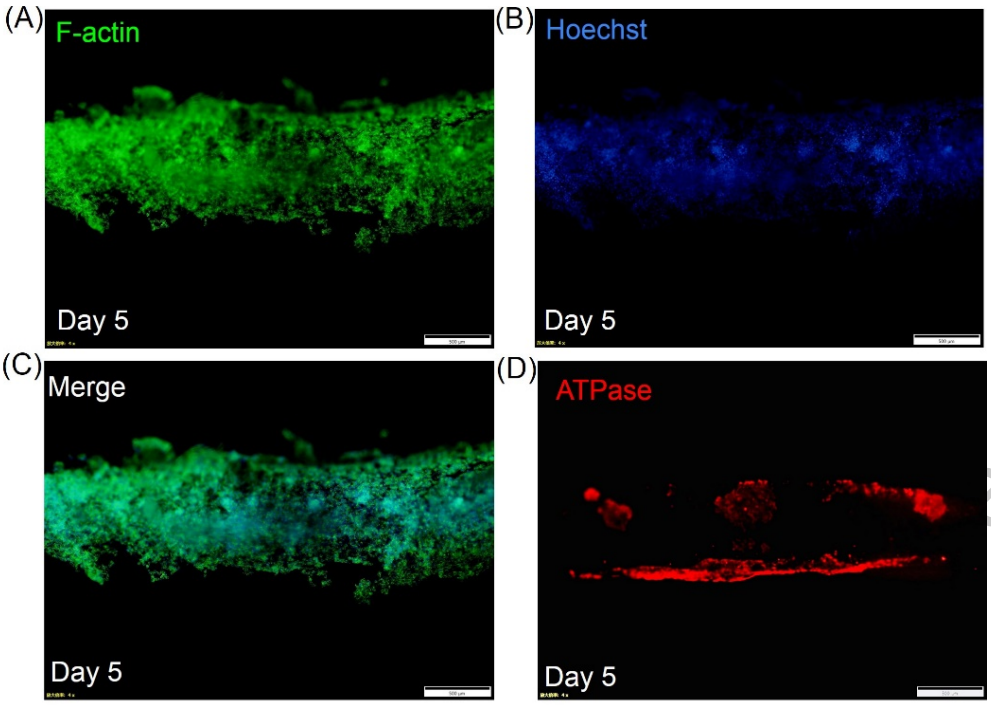

Figure 9. Cell structure and function. Images of RECs that were stained with (**A**) Phalloidin and (**B**) Hoechst 33342, (**C**) merged image of F-actin and Hoechst stained cells, and (**D**) image of RECs that were stained with ATPase antibody (scale bar is 500 μm).

The filamentous actin (F-actin) cytoskeleton regulates the change of cell shapes and the mechanical force generation in cell division and migration [10]. The staining image of F-actin in **Figure 9A** showed that RECs had a polygonal cytoskeleton structure. Meanwhile, cell nuclei of RECs on the alginate tube could be seen clearly (**Figure 9B**), and these cell nuclei were located in the F-actin networks (**Figure 9C**). These results showed that RECs cultured on the alginate hollow tube had a complete cytoskeleton structure.

ATPase can decompose adenosine triphosphate (ATP) into adenosine diphosphate (ADP), which is crucial for the energy generation in cells. The ATPase of RECs was successfully stained (**Figure 9D**), indicating that RECs had the ability to consume energy. Thus, the cumulative results demonstrated that RECs cultured on the alginate tube had intact cellular structure and demonstrated routine cellular function; in other words, the alginate that was chosen as sacrifice material to construct FSSRT had little influence on the cytoskeleton and function of RECs.

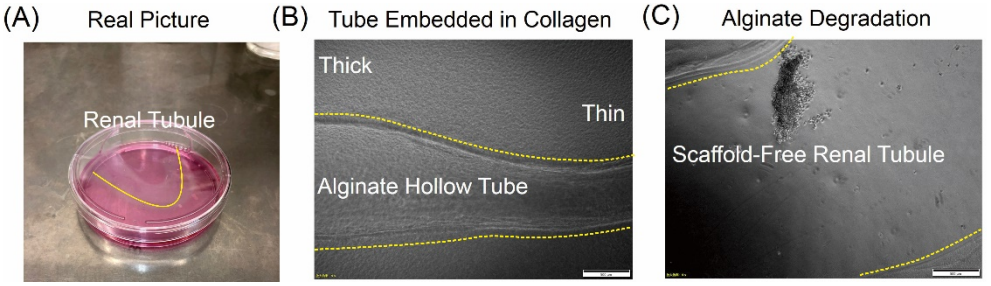

Figure 10. Construction of FSSRT: (**A**) real picture of the embedded tubule, (**B**) alginate hollow tube embedded in the collagen gel, and (**C**) image of the FSSRT after alginate tube degradation (scale bar is 500 μm).

3.5 FSSRT Formation

To construct the FSSRT, the alginate tube embedded in collagen gel was degraded with EDTA after the RECs on the outer surface of the tube were cultured (**Figure 10A**) and attached to the collagen gel. The alginate tube embedded in the collagen gel is shown in **Figure 10B** while the FSSRT we constructed is illustrated in **Figure 10C**. It can be seen that alginate tube was fully embedded in the collagen gel and some RECs were located in the inner surface of the FSSRT. However, the constructed FSSRT still needs further optimization. In experiments, the lumen of the FSSRT became much wider and the number of cells in the lumen was small after the alginate hollow tube was degraded. This is probably because the high flow of EDTA solution washed some of the collagen and cells away. In our future work, we will optimize the experiment method of alginate degradation to improve the FSSRT structure and shorten the time of cell growth and assembly. In addition, we will culture different cells on specific segments of the alginate tube (*e.g.*, renal chief cells for distal tubule, and renal epithelial cells for proximal tubule and loop of Henle), implant the renal tubule-associated peritubular capillary to further mimic human renal tubule region and make sure that the reabsorption and regulation functions of the developed renal tubule can be presented successfully.

4. Conclusion

In this study, we, for the first time, fabricated a fully structured scaffold-free renal tubule using cell-laden alginate hollow tube with thin middle segment and thick segments at both ends as sacrifice material. The rate-tunable coaxial coextrusion technology was used to develop the alginate hollow tube with different diameters of the tubule. Renal epithelial cells were cultured on the outer surface of the alginate hollow tube, and then the tube was embedded in the collagen gel. After the alginate tube was degraded, the renal epithelial cells attached to the collagen and proliferated to form the bionic renal tubule. This study provides novel ideas for the construction of *in vitro* renal tubule model.

References

[1] Urry LA et al. Campbell Biology, Eleventh edition. Pearson Higher Education, 2016.

[2] Liu BC, Tang TT, Lv LL, Lan HY. Renal tubule injury: a driving force toward chronic kidney disease. Kidney International. 2018 Mar; 93: 568-579.

[3] Lin NYC, Homan KA, Robinson SS, Kolesky DB, Duarte N, Moisan A, Lewis JA. Renal reabsorption in 3D vascularized proximal tubule models. Proceedings of the National Academy of Sciences of the United States of America. 2019 Mar; 116: 5399-5404.

[4] Gao Q, He Y, Fu JZ, Liu A, Ma L. Coaxial device-assisted 3D bioprinting with built-in microchannels for nutrients delivery. Biomaterials. 2015 Aug; 61: 203-15.

[5] Homan KA, Kolesky DB, Skylar-Scott MA, Herrmann J, Obuobi H, Moisan A, Lewis JA. Bioprinting of 3D Convoluted Renal Proximal Tubules on Perfusable Chips. Scientific Reports. 2016 Oct; 6: 34845.

[6] Singh NK, Han W, Nam SA, Kim JW, Kim JY, Kim YK, Cho DW. Three-dimensional cell-printing of advanced renal tubular tissue analogue. Biomaterials. 2020 Feb; 232: 119734.

[7] Fransen MFJ, Addario G, Bouten CVC, Halary F, Moroni L, Mota C. Bioprinting of kidney in vitro models: cells, biomaterials, and manufacturing techniques. Essays in Biochemistry. 2021 Jun; 65: 587–602.

[8] Rastogi P, Kandasubramanian B. Review of alginate-based hydrogel bioprinting for application in tissue engineering. Biofabrication. 2019 Sep; 11: 042001.

[9] Gungor-Ozkerim PS, Inci I, Zhang YS, Khademhosseini A, Dokmeci MR. Bioinks for 3D bioprinting: an overview. Biomaterials Science. 2018 May; 6: 915-946.

[10] Stricker J, Falzone T, Gardel ML. Mechanics of the F-actin cytoskeleton. Journal of Biomechanics. 2010 Jan; 43: 9-14.

Acknowledgements

This work was conducted under the supervision of Prof. Weiping Ding in his lab at USTC. Therefore, I would like to first thank Prof. Ding for offering the opportunity to do this research, helping me select the research topic, supplying me the lab facility for experiments, and providing kind guidance throughout the study (especially for the paper writing). Meanwhile, I also would like to thank the graduate students and postdocs in Prof. Ding's group, especially Dr. Chengpan Li, for their kind help on experiments (they taught me many experimental skills and helped me solve a lot of problems in experiments) and paper writing.