Reconstruction of tracheal window-shape defect by 3D printed polycaprolatone scaffold coated with Silk Fibroin Methacryloyl

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Abstract

Tracheal resection and end-to-end anastomosis has been the standard clinical approach for the treatment of most airway diseases, especially invading the lower trachea or carina. However, when long-length (exceeding 2 cm in children or 5 cm in adults) tracheal circular resection is performed, tracheal replacement therapy is often required. In this study, we aimed to utilize autologous tracheal epithelia and bone marrow mesenchymal stem cells (BMSCs) as the seeding cells, utilize polycaprolactone (PCL) coated with Silk Fibroin Methacryloyl (SilMA) as the scaffold to carry the cells and Kartogenin (KGN). Firstly, SilMA with the concentration of 10%, 15% and 20% was made, and the experiment of swelling and degradation was performed. With the increase of the concentration, the swelling ratio decreased, the degradation progress slowed down. Upon the result of CCK-8 test and HE staining of 3D co-culture, the 20% SilMA was selected. Next, SilMA and the cells attached to SilMA were characterized by scanning electron microscopy (SEM). Furthermore, in vitro cytotoxicity test shows that 20% SilMA has good cytocompatibility. The hybrid scaffold was then made by PCL coated with 20% SilMA. The mechanical test shows this hybrid scaffold has better biomechanical properties. In vivo tracheal defect repair assays were done to evaluate the effect of the hybrid substitution. H&E staining, immunohistochemical (IHC) and immunofluorescence (IF) staining showed that this hybrid substitution ensured the viability, proliferation and migration of epithelium. This study is expected to provide new strategies for the fields of tracheal replacement therapy needing mechanical properties and epithelization.

1. Introduction

Long tracheal injury or stenosis have always been a difficult problem to be solved in surgical reconstruction.[1, 2] When the resections of the diseased tissue or stenosis and end-to-end anastomosis can not achieve the clinical curative effects, tissue-engineered trachea provides a new inspiration for tracheal replacement therapy.[3] Scaffolds, seed cells and growth factors are the three elements of tissue engineering. Scaffolds provide the guarantee for the adhesion and growth of cells and the role play of cytokines.[4] Therefore, appropriate tracheal scaffolds are the key to the successful implementation of tissue-engineered trachea, and the selection of scaffold materials has become the important part.[5] Autologous tissue has good vascularization and does not need immunosuppressive treatment, but its long-term application is restricted by large surgical trauma, limited scope of use and lack of epithelialization. After allogeneic transplantation, there are serious immunological rejection and lack of biological function.[6, 7] With the deepening of the interdisciplinary concept, synthetic polymer materials have been continuously developed and applied. Among them, PCL occupies a place in the field of scaffold materials for its slow rate of biodegradation and perfect biomechanical properties.[8] In previous study, we have successfully manufactured tracheal graft using PCL as the material by 3D printing technology. Biomechanical and biocompatible test proved that 3D printed tracheal graft was equipped with favorable cellular biocompatibility and biomechanical properties.[9] However, the surface hydrophobicity of PCL greatly affects cell adhesion. Therefore, we performed nano-silicon dioxide surface modification to make the surface smoother and significantly improve the cytotropism. In

addition, the porous structure of 200 μm made it more conducive to cell adhesion and proliferation.[10] Even so, PCL scaffolds are still difficult to load seed cells and cytokines, and thus cannot achieve subsequent cartilaginification, vascularization and epithelization.

In recent years, hydrogels have been widely used as carriers of cells and growth factors, and their 3D network structure is conducive to the transport of seed cells, growth factors, nutrients and the discharge of metabolic wastes.[11] Silk fibroin (SF) is a natural biomaterial with good biocompatibility, excellent mechanical properties, and tunable degradability.[12, 13]Silk Fibroin Methacryloyl is the product of SF, which was modified by methylacrylylation. Its rapid solubilization in water allows SilMA to be photocurable as a hydrogel. KGN is a kind of small-molecule drug, which was discovered in 2012 for the first time.[14] It could selectively stimulate chondrogenic differentiation of endogenous bone marrow mesenchymal stem cells.[15]

Here, the purpose of this study was to prepare 3D printed hybrid tracheal graft fabricated by PCL coated with SilMA, in which BMSCs, KGN and epithelia were co-cultured, and to select the appropriate concentration. So that, the effect on cell behavior can be explored. BMSCs were isolated from tibial plateau and epithelia were cultured from autologous tracheal mucosal epithelium, which was extracted by biopsy forceps in endoscope. Biocompatibility and mechanical properties were evaluated by in vitro experiments. What's more, the role of epithelization and cartilaginization of this hybrid tracheal graft were evaluated via in vivo window-shape defect repair**(Figure 1)** . This study provides the theoretic and experimental basis for further research and practice in tracheal reconstruction.

2. Experimental Section

All the experiments were conducted in accordance with the Guidance suggestions of caring and using laboratory animals, issued by the Ministry of Science and Technology of the People's Republic of China in 2006.[16] The study protocol complied with all committee regulations approved by the Ethics Committee for Animal Experimentation of Yangzhou University (Yangzhou, China. Ethical study number: 202205001).

2.1 Preparation of Silk Fibroin Methacryloyl

2.1.1 Preparation of photoinitiator LAP

The product of SilMA was purchased from Engineering For Life (Suzhou, China). Firstly, we should prepare the solution of LAP according to the instructions. In brief, 20 mL PBS was added into the brown bottle containing 0.05 g photoinitiator LAP in solid state. The solution was dissolved in 40-50 water bath for 15 min, and be shaken several times during the period. The standard solution of LAP was successfully prepared and stored in the refrigerator (4), protected against exposure to light.

2.1.2 Preparation of SilMA hydrogel

As recommended, the SilMA with concentration of 10%, 15% and 20% were prepared. The required mass of solid SilMA was taken and put into the centrifuge tube, then the corresponding volume of standard solution of LAP was added. The SilMA solution was dissolved at room temperature for 0.5-1 h, and be shaken several times during the period (avoid violent ultrasound, high temperature and strong shear). The sterile SilMA solution could be prepared by filtered with syringe filter of 0.22 μM pore size (Biosharp, Hefei, China). The mechanical properties, especially the hardness of hydrogel can be readily adjusted by changing the UV irradiation time to meet the needs of different tissues and experimental requirements,[17] so is SilMA hydrogel. In this study, the solution was irradiated with 405 nm light source for 1 min to make SilMA hydrogel.

2.2 Characterization of SilMA hydrogel

2.2.1 Swelling test

The swelling ratio of SilMA hydrogel is evaluated by a weighing method.[18] Firstly, the prepared hydrogel was weighed and recorded as M0. Then, the hydrogel was soaked in deionized water (dd H_2O) for 24 h at 37°C. After swelling equilibrium, the hydrogel was collected from dd H_2O and excess fluid on the surface

was removed with the filter paper, then the hydrogel was weighed again and recorded as Mt. The swelling ratio was calculated as follows: Swelling ratio (%)=(Mt-M0)/M0x100%. Experiments were done thrice per composition.

2.2.2 Degradability test

The prepared hydrogel was soaked in dd H_2O to reach swelling equilibrium as described above, weighed as W0. Then, the hydrogel was soaked in 24-well culture plate containing 1.5 mL type II collagenase (Absin, Shanghai, China) solution with the concentration of 0.5 mg/mL (37degC), and taken out at 2, 4, 6, 8, 10, 12, 18, 24, 36, 48, 60 and 72 h, respectively. At each point of time, the filter paper was used to remove the excess liquid on the surface and the hydrogel was weighed as Wt. The degradation ratio was calculated as follows: Degradation ratio (%)=(W0-Wt)/W0x100%. Remaining mass (%)=Wt/W0x100%. Same as swelling test, experiments were done thrice per composition.

2.3 Isolation and culture of cells

2.3.1 Bone marrow mesenchymal stem cells

Bone marrow mesechymal stem cells (BMSCs) were isolated from tibial plateau with whole bone marrow adherent culture method of young New Zealand white rabbit under aseptic conditions according to our previous protocol.[5, 9] Through this method, non-adherent cells were washed and the culture medium was changed after 48 h for the first time. Thereafter, the culture medium was replaced every 2 days and the cell growth was observed until cells were subcultured at 80% confluence. The 2^{nd} passage BMSCs were obtained to be used in the follow-up experiment, in order to have better differentiation ability. Cell surface markers were examined by flow cytometry.

2.3.2 Autologous tracheal epithelia

The extraction and culture of autologous tracheal epithelia were improved on the basis of the reference.[19] In brief, the experimental rabbits were anesthetized by inhalation of isoflurane (RWD, Shenzhen, China). Then, the mucosal epithelium of trachea was extracted by biopsy forceps in endoscope and digested by 100 U/mL type I collagenase (Absin, Shanghai, China) at 37degC for 12 h. Digested solution was filtered by 200-mesh sieve, followed by centrifugation at 800 rpm for 5 minutes. After the supernatant was removed, and the remnants were collected and seeded in cell culture dish (Corning, New York, USA) containing DMEM-F12 (1:1) (Hyclone, Utah, USA) supplemented with 10% fetal bovine serum (FBS, Clark, USA) and 1% penicillin/streptomycin (Beyotime, Shanghai, China) for 2 h and then the culture medium was transferred to 25 cm^2 cell culture flask. The following culture method was the same as BMSCs.

2.4 Selection of theoptimum concentration

2.4.1 Cell Counting Kit-8 test

The 150 μL of sterile SilMA solution was injected to the sterile curing ring, under the 405 nm UV irradiation, to made SilMA hydrogel. Afterwards, SilMA hydrogel on the curing ring was transferred into a 48-well culture plate, 1 mL of the 2^{nd} passage BMSCs suspension was gradually dropped onto the surface of SilMA hydrogel with a density of 5×10^4 cells/piece and incubated for 4 h, followed by transfer to another 48-well culture plate. Then, the culturte medium was exchanged every 48 h, and removed from each well at 1, 3, 5, and 7 day. 200 μL of Cell Counting Kit-8 (CCK-8) (APExBIO, Houston, USA) working solution (dilution ratio: 1: 10 with culture medium under dark conditions) was added to cover the hydrogel. After incubating for 2 h, 100 μL of the supernatant was pipetted to a 96-well plate and the OD450 was measured by a microplate reader (BioTek, Vermont, USA). So that, the adhesive and proliferative effects of cells on SilMA hydrogel of different concentrations could be quantified.

2.4.2 3D co-culture and H&E staining

500 μL of the 2^{nd} passage BMSCs suspension with a density of 5×10^4 cells/mL was added in the sterile SilMA solution. To prepare the hydrogel as described in section 2.4.1, so that BMSCs could be 3D co-cultured

with SilMA hydrogel. The hydrogel was cultured with the conventional culture method in incubator (RS232, Thermo Fisher) at 37/5%$CO_2$for 7 days. The samples were taken out and fixed in 4% paraformaldehyde (Biosharp, Hefei, China) for 1 h. After fixation, washed in the PBS solution for three times (5 minutes each time), then dehydrated them in gradient ethanol from 30% to 100%, embedded them in optimal cutting temperature (O.C.T.) compound (SAKURA Tissue-Tek, Tokyo, Japan) at 4 overnight, and successive sectioned them at 6 mm thickness with freezing microtome (Leica, Wetzlar, German). Hematoxylin and eosin (H&E) (Solarbio, Beijing, China) staining was used to evaluate the 3D network structure of SilMA hydrogel and count the alive cells.

2.5 In vitro test

2.5.1 Scanning electron microscopy

The morphology of the 3D printed PCL scaffold which was prepared as precious study,[5] the microstructure of SilMA hydrogel and BMSCs attached to it were observed by SEM (S4800, Hitachi, Tokyo, Japan) operated at 15 kV. PCL samples could be attached to the test table by conductive tapes directly, while hydrogels should be freeze-dried in advance. All of the samples were observed after spraying with gold.

2.5.2 Cytotoxicity test

The cytotoxicity of the hydrogel was evaluated by Giemsa staining (KeyGen, Nanjing, China). In brief, the 2^{nd} passage BMSCs suspension was added into the 48-well culture plate and cultured for 24 h to make cells adhere to the plate. The cells were observed under an inverted microscope. Then, sterile SilMA hydrogel on the curing ring and PCL which was treated as negative control were put into the medium. The blank and positive controls for cytotoxicity experiments were performed with pure medium in wells and coated with cyanoacrylate. The samples were co-cultured with BMSCs for 48 h, follow by Giemsa staining. The working solution was executed according to the instructions in order to observe the morphology of the cells.

2.5.3 The mechanical test of the hybrid scaffold

In this section, the PCL scaffold was submerged in optimum SilMA solution overnight to prepare the hybrid scaffold. The native trachea and the bare PCL were treated as the control group. The length, outer diameter and inner diameter of each sample were measured using vernier caliper. Biomechanical properties, including the stretched properties, the compression properties and three-point bending properties, were measured by the Autograph AGS-X series of universal material testing machines (Shimadzu, Japan).

2.6 In vivo experiment

2.6.1 Establishment of a rabbit model for tracheal partial window-shape defect

Adult male New Zealand white rabbits (n=6) were selected as recipients. Intravenous inhalation combined anesthesia was performed during the operation with isoflurane inhalation by mask, follow by ethyl carbamate (Aladdin, Shanghai, China) injection (20%, 2.5 mL/kg) through the ear vein. Keep continuous inhalation anesthesia until the operation was done. Surgical procedures refer to previous study,[20] a median vertical neck skin incision was made to dissociate the fascia and neck muscles, and the irrelevant tissues around the trachea were dissected layer by layer carefully, in order to prevent bleeding and retain as much fascia as possible. After exposing the cervical trachea completely, the window-shape defect with a size of 5 x 5 mm was established at the position of 2 cm away from the cricoid cartilage. The tracheal patch, which was hybridized by PCL and SilMA hydrogel loaded with autologous tracheal epithelia, was used to repair the defect and was sutured intermittently with 4-0 absorbable surgical sutures. After suturing, the hydrogel carrier was injected in the form of droplets with ingredients of BMSCs ($5x10^4$/100 μl) and Kartogenin (10 mM, MCE, New Jersey, USA) on the outer surface of the patch. Next, the tissues, including fascia and muscles, were sutured tightly. In the end, the skin sutures were operated via the continuous suturing method. In the first 2 days postoperatively, the recipients were treated with daily intramuscular injection of penicillin (5×10^4 U/kg, Mingyue, Suzhou, China).

2.6.2 Bronchoscopy, H&E, IHC and IF staining

After surgery, the experimental rabbits were examined daily for 60 days or until death. On the 30th day, X-ray was performed to observe whether there is obvious airway stenosis. After 60 days, the rabbits were anesthetized with inhalation anesthesia as described above, then the airway patency and repair patch could be observed and photographed under bronchoscopy (Jiechong International Trade Co., Ltd, Shanghai, China). Afterwards, they were euthanized by aeroembolism through ear veins and the patch specimen could be obtained. Specimens were washed three times with PBS gently and embedded in O.C.T. compound, and then sectioned successively at 6 mm thickness using freezing microtome. H&E staining was performed to observe the tissue structures of the patch. To confirm the sub-regional structural morphology, especially the epithelial cells' creeping condition and the differentiation effects of BMSCs on the patch, IHC and IF staining of cytokeratin-18 (CK-18) (Absin, Shanghai, China) and type-II collagen antibodies (Huabio, Woburn, USA) (dilution ratio was 1:200) were performed.

2.7 Statistical analysis

All data were expressed as means ± standard deviations and analyzed by SPSS 20.0 statistical software. Comparisons between two groups were evaluated using a student's t-test whereas those among three or more groups were analyzed using ANOVA. Differences were considered significant at the 95% level (P <0.05).

3. Results

3.1 Fabrication and physical properties of SilMA Hydrogel

According to the fabrication method in the manual, SilMA hydrogel was prepared successfully and the macroscopic appearance was shown in Fig 2. Fig 2B showed the pure SilMA with concentration of 10%. Fig 2C and 2D was the morphology of 15% SilMA and 20% SilMA respectively after culturing in DMEM-F12 medium for CCK-8 test. From the side, the color changes of the hydrogel reflect the capacity of water absorption, which can affect the adhesion and growth of the cells. Furthermore, the swelling and degradability of SilMA was shown in Fig 3. Fig 3A and 3D represent the data of pure SilMA, while Fig 3B and 3E represent the freeze-dried SilMA. With the increase of the concentration, the swelling ratio of SilMA decreased, and the degradation progress slowed down. After drying, the moisture is removed. Whether swelling or degradation experiment, it must firstly go through a process of water absorption. As a result, the swelling ratio of freeze-dried SilMA were several times than the pure SilMA, also the degradation would be further slowed down.

3.2 Selection of optimum SilMA hydrogel

3.2.1 Culturing and Identification of BMSCs

BMSCs have a characteristic of adherent, so that the non-adherent cells were removed when the culture medium was changed. The typical spindle shape of isolated BMSCs were presented by optical microscope image in Fig 4A_1 and A_2. Flow cytometry analysis in Fig 4A_3 revealed BMSCs matched the characteristics of MSCs that CD29 (98.5%), CD44 (99.0%) and CD90 (99.4%) were highly expressed and few cells were positive in CD34 (0.93%). The results indicated that BMSCs could be passaged stably in vitro and maintain their phenotypes.

3.2.2 Culturing and morphologic observation of autologous tracheal epithelia

Optical microscope image of autologous tracheal epithelia was shown in Fig 4B. On the fifth day of culture, the cells have already adhered to the culture flask, became larger, and became polygonal. The cells gathered into clusters, which were like paving stones (Fig 4 B_1 and B_2). Cells reached 90% confluence at day 13 (Fig 4 B_3 and B_4). The cells grew vigorously and uniformly, and more large flat and polygonal cells can be observed, which were densely distributed like paving stones.

3.2.3 Cell proliferation

As described above, the SilMA hydrogel with concentration of 10%, 15% and 20% were prepared and used in CCK-8 test. At the third day, the 10% SilMA hydrogel was degraded, which was consistent with the result

of degradation test. Therefore, Fig. 5A only showed the results of the concentration of 15% and 20%. The cells attached to the surface of the hydrogel continued to proliferate during the test, especially at the fifth day. Compared with conventional culture, there is still a certain gap in the number of cells, but the hydrogel with concentration of 20% is more suitable for cell growth than 15% and the difference was statistically significant.

3.2.4 H&E staining analysis of 3D co-culture

In order to select the appropriate concentration ulteriorly and evaluate the growth of cells in the hydrogel, 3D co-culture experiments were conducted. Same as section 3.2.3, the 10% SilMA hydrogel was degraded. As a result, Fig. 5B represented the H&E staining of 15% SilMA and Fig. 5C represented 20%. SilMA hydrogel with 20% concentration possessed a more compact microstructure and smaller pore size. After 7 days of 3D co-culture, the number of alive cells decreased significantly, which was different from the result of 2D co-culture in section 3.2.3. However, the gratifying result was that under the same field of vision, more cells survive in the 20% SilMA hydrogel and the difference was statistically significant. The results were further quantified and plotted into a column chart, as shown in Fig. 5D.

3.3 In vitro cell contact toxicity of optimum SilMA

After the above procedures, the concentration of 20% was selected. The cell contact toxicity test was operated to evaluate the cytocompatibility of optimum SilMA. The fusiform adherent cells were obviously observed 24 hours after cell seeding, as shown in Fig. 6a-d. After 48 hours of co-culture with the samples, the morphology of cells began to appear difference. The cells around the PCL and SilMA grew well and showed a long shuttle clustered growth [Fig. 6(f, g)]. Compared with blank control (Fig. 6e), no matter in morphology or density of cells, there was no obvious difference. However, there were almost no adherent cells in the positive control group (Fig. 6h). This result was more obvious after Giemsa staining, which can dye the nucleus of the cells purple or blue purple, while the cytoplasm became pink (Fig. 6i-k). In Fig. 6l, the remaining cells showed the round or irregular shape, vacuoles were observed in the cytoplasm, and the nuclei were pyknotic.

3.4 Fabrication and physical properties of hybrid scaffold

3.4.1 The microstructure of PCL, PCL/SilMA, SilMA, SilMA/BMSCs

As the previous study, the crisscross structure of PCL scaffold forms the pore designs (Fig. 7a). It is widely known that inter-connected pore networks of a biological scaffold is a critical index affecting cell adhesion, proliferation and differentiation. SilMA hydrogel possessed highly 3D porous structure with moderate thickening of the hole wall (Fig. 7d, e). Compared with the surface of PCL (Fig. 7b), SilMA hydrogel is glossier (Fig. 7c). The surface of PCL coated with SilMA hydrogel clearly changed under the observation of SEM (Fig. 7f), the pores and gaps were filled with a thin layer of hydrogel, as well as the longitudinal section (Fig. 7g, h). After seeding cells to SilMA hydrogel, the surface was covered with underlying cells, which were mostly flat polygonal or long shuttle shapes (Fig. 7i, j). The cells were distributed in clusters and extended antennae to connect with each other.

3.4.2 Mechanical properties of hybrid scaffold

The morphology of each group was similar and there were no significantly differences in physical measurements between them. With the increase of stretched tension, the native trachea broke at the point of the cartilage rings, and remained in the original structural state. PCL and PCL/SilMA showed segmental changes with the samples were significantly elongated. During the stretched process, the tensile force reached the maximum value when the samples broke. The compression process could continue all the time, so in order to unify the standard, maximum load and elastic modulus were recorded at the 50% of the compression deformation. During three-point bending test, the data were recorded when the samples slided from the fulcrums on both sides. When PCL was coated with SilMA hydrogel, there were certain changes in structure, leading to better mechanical properties.

3.5 Trachealwindow-shape defect repair in vivo

3.5.1 Intraoperative anastomosis, postoperative examination and specimen acquisition

Last but not the least, this hybrid scaffold was applied to repair rabbit tracheal window-shape defects and seeded them with epithelia, BMSCs and KGN during the operation. As shown in Fig. 9a, the window-shape defect of 5 × 5 mm was established, with excision of two cartilage rings. Then, the hybrid scaffold seeding with epithelia was sutured the defect (Fig. 9b). After that, SilMA solution containing BMSCs and KGN was injected under the UV irradiation in the form of droplets to cover the outer surface of the patch (Fig. 9c).

Six rabbits survived well after the operation. There was slight sputum murmur in the first two days and disappeared from the fifth day. One rabbit died two weeks after operation, and a large amount of pleural effusion was detected through autopsy. Another died more than one month after operation and the other four all survived for two months, without sputum obstruction, stenosis, wheezing, cyanosis or infection. On the 30^{th} and 60^{th} day, X-ray examination demonstrated that there was no obvious stenosis or collapse (Fig. 9d, e). The airway, especially the point where the patch was sutured, was firstly examined via bronchoscopy, prior to acquisition of specimens. The picture of bronchoscopy on the 60^{th} day (Fig. 9f) revealed that the transplantation site was covered with mucosa and maintained complete unobstruction of the lumen. It was consistent with the macroscopic appearance of the specimen, and there was no obvious abscess or necrosis at the patch site (Fig. 9g-i).

3.5.2 H&E staining analysis

H&E staining helped to recognize the transplantation site between the two segments of the cartilage ring of the graft, the morphology and structure of which were intact with few inflammatory cells infiltration. Residual SilMA hydrogel and undegraded PCL were also observed on the outside of the graft, while the continuous epithelial structures were detected from native area to patch site on the inside of the graft (Fig. 10a, c). What is more gratifying and unexpected is that a few vascular-like structures were observed near the repair site (Fig. 10b).

3.5.3 IHC and IF staining analysis

In order to further confirm that the tracheal transplantation site was covered by epithelial cells, IHC and IF staining were performed. IHC staining of CK-18 (Fig. 10d-f), which was specifically expressed by epithelial cells, revealed that the continuous brown-colored antigen was expressed on the inner surface of the repair site, which was similar to normal trachea, indicating that the hybrid scaffold has good epithelization performance. Similarly, IF staining of CK-18(Fig. 10g-i), which was expressed by green fluorescence, confirmed the positive result again.

4. Discussion

Effective repair of tracheal diseases caused by tumor, trauma, infection or congenital abnormalities is still an urgent clinical demand. When the length of trachea lesions is less than half of the total length of adults or one-third of children, end-to-end anastomosis is feasible. However, when the length exceeds this proportion, trachea replacement therapy is often required.[7, 21] Although the reported incidence rate of tracheal diseases seems to be low, factors such as prehospital death and common symptoms similar to other diseases, for example asthma, conceal the true incidence rate of tracheal diseases worldwide. In addition, this misdiagnosis often leads to high mortality.[22] Therefore, the study of tracheal transplantation and regeneration has important clinical significance. The first attempt of tracheal transplantation and regeneration can be traced back to the end of the 19th century, and in long-term research, the replacement treatment of long segment tracheal defects is still unsatisfactory.[23] Serious postoperative complications and difficulties in obtaining substitutes need to be solved urgently in clinical treatment. Postoperative infection, necrosis, collapse, stenosis, air leakage, rejection and other problems are important reasons for transplantation failure. In recent years, tracheal replacement research has turned to the field of tissue engineering, which has developed rapidly in the past decades. Together with regenerative medicine, they have injected new impetus into the development of clinical medicine and pointed out new directions for tracheal replacement therapy.[24, 25]

As is known to all, scaffold, seed cell and cytokine are the three elements of tissue engineering, and the same

is true for tissue engineering trachea. On this basis, three major research directions are mainly focused on epithelization, vascularization and cartilaginization. The ideal tissue engineering tracheal scaffold should be equipped with the following capabilities: 1) Specific tubular structure to maintain ventilation; 2) Sufficient mechanical properties to prevent collapse; 3) Biomimetic extracellular matrix structure for cell adhesion; 4) Bioactive environment for epithelial regeneration, cartilage regeneration and angiogenesis.[26] Until now, a variety of scaffolds, including synthetic scaffolds and natural scaffolds, have been used for tissue engineering trachea. However, natural derived materials are limited by mismatched shapes, poor mechanical properties and rapid degradation rate.[27] While the use of synthetic materials is hindered by their low adhesion rate, limited biological activity and aseptic inflammation.[28] Therefore, the composite scaffold prepared by combining natural materials with synthetic materials has become a better strategy.

Silk fibroin, a natural biological material, has attracted extensive attention in the fields of regenerative medicine and tissue engineering. Various forms of scaffolds, including foam, film, electrospun fibers and hydrogels, all can be prepared via SF.[13] Among them, the application of hydrogel is gradually increasing for its three-dimensional structures, which are equipped with suitable porosity and proper pore size for cell migration, survival and tissue regeneration and repair.[29] Kim's resarch group developed a methacrylated photocurable silk fibroin (SilMA) bioink, which can be modulated by digital light processing (DLP) 3D bioprinting. 3D hydrogels using the bioink are characterized in terms of printability, mechanical and rheological properties, and biocompatibility. The versatile bioink can be used broadly in a range of applications, including trachea tissue engineering.[30, 31] Hong's study promised that the fabricated SilMA hydrogel using DLP 3D printer played an important role in ensuring of viability, proliferation and differentiation to chondrogenesis of encapsulated cells and could be applied to the fields of tissue engineering needing mechanical properties like cartilage regeneration.[32] Wu's and Rajput's research both demonstrated that the scaffolds prepared by silk fibroin-derived bioinks for DLP-based 3D bioprinting could be applied for tissue engineering.[33, 34] However, the disadvantage that the mechanical properties are insufficient to support the load-bearing structure has become an obstacle to its application under specific requirements.[35] PCL has been approved by the Food and Drug Administration for internal use in the human body and been widely used in various fields due to its several advantages;[36] our research group has also fulfilled some researches in tracheal scaffold using PCL.[5, 9, 10, 37] In this study, the hybrid scaffold was prepared by 3D printed polycaprolatone coated with Silk Fibroin Methacryloyl hydrogel, which was also used as carrier of seed cells and growth factors.

Cell adhesion, an index of biocompatibility, is the basis of cell proliferation, migration and differentiation. The pore size, mesh distribution and other properties of hydrogel, which are related to concentration, can affect the cell adhesion. As a result, the reasonable concentration of SilMA hydrogel should be selected first. The swelling and degradation test, CCK-8 test and H&E staining of 3D co-cultured test all suggested that the 20% SilMA hydrogel was a better choice. The cytocompatibility of biomaterials, another component of biocompatibility, is usually tested by the method of co-culturing with cells in vitro. The result in this study indicated satisfactory cytocompatibility of the 20% SilMA hydrogel.

The mechanical property of biomaterials is an important indicator to evaluate whether they can be used for tracheal reconstruction in vivo. The maintenance of longitudinal tension and radial compression mechanical properties is an important factor to prevent airway collapse.[38] The stretched and compression test results of this study showed that the longitudinal and radial mechanical properties of 3D printed PCL scaffold and the hybrid scaffold were significantly better than native trachea. However, in the stretched process, the longitudinal ductility of native trachea was better than PCL and the hybrid scaffold, which may be related to the own physical properties of the material. The trachea scaffold should also have a certain degree of toughness on the side to bear the pressure during neck movement, so three-point bending test was carried out in this study. There is no doubt that the hybrid scaffold showed better mechanical properties. Therefore, it is considered that the hybrid scaffold can bear the changes of intrathoracic pressure and the movement of surrounding muscles during respiratory movement in vivo, so as to maintain the patency of the tracheal lumen.

It is not sufficient to examine the properties in vitro, so the tracheal partial window-shape defect and repair

was constructed by using the hybrid scaffold, which was loaded with seed cells and small molecule drug, KGN. Although the trachea seems to be a simple tube, it actually has complex biological and anatomical characteristics. The innermost layer at the junction between the trachea wall and the lumen is the mucous membrane, which is composed of respiratory epithelium and lamina propria. The ciliated pseudostratified columnar epithelium is responsible for clearing mucus, attracting inflammatory cells and secreting various mediators when the airway suffers injury, and is the first barrier against pathogens and particles when breathing.[23] For this reason, the potential to regenerate respiratory epithelium is an understood thing.[39] In this study, autologous epithelial cells was cultivated in the hybrid scaffold. Bronchoscopic images and gross view of specimen two months after transplantation showed that this hybrid scaffold maintains the luminal structure. At two months, the results suggested that the structure around the patch was complete without pale necrosis, purulent exudation, and obvious stenosis and collapse. The overall structure could be clearly detected under H&E staining, including the native area and anastomosis area. At the same time, it could be observed that the cilia were arranged neatly on the inner surface, similar to the native cilia in morphology, even with inflammatory cell infiltration. Furthermore, IHC and IF staining of CK-18 confirmed the result again. Obvious deepening of the brown-color at the anastomotic region and obvious enhancement of antigen expression could be observed in IHC staining. Similarly, IF staining indicated that the intensity of green fluorescence in the anastomotic region is similar to that in the primary region. Taken together, this hybrid scaffold is conducive to the growth and crawling of epithelial cells and regeneration of ciliary structure.

The mechanical support of native trachea is mainly provided by C-shaped cartilage ring. The implanted tracheal scaffold possesses better mechanical properties, which is beyond doubt, but it cannot be implanted permanently. Therefore, when the stent is gradually degraded and absorbed in the body, the regenerated cartilage structure becomes more and more significant. Therefore, the SilMA hydrogel containing BMSCs and KGN were dropped on the outer surface of the patch in this study to promote cartilaginization. Alcian blue staining, modified Saffron-O and fast green staining and IHC staining of type II collagen were performed on the postoperative specimens, but the results were a pity (Not shown in the results section). Therefore, the following research will focus on how to promote chondrogenesis, including biomimetic structure, selection of hydrogel, optimization of experimental plan, etc. In addition, how to achieve long tubular orthotopic transplantation based on the patch is another challenge.

In conclusion, Silk Fibroin Methacryloyl is seized of good gelling property and the SilMA hydrogel can maintain a stable status. Meanwhile, SilMA hydrogel possesses appropriate 3D pore structures and the concentration of 20% is more suitable for cells adhesion and proliferation. In vitro cytotoxicity test showed that 20% SilMA has good cytocompatibility and the mechanical test suggested the hybrid scaffold prepared by PCL coated with 20% SilMA own better biomechanical properties than native trachea. What is more important, the cell loaded hybrid scaffold could be applied for tracheal window-shape defect repair to successfully realize the crawling of epithelial cells at the transplantation site.

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Conflict of interests

The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

Data availability statement

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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Figure legends

Figure 1: Schematic illustration of the designs of the hybrid scaffold combining 3D printed polycaprolatone coated with Silk Fibroin Methacryloyl, epithelium, BMSCs and Kartogenin for reconstruction of rabbit tracheal window-shape defect.

Figure 2: The macroscopic appearance of SilMA in different status (A: curing ring; B: pure 10% SilMA; C, D: 15%, 20% SilMA after culturing in DMEM-F12 medium; E: freeze-dried status of Fig. D).

Figure 3: The swelling ratio and remaining mass of SilMA in different concentration and different status (A, D: The swelling ratio and remaining mass of pure SilMA; C: The degradation process of pure SilMA; B, E: The swelling ratio and remaining mass of freeze-dried SilMA). Data represent mean ± SD; $^{**}P < 0.01$.

Figure 4: A1: At day 4, almost no RBCs left and BMSCs grew well and fast; A2: The 2^{nd} passage BMSCs; A3: Flow cytometry analysis revealed BMSCs matched the characteristics of MSCs that CD29, CD44, and CD90 were highly expressed and few cells were positive in CD34. The epithelial cells were cultured by the tissue extracted from autologous trachea mucosa. B1, B2: At day 5, the cells gathered into clusters; B3, B4: Cells reached 90% confluence at day 13. (Magnification: A1, A2: ×40; B1, B3: ×100; B2, B4: ×200).

Figure 5: A: CCK-8 test of SilMA in different concentration. B represented the H&E staining of 15% SilMA and C represented 20%, red arrows indicate the alive cells (Magnification: B, C: ×100; b, c: ×200). D represented the quantified column chart. Data represent mean ± SD; $^{*}P < 0.05$, $^{**}P < 0.01$.

Figure 6: The appearance and Giemsa staining of BMSCs around the scaffold of each group after inoculation for 48 h. In vitro cytotoxicity test shows 20% SilMA has good cytocompatibility (Magnification: a-l: ×40).

Figure 7: Scanning electron microscopy (SEM) observation of PCL, 20% SilMA, PCL+20% SilMA and SilMA+BMSCs (Magnification: a: ×50, d and g: ×500, b, c, e, f, h and i: ×1000, j: ×3000).

Figure 8: Mechanical test shows hybrid scaffold has favorable biomechanical properties. In the upper left corner of the figure, from left to right are native trachea, PCL scaffold and hybrid scaffold (PCL+20% SilMA).

Figure 9: a-c: Intraoperative images of tracheal partial window-shape defect and repair. Black arrow indicates the SilMA hydrogel droplet containing BMSCs and KGN; d, e: X-ray images of 1, 2 months after operation; f: Bronchoscopic images of the luminal area of the graft at 2 months, white arrow indicates the transplantation site; g-i: Gross view of native trachea and the patch after tracheal reconstruction for 2 months. Red arrow and white dotted boxes indicate the transplantation patch.

Figure 10: a-c: H&E staining (S: SilMA hydrogel; P: PCL; C: cartilage rings); d-f: Immunohistochemistry staining of CK-18; g-i: Immunofluorescence staining of CK-18. Red arrow indicates the vascular-like structures, white dotted lines mark the anastomosis, green arrows indicate the native tracheal epithelium and yellow arrows show the nascent epithelial cell layer on the inner surface of the repair site (Magnification: a, d: ×40, e: ×100, b, c, f: ×200, g, h, i: ×200).

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