Chapter 11

Tissue Engineering of Esophagus

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Additional information is available at the end of the chapter

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Abstract

The incidences of esophageal diseases like atresia, tracheoesophageal fistula, esophagitis, and even carcinoma rise rapidly worldwide. Traditional therapies such as surgery, chemotherapy, or/and radiotherapy, etc. always meet problems, leading to deterioration of the patients' life quality and sometimes the reduced survival rate. Tissue-engineered esophagus, a novel biologic substitute with tissue architecture and bio-functions, has been believed to be a promising replacement in the future. However, the research of esophageal tissue engineering is still at the early stage. Considerable research has been focused on the issues of developing ideal scaffolds with optimal materials and fabrication methods. The *in vivo* tests and clinic attempts are being progressed.

Keywords: materials, scaffolds, fabrication technology, tissue engineering, regeneration

1. Introduction

The incidences of esophageal diseases like atresia, tracheoesophageal fistula, esophagitis, and even carcinoma rise rapidly worldwide. For example, Barrett's esophagus, a complication of chronic gastroesophageal reflux disease (GERD), is a metaplasia of epithelial cells and often causes adenocarcinomas at a rate of approximately 1%. Among them, only 5–10% patients had chance to survive for 5 years if they do not receive treatment at the earliest stage [1]. The atresia of esophagus is a relatively common malformation occurred with a frequency of one in 2500 births [2]. Recently, more than 500,000 individuals are diagnosed with esophageal cancer each year with possibility of 850,000 by 2030 [3]. Esophageal cancer (EC) is a destructive disease. The treatment is usually tough and protracted, so as to inevitably reduce the patients' life quality, and may indirectly contribute to the mortality rate. Badly, the rate of esophageal cancer is 10–100 times higher in Iran, India, Northern China, and Southern Africa than the people in other place of the world [4]. The traditional therapies like

surgery, radiotherapy, or/and chemotherapy, and surgically replacing with stomach, colon, small intestine, etc. did not improve greatly the survival rate. In addition, esophagus donor is too rare to get autologous/allogeneic replacement from human body. A tissue-engineered substitute with integrated structure and function is thought to be a promising and effective alternative for treating esophageal disease, which will eliminate the need to harvest replacement tissues from the patients' own body or other human body.

The esophagus is a muscular canal extending from pharynx to stomach and has functions to transport food and water from mouth to stomach. There are three types of cells, i.e., stratified squamous epithelial cells, fibroblasts, and smooth/skeletal muscle cells, which constitute four layers of this tissue, namely the mucosa, submucosa, muscularis externa, and adventitia. **Figure 1** shows the sketch and histological structure of human esophagus, in which a folding lumen is observed in a resting state (**Figure 1a**). The stratified squamous epithelial cells (E) compose the lumen epithelium that serves as a barrier or protective layer against mechanical stresses produced by food bolus. The epithelial cells are supported by the underlying basement membrane (**Figure 1b**, arrows). The topography of the basement membrane is a rugged and uneven stripe that consists of interwoven fibers. The diameters of these fibers were measured to be from 28 to 165 nm with an average of 66 ± 24 nm. The pores displayed between fibers with unequal size (**Figure 1**c). The molecular components of the basement membrane

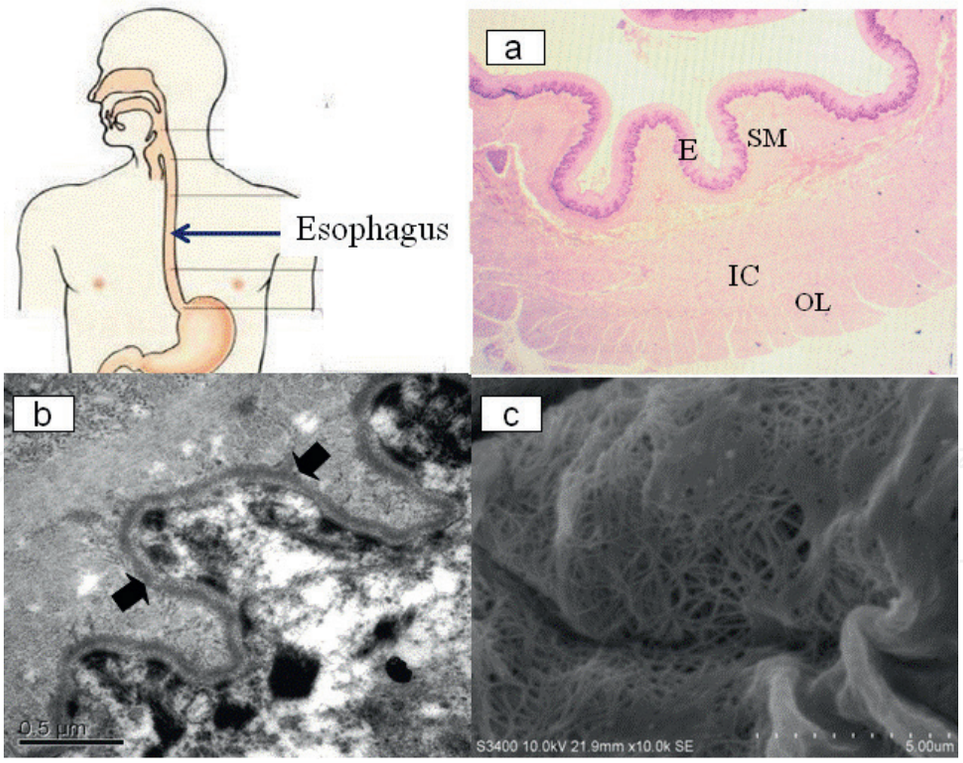

Figure 1. Overview and histological structure of esophagus (a). There are four tissue layers, i.e., mucosa containing epithelium (E), lamina propria and muscularis mucosae, submucosa (SM), muscularis externa consisting of two sublayers of inner circular (IC) and outer longitudinal (OL) muscle, and adventitia in esophagus organ. The stratified squamous epithelial cells (E) lined the esophagus lumen (H&E staining). Cross-section and topography of basement membrane were observed under transmission electron microscope (TEM) (b, c).

were detected to be collagen IV, laminin, entactin, and proteoglycans, mainly; among them collagen IV is slightly less than that of laminin but ~50 times more than that of entactin, but the quantity of proteoglycans was ~5 times more than that of entactin [5].

The muscle component in esophagus is responsible for motor function via peristalsis longitudinally and circumferentially. It consists of striated skeletal muscle in upper third, mixture of skeletal and smooth muscle in the middle third, and pure smooth muscle in the lower third. The muscle exhibits a bilaminar arrangement. The endo-circular and exo-longitudinal myofibrils (**Figure 1a**, IC and OL) are packed bilaminarly in order to propel the swallowing food and water into stomach through sequential contraction of the circular muscles via occluding the esophagus lumen, and longitudinal muscle by shortening the duct and enlarging the lumen, or enhancing the fibril density of the circular muscle, which in turn improves the contracting efficiency of the circular muscle [6–8].

Tissue engineering is an interdisciplinary field that applies the principles of engineering and life sciences toward the development of biological substitutes that restore, maintain, or improve tissue function, proposed by Langer and Vacanti. Scaffolds, cells, and their combination are the main three topics of tissue engineering research. Material is the necessary substratum in scaffold fabrication. There are various types of materials that have been developed as scaffold matrices to constitute esophagus tissue, for example, resorbable substances, decellularized matrices, acellular patches, and the composites from natural and/or synthesized polymers. Among them, a number of tissue-derived extracellular matrix (ECM) like decellularized urinary bladder submucosa, gastric acellular matrix, aortal acellular matrix, acellular dermal grafts, and decellularized esophagus have been much investigated for the applications as esophagus replacement. Some have also been tested for healing injury of esophagus in animal models or even human trials. Alternatively, synthesized and/or natural materials or their composites also attracted more and more attentions in researches of tissue engineering and regenerative medicine. Refs. [9, 10] reported that poly(glycolic acid) (PGA) and silicone with collagen coating were applied to constitute tubular scaffold. Small intestinal submucosa (SIS) was used as the replacement of tubular organs, for example, esophagus and large-diameter vascular grafts, as literature reports. Nonetheless, the complications or postoperative problems like inflammation, leakage, stenosis, and extrusion in long-term implantation are still presented when the materials or scaffolds are implanted into bodies.

2. Materials for scaffold constitution

Since the ancient times, some allogeneic materials have been adopted for medicinal purposes. In the past decades, after the concept of tissue engineering was submitted, many natural and artificial materials were adopted to produce scaffolds. Some achievements have been obtained toward improving tissue regeneration in laboratory scaffold, serving as a temporary platform to support or promote the growth of cells or/and tissues, which is one of the key issues in the research of tissue engineering. For esophagus, a variety of natural or/and artificially synthesized materials have been investigated as scaffold substrate.

2.1. Natural biomaterials

Natural biomaterials, for example, collagen, chitosan, gelatin, decellularized extracellular matrix (ECM), etc., all of which are derived from animal sources, have been widely studied in scaffold constitution, since all these materials possesses good biocompatibility and have biospecific signals cued from the molecules secreted by the resident cells. Thus, they are believed to be able to direct the *in vivo* remodeling process. These natural materials also have been used in a variety of tissue engineering applications, such as the grafts of heart, heart valve, skeletal muscle, skin, cardiovascular grafts, etc. For the research of esophageal tissue engineering, some ECMs like acellular dermal grafts, gastric acellular matrix, aortal acellular matrix grafts, and decellularized esophagus and urinary bladder submucosa had once been tested to repair esophagus in animal models [11–14]. For example, Marzaro et al. seeded porcine esophageal smooth muscle cells (SMC) on the acellular esophagus aiming at healing the defected porcine esophagus. They got results that SMCs grew on the ECM without obvious inflammation and rejection after implantation for 3 weeks [14].

The research team led by Professor Badylak pioneers the scaffold fabrication using decellularized ECM like porcine urinary bladder matrix (UBM) and small intestinal submucosa (SIS) toward *in vitro* and *in vivo* repairing of esophagus organ [15–17]. For example, they implanted the acellular UBM at the esophagus defects of female mongrel dogs, where the circumferential endo-mucosa/submucosa had been resected. The results showed that complete epithelialization took place on the scaffold surface at day 35, neovascularization and formation of muscle bundles took place at day 50, and the immature nerves and Schwann cells were observed at day 91. After implantation for 230 days, neonatal esophagus with the formation of well-organized tissue laminar and tissue motility had grown [12]. Another important application is skeletal muscle ECM. They decellularized skeletal muscle with enzymes and chemicals to obtain acellular ECM. This ECM was verified to contain the growth factors, glycosaminoglycans, and basement membrane structural proteins. Expectedly, these components greatly promoted myogenic cells' growth and proliferation *in vitro*, and also promoted the myogenesis when the ECM was implanted into a rat abdominal wall. The ECM scaffold was found to degrade gradually at the implant site [16]. The xenogeneic ECM derived from porcine SIS combined with endoscopic technique was adopted to repair dysfunctional esophagus of five male patients who had esophageal adenocarcinoma, Barrett's or/and high-grade dysplasia (HGD). After 24 months, patients restored the mature squamous epithelium and returned a normal diet without significant dysphagia. Unfortunately, among these five patients, some recurred Barrett's esophagus, mainly at the gastroesophageal junction [17]. It was the first and very important report about clinical application of tissue-engineered esophagus in human body. A model of the human esophageal mucosa was reported recently by the MacNeil laboratory [18]. Unlike conventional 2D cell culture systems, they seeded primary human esophageal fibroblasts and epithelial cells in a porcine-derived acellular esophageal scaffold and discovered an esophageal mucosa recapitulation after 20 days. It provided a biologic-relevant experiment model of human esophageal mucosa.

Some literatures reported that three-dimensional biological scaffolds made from nonautologous extracellular matrix (ECM) can act as an inductive template for tissue and organ

reconstruction after the ECM was recellularized with autologous stem cells or differentiated cells. This kind of ECM/cells was tried to repair and reconstruct some complex tissues like esophagus, trachea, and skeletal muscle in animal models. Porcine SIS was once attempted to be used as the scaffold by Wei et al., on which the canine oral epithelial cells were pre-seeded before transplantation into animal body followed by suturing across an esophageal gap in the cervical portion (~5 cm). Not only reepithelization but also muscle formation was discovered in the cell-seeded SIS after implantation in animal body for 8 weeks [19]. Urita et al. used the decellularized stomach tissue to evaluate the esophageal mucosa regeneration [13]. Isch et al. implanted a commercial decellularized product, AlloDerm® (LifeCell™), for the esophagoplasty of canine cervical esophagus. Complete epithelialization on the membrane surface was achieved after 2 weeks, without obvious anastomotic fistula or stenosis [20]. Bhrany compared the growth of rat epithelial cells on esophageal ECMs decellularized by deoxycholic acid and Triton X-100. The results indicated that treatment with deoxycholic acid was better than Triton X-100 treatment in epithelium regeneration [11]. Koch et al. decellularized a porcine esophagus and implanted subcutaneously into Sprague-Dawley rats. The decellularized esophagus was shown to maintain its native matrix morphology and extracellular matrix composition [21]. Considering the findings in these literatures, we believed that the decellularized ECM is a good scaffold candidate in esophageal tissue engineering.

Proteins or/and proteoglycans derived from animal ECM are also the important materials that have been actively researched previously. Saxena et al. seeded rat esophageal epithelial cells on the collagen-based scaffolds (OptiMaix-3D001315). After cultured *in vitro*, cells were tested to display positive pan cytokeratin PCK-26 which broadly recognizes the epitopes present in most human epithelial tissues [22]. Qin et al. fabricated a cross-linked collagen-chitosan sponge and implanted into the latissimus dorsi of nude mice after it was preseeded with fetal canine esophageal epithelial cells. Ten layers of mature epithelial cells formed after 2 weeks and the collagen-chitosan implant degraded totally after 4 weeks [23]. Saito et al. implored the feasibility of collagen that used to be the substrate of tissue-engineered esophagus [24]. They constituted an artificial esophagus using collagen sponge together with a latissimus dorsi muscle flap and split-thickness skin and replaced the esophagus of rabbits. Five in 12 total experimental rabbits survived without anastomotic leakage or stenosis. The longest survival period in these rabbits was 16 days.

Although many interesting achievements about natural biomaterials have been obtained in some *in vitro* or *in vivo* experiments, problems like weak mechanical strength, fast degradation, and source limitation of these natural biomaterials are still to be worked out.

2.2. Polymeric materials

Artificial polymers have been much investigated as scaffold substrates in tissue engineering because these materials possess many attracting features, for example, good availability, low cost, and high possibility of designing and production. Polyethylene (PE) tube was the earliest example to be an artificial conduit for esophageal replacement in a dog model in 1952 [25]. However, 6 in 20 experimental dogs died after the PE tube was replaced. Leakage and stricture in some dogs were discovered at the site of the PE junction. A fibrous sheath around

this plastic tube was developed in all dogs, likely because of the nonbiodegradability of PE materials. Despite the failures, this study is the pioneering experiment of tissue engineering research of esophagus.

Since the concept of tissue engineering was put forward, the interests in using biocompatible and biodegradable polymers, such as polyglycolic acid (PGA), polycaprolactone (PCL), poly-L-lactic acid (PLLA), and their copolymers, has been increasing greatly, particularly after the regulatory approval from the U.S. Food and Drug Administration (FDA). Various kinds of biodegradable polymers have been investigated specifically for the tissue engineering of esophagus. For example, vicryl, made from PGA and collagen coating, is one type of biodegradable and absorbable scaffold with good biocompatibility. It has been tested as a porcine thoracic esophageal replacement in 1991 [26]. No matter what, it failed when the material was implanted into animal body. It was because the reflux gastric fluid dissolved the grafted tube, resulting in severe mediastinitis, leakage, and stenosis. In 1998, Shinhar used vicryl mesh to repair the porcine cervical esophagus. Here, stenosis still took place, though the stump fistula disappeared [27]. Miki et al. fabricated a tube using PGA mesh as a frame, and collagen containing esophageal fibroblasts and epithelial cells as the outer and inner layer, respectively. The fibroblasts were found to be able to accelerate the proliferation and differentiation of epithelial cells due to the keratinocyte growth factor secreted by fibroblasts. After the tube was implanted into muscle flaps of athymic rats for 14 days, nonstenosis was observed in the tube's lumen, but also 20 layers of stratified epithelium were developed from histological examination [28].

One key issue of those synthetic materials used as scaffold matrices is the materials' hydrophobic and biologically inert surface, which will inevitably lead to the inferior reactions between material and cells when the host cells come into contact with the scaffold surface upon implantation. Zhu et al. developed some methods to modify the surface chemistry aiming at enhancing cell-polymer interactions. In order to graft proteins or other biomolecules onto polymer surface, a reaction of ester groups from the substrate polyesters (e.g., PLLA, PU, PCL, and their copolymers) and amino groups (-NH_2) of hexanediamine was firstly introduced to produce pendent amino groups on polyester surfaces through formation of amide bonds. This reaction was called as aminolysis. The density of amino group produced from the aminolysis reaction was quantified using ninhydrin method and fluorescein labeling. Second, this pendent -NH_2 reacted with one aldehyde group (-CHO) from glutaraldehyde (GA). Third, the other aldehyde of GA was used in covalently bond proteins or other biomolecules. Collagen, gelatin, chitosan, fibronectin, polypeptides, growth factors, etc. were thus grafted on the polymeric scaffold surface. Finally, the protein or other biomolecule-grafted surfaces were produced. The water soluble carbodiimide (WSC) can also induce the reaction between the pendent -NH_2 on the aminolyzed surface and –COOH of target proteins, so that the proteins were covalently bonded to the material surface as the **Scheme 1** demonstrated [29–31].The introduction of the amino groups also allows layer-by-layer (LBL) assembly on the polymer surface, because the aminolyzed polyester can be used as a polycationic substratum, on which polyanions can be assembled by means of electrostatic attraction. For example, LBL assemblies of poly(styrene sulfonate, sodium salt) (PSS)/chitosan and chondroitin sulfate (CS)/collagen were performed on the aminolyzed poly-L-lactide (PLLA-NH_2) surface (**Scheme 1**) [32, 33].

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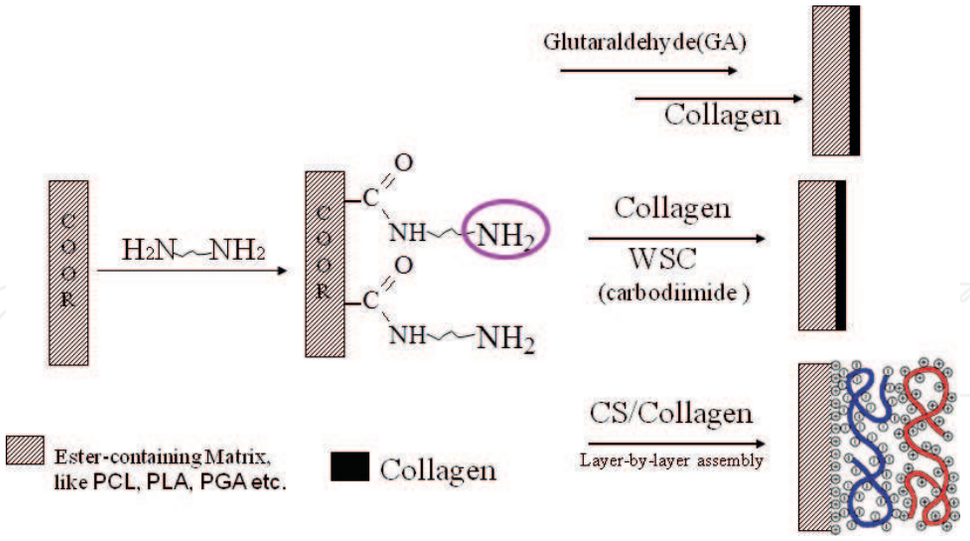

Scheme 1. Diagram of reactions between ester groups from synthesized polyesters and amino groups from diamine, aiming at introducing pendent amino groups onto substrate surface, through which many biomolecules containing amino or/and carboxylic groups can be bonded via crosslinking reagents or layer-by-layer assembly technology.

We tried another method, photo-oxidation plus copolymerization, to modify the material surface chemistry. This method was processed under UV initiation to introduce carboxylic groups (-COOH) onto material surface. Through these carboxylic groups, molecules like protein or other bio-molecules (containing COOH) will be bonded onto the surface under the catalysis of 1-ethyl-3-(3-dimethylamino propyl) carbodiimide hydrochloride (EDAC). As a result, covalent immobilization of proteins onto the material surface took place [34]. The optimal conditions for each method with respect to cell functions have been elucidated by *in vitro* evaluation of endothelial cells or esophagus cells including epithelial cell, fibroblast, and smooth muscle cell [30, 35–39].

Another important issue regarding polymers is the catalyst used during synthesis. Those ester-containing polymers are usually synthesized under catalyzing of stannum compounds [40, 41]. However, this kind of catalyst covalently links to the molecular chain of the ultimate products. We know, these stannum-containing materials are harmful to human body when it is implanted *in vivo* as a scaffold substrate, because the bonded stannum will release and accumulate in body as the material gradually degrades. Thus, it is necessary to develop new methods to catalyze efficient polymerizations but no toxicity giving off. Stolt et al. explored the reaction of L-lactide ring-opening polymerization using catalysts generated from iron and acetic acid, isobutyric acid, butyric acid, trifluoroacetic acid, dichloroacetic acid, etc. They discovered that the iron acetate, iron isobutyrate, and iron trifluoroacetate were the efficient catalysts for ring-opening reaction to yield poly(l-lactide) (PLLA) with a molar mass (weight average molecular weight, Mw) of 150 kDa. The monomers' conversion was up to over 85% under the optimum reaction conditions [42]. After this, they produced lactic acid-based poly(ester-urethane) (PEU) using iron monocarboxylates as the initiators, which were prepared from the reaction between iron powder and acetic acid, trifluoroacetic acid, or isobutyric acid. These iron monocarboxylates were considered as catalysts in reactions of

hydroxyl-terminated prepolymers and further linking with hexamethylene diisocyanate. The final product, PEU with high Mw, was achieved under the catalyst of fluorinated iron acetate [43]. Zhu group investigated the polymerizing of ester-containing monomers like lactide, caprolactone, glycolic acid, etc. using ferric chloride ($FeCl_3$), ethanol iron ($Fe(OC_2H_5)_3$), iron (III) acetylacetonate ($Fe(acac)_3$), or iron (II) acetylacetonate $Fe(acac)_2$, as the catalyst. The result was that $Fe(acac)_3$ was the most efficient catalyst among them to yield products with high monomer conversion and number average molecular weight [44]. Based on these studies, an oligomer, poly(ethylene glycol-co-lactide) dimethacrylate (PLEGDMA) was further synthesized via ring-opening polymerization of L-LA and polyethylene glycol (PEG) under $Fe(acac)_3$ initiation. After cross-linking with PEG diacrylate and NIPAAm, or with linear prepolyurethane in the homemade mold, biodegradable tubular scaffolds with good mechanical properties were fabricated (**Figure 2**). These scaffolds were verified to be good enough to support the growth of porcine esophageal cells like epithelial, fibroblast, and muscle cell.

3. Fabrication of 3D scaffold for esophageal tissue engineering

Biodegradable 3D scaffolds serve as analogues of extracellular matrix (ECM) in the engineered tissue or organ. Therefore, the scaffold's chemistry and macro and/or microscale architecture must be helpful to maintain cell's functions including cell-matrix adhesion, cell-cell adhesion, cell migration, proliferation, differentiation, etc. On the other hand, 3D scaffold should provide spatial cues for cell infiltration, so that cells are capable of integrating with the underlining substrate. People are always seeking techniques to fabricate spatial scaffolds. Some technologies like foaming, porogen leaching, electrospinning, or other fiber processing, phase separation, 3D microprinting, etc. were developed to construct 3D porous scaffolds. In particular, electrospinning technology and thermally induced phase separation (TIPS) method have been extensively studied to constitute 3D scaffolds in tissue engineering of esophagus.

3.1. Phase separation

Phase separation of polymeric materials is often induced by thermal alteration. That is called thermally induced phase separation, shortened as TIPS. TIPS is one of the most practical

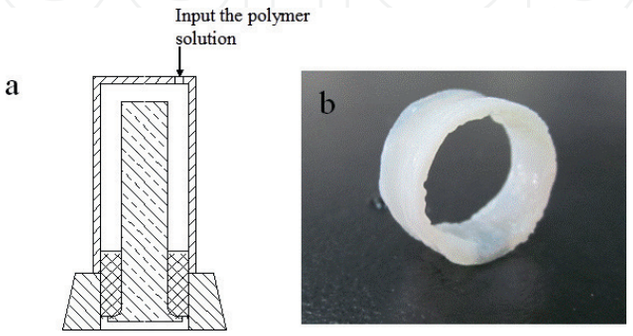

Figure 2. (a) A tubular mold; (b) overview of the tubular scaffold made from crosslinking of PLEGDMA, PEG diacrylate and NIPAAm.

techniques to prepare 3D scaffolds with optimal pore profiles by modulation of process parameters. It was firstly proposed by Castro as early as in 1981 [45] and extended to scaffold making after commencing of tissue engineering [36, 46–48]. The TIPS procedure usually involves four steps: (1) polymer dissolution in some solvent, (2) occurrence of phase separation, (3) polymer gelation, and (4) solvent extraction [49]. There are many advantages of TIPS over other traditional methods such as porogen leaching and foaming. For example, TIPS has the abilities to create a variety of pore structures by employing different parameters, and abroad applications for substrate sources because of its universality for various materials like crystal or noncrystal synthesized polymers [50, 51].

Zhu et al. prepared porous scaffolds with ≥100 μm pore size and good pore interconnectivity using TIPS technique [52, 53]. In order to simulate the ECM architecture, the original TIPS was modified, and thus a scaffold with an asymmetrical pore structure in a hierarchical order was created (**Figure 3**). The pore size on the scaffold surface was small, 1–10 μm, while that of the scaffold bulk was large, ≥100 μm (**Figure 3b**). Primary porcine epithelial cell was cultured on this asymmetrical scaffold lumen surface and primary fibroblast was cultured in the scaffold bulk. The coculture results verified that the bulk with large pores allowed fibroblast migration and infiltration while the lumen superficies with micropores supported the growth of epithelial cells and served as a barrier against fibroblast penetration. The immuno-fluorescent staining (nuclei displayed as blue and keratin as green) of epithelial cells exhibited that several layers of epithelial cells had formed after *in vitro* culture for 14 days on the scaffold lumen (**Figure 3**, **a2** and **b2**). The *in vivo* test showed that a complete layer of epithelium was regenerated on porcine esophagus lumen while the scaffold was being degraded after implantation for 5 months [53].

Beckstead et al. prepared porous sheets with salt-leaching/gas foaming method. The ammonium bicarbonate salt with size from 38–75 to 150–250 μm was used as the porogen reagent. A mixture of 10% chloroform and 90% ethanol was adopted as the polymer solvent. After dissolved in the solvent completely, the polymer solution was evaporated while 50% aqueous citric acid solution was used to initiate gas foaming accompanying with salt leaching [54]. They evaluated the cell (rat esophageal epithelial cell) behaviors on the scaffolds derived from natural material (exemplified AlloDerm), and synthetic materials like poly(lactic-co-glycolic acid) (PLGA) and PCL/PLLA. The results exhibited that AlloDerm scaffold had superior epithelial organization and stratification over other artificial scaffolds. Further modification to the artificial scaffolds would be a necessary way to polish their chemistry and to improve the cell behaviors.

3.2. Electrospinning

The technique of electrospinning was first proposed by Formhals in 1934 [55]. After that, it was gradually applied in diverse regions, for example, filtration industry, wound dressings, controlled drug releasing, and scaffold making in tissue engineering, and so on. In particular, this technique has gained popularity in tissue engineering fields, as a means of making scaffold. The fiber sheets obtained from electrospinning process possesses many features similar to natural ECM, for example, fibers were loosely connected with nano to microscale diameters; the sheet has high porosity and high surface area to volume ratio. Therefore, this technology became an interesting and valuable way to constitute scaffolds for esophageal tissue engineering.

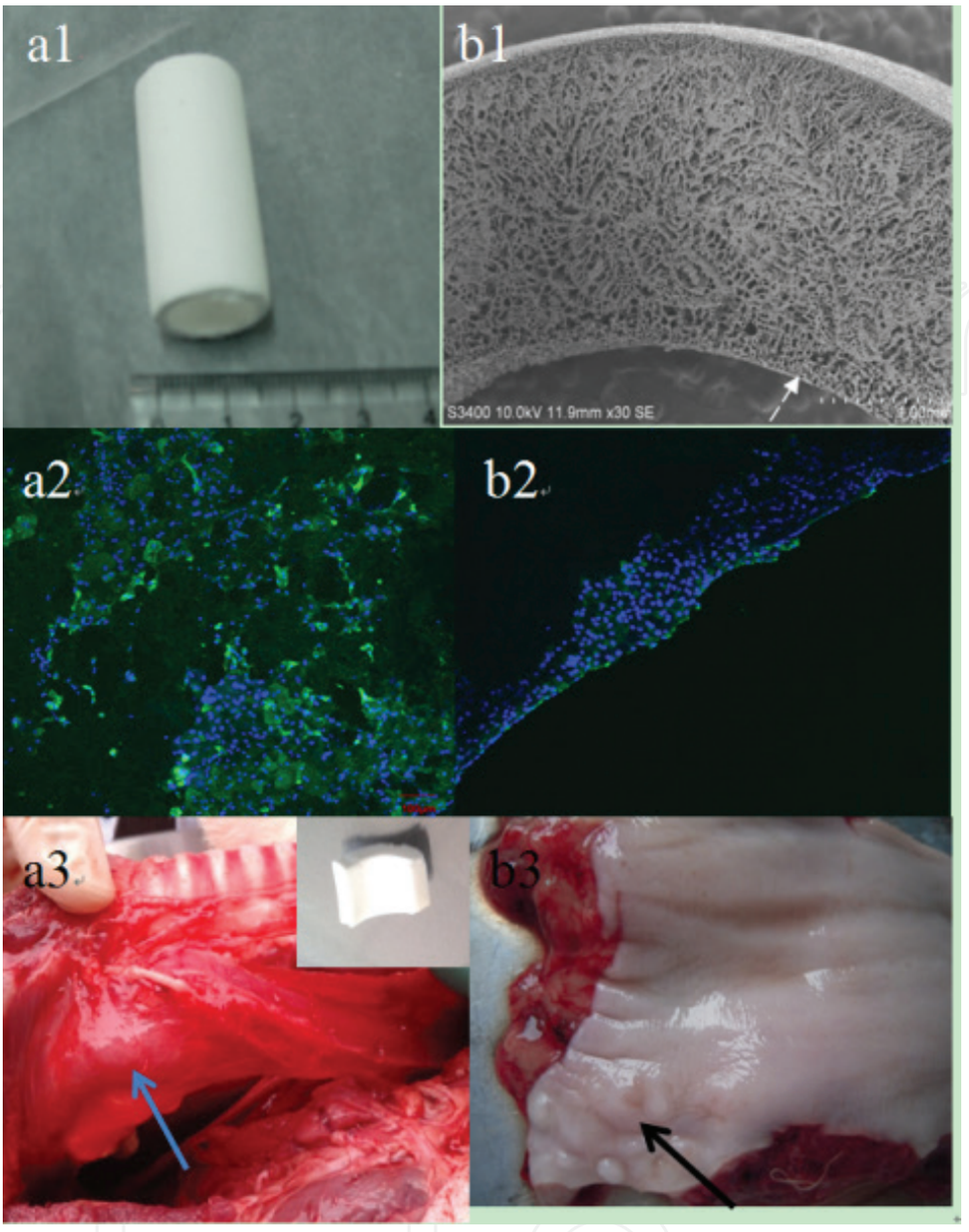

Figure 3. Overview of tubular scaffold (a1) and cross-section structure (b1). The scaffold was prepared with TIPS technique using biodegradable poly(l-lactide-co-caprolactone) as the substrate material. Primary epithelial cells were cultured on the scaffold lumen for 14 days and the immunofluorescence staining (nuclei displayed as blue and keratin as green), a2 is surface scanning and b2 is cross-section observation. The scaffold was surface grafted with fibronectin and implanted in porcine esophagus for 5 months (a3 and b3). Arrows in b1 indicate scaffold lumen, and in a3 and b3 referred the nude esophagus and scaffold-implanted site.

A typical electrospinning setup includes a syringe pump, a metallic collector, and a high-voltage generator. The parameters of this electrospinning system, including process parameters (e.g., electric potential, solution flow rate, distance between the spray nozzle and collector, etc.), polymer solution properties (e.g., solvents, solution viscosity, and concentration), and ambient parameters (e.g., temperature and humidity) influence the fiber features and internal construction.

Leong et al. yielded poly(D,L-lactide) fibers with the diameter of ~1 μm and nanoscale pores on each fiber through the method of electrospinning combined with phase separation. Large pores between the fibers in the whole sheet were also formed. Such multiporous structure greatly enhanced the cell-matrix interactions and thus promoted the adhesion of porcine esophageal epithelial cells onto the fibers [56].

We have set up a programmable electrospinning system (China Patent ZL 200810062323.8) to upgrade the apparatus's versatility. Besides the basic components, i.e., high-voltage generator and syringe pump, an electronic controller that allows manipulating the nozzle and metallic collector was automatically incorporated into the system. Two nozzles were applied in this system. They can be connected independently to two syringe pumps via silicone tube and operated under programmable monitor to spray polymer fibers individually, sequentially, or simultaneously. Using this upgrade system, a uniform composite fiber sheet consisting of polymers and natural biomaterials with the diameter ranging from 1 to 600 nm was created. These composite sheets derived from proteins and polymers showed good biocompatibility and good mechanical properties. Furthermore, a PCL fiber mesh with macroscopically alignment was electrospun on this setup. The interesting discovery is that this aligned fiber was able to switch smooth muscle cells from synthetic to contractile phenotype and hopefully to maintain the biological function of the cultured muscle tissue [57].

Grafting with ECM molecules is a good way for synthetic scaffolds to improve their bioactivity. For example, poly(L-lactide-co-caprolactone) (PLLC) was electrospun to form fibers. After then, they were grafted with fibronectin in order to promote epithelial cell growth [58]. According to the findings about the topographic features and protein quantifications of the basement membrane of porcine esophagus [4], an electrospun scaffold was fabricated using fibroin (extracted from pregnant silkworm originated in Zhejiang province, China) and polymer as the materials. In order to simulate the architecture of the basement membrane, proteins including collagen IV, laminin, entactin, proteoglycans (PG) extracted from porcine esophagus were coated on the above fibers, aiming at enhancing epithelium regeneration

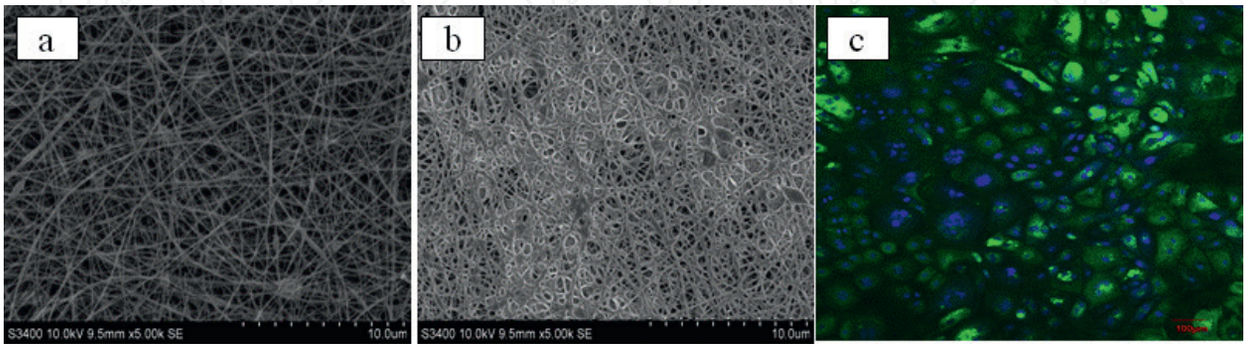

Figure 4. Scaffold morphology of PCL/SF (a), scaffold coated with basement membrane proteins that were extracted from porcine esophagus (b), and cell phenotype immune-histochemically stained with CK14 antibody (green) and nuclei with DAPI (blue) (c).

(**Figure 4**). Silk fibroin is known to have good physical and mechanical properties, and also good biocompatibility. Electrospun fibroin scaffolds together with optimal pore parameters and protein coating extracted from animal esophagus could be great candidates in esophageal tissue engineering [59].

4. Constitution of muscularis propria of the esophagus

The muscle tissue of esophagus consists of striated muscle (skeletal) in the upper third, mixture of skeletal and smooth muscle in the middle third, and pure smooth muscle in the lower third. These muscle contents arrange into endo-circular and exo-longitudinal sub-bilayers to play an important role in propelling the swallowed food or fluid into the stomach via muscle peristalsis. Generating an oriented muscle architecture to mimic the tissue of muscularis externa is an important issue to restore the functions of tissue-engineered esophagus. Many researchers studied the relationship between scaffold's chemistry and microstructure and muscle cells' phenotype. Stegemann once verified that the behaviors of smooth muscle cell (SMC) were positively correlated to the scaffold geometry (2D and 3D) [60]. Li et al. believed that the scaffold geometry played an important role in modulating SMC phenotype. They cultured SMCs and discovered that cells in 3D collagen (type I) gels had lower proliferation and higher collagen synthesis than the cells in 2D collagen substrate [61]. Chan-Park verified that smooth muscle α-actin of SMCs cultured in microchannels upregulated greatly, suggesting a phenotype shift from synthetic to contractile state of cells [62]. They thus believed that 3D microchannels could encourage cells to reorganize into orientation patterns because SMC have a natural self-arrangement propensity. Moreover, the narrow space of channels around 100 μm or less helped cells to achieve more uniform orientation. We also fabricated scaffolds with circular and longitudinal microchannel patterns (**Figure 5**). Further, the scaffold surface was grafted with silk fibroin using our method of diamine aminolysis and GA crosslinking. The primary esophageal SMC was cultured in these 3D protein-grafted channels in order to achieve SMC phenotype regulation and *in situ* muscle formation [63]. The results confirmed that primary esophageal smooth muscle cells exhibited fine alignment in all types of microchannels while SMCs in the interval channels communicated well through the gaps (**Figure 5**).

Some researchers had considered and investigated that mechanical stimulation might be an effective way to regulate SMC phenotype. Ritchie et al. designed a system to exert mechanical forces on esophageal smooth muscle cells. They discovered that cells on the flexile polyurethane membrane displayed alignment parallel to the force direction when low cyclic strains (2%) was used, but alignment perpendicular to the force direction when high strains (5 and 10%) used [64]. Cha et al. reported that muscle cells would orient according to the optimal movement of the tissue. They adopted cyclic mechanical strain (a homemade stretching chamber) on primary myofibroblasts, and promoted the cell differentiation, and further modulated the orientation and proliferation of the differentiated smooth muscle cell. Their conclusion was that myofibroblast/scaffold hybrids with cyclic strain could be applied to organize smooth muscle cells with muscle tissue functions [65].

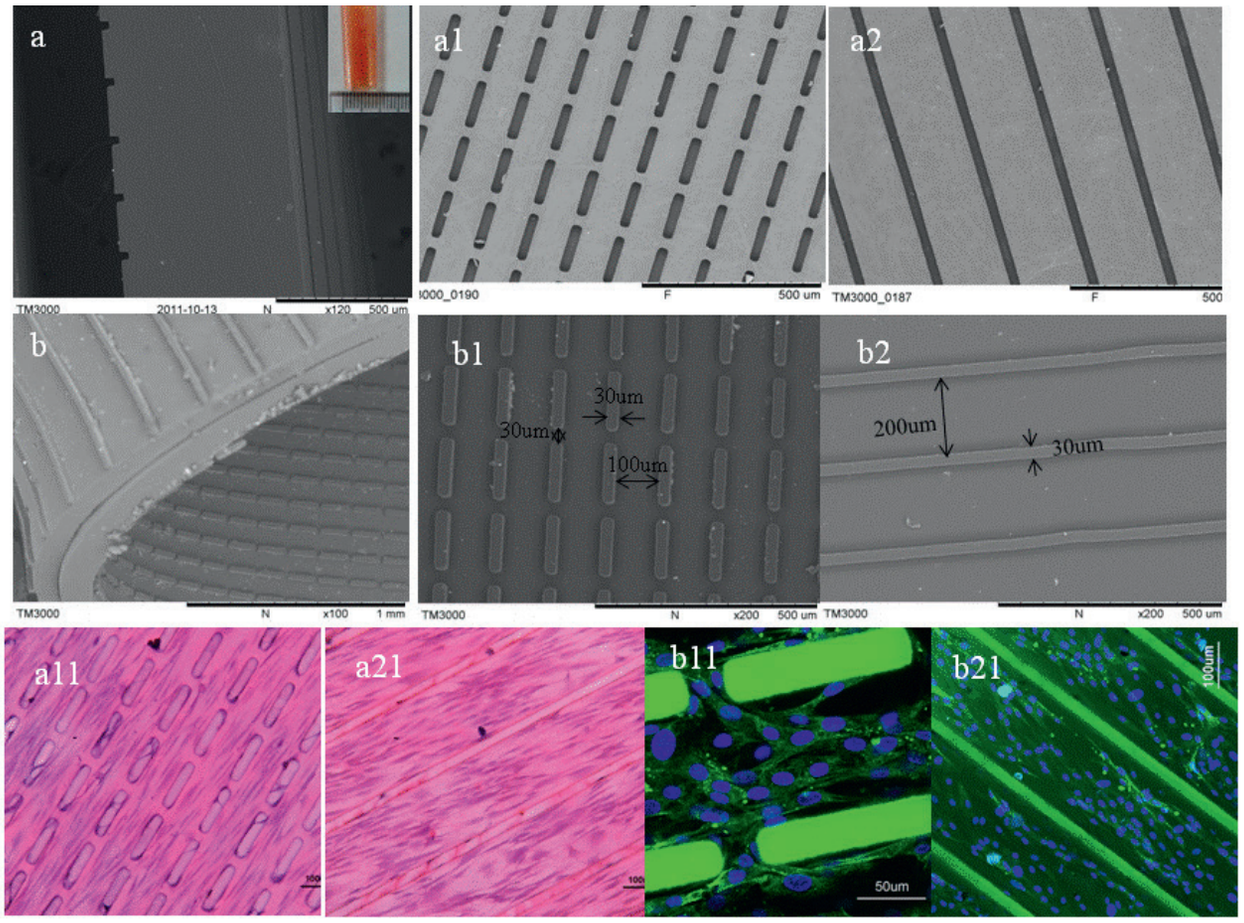

Figure 5. Overview of tubular scaffold (a, inserted) and tube wall's cross-section structure observed under SEM (a). (a1) Scaffold's morphologies of tube lumen and outer face, containing microchannels of 100 μm width with discontinuous channel walls intermitted by 30 μm gap, and both the wall thickness and depth are 30 μm; (a2) microchannels of 200 μm width with noninterval slits. (b) SEM picture of tubular scaffold with bulge wall (b1 and b2). The height of wall and gap between wall intervals is 30 μm. Esophageal smooth muscle cells were seeded and aligned in all scaffolds' channels while cells in the interval channels communicated through the wall gaps (a11, a21 and b11, b21). The scaffold was constructed through silica mold with predetermined patterns using biodegradable poly(ester-urethane) as the substrate material. The surface was grafted with silk fibroin via the method of diamine aminolysis and GA crosslinking. Cells of a11 and a21 were stained by H&E and cells of b11 and b21 were immuno-fluorescently stained with anti-α-smooth muscle as the primary antibody.

5. Clinical potential and future perspectives

With the development of stem cell technology, some kinds of stem cells, for example, embryonic stem cell, mesenchymal stem cell, progenitor stem cell, induced pluripotent stem cell, etc., are adopted to be the seeded cells in tissue reconstruct. In case of esophagus, bone mesenchymal stem cell (bMSC) is more often used to seed on scaffolds than other kinds of stem cells to regenerate or remodel the engineered esophagus. Taylor and Macchiarin reported that allogeneic mesenchymal stromal cells were seeded on the decellularized rat esophagi to orthotopically replace the entire cervical esophagus. After 14 days, the explanted grafts showed regeneration of all the major cell and tissue components of the esophagus including

functional epithelium, muscle fibers, nerves, and vasculature. Thus, this tissue-engineered esophageal scaffold was considered as a significant step toward the clinical application of bioengineered esophagi [66].

In summary, the research of substrate materials and scaffold fabrications in esophageal tissue engineering has made great progress in past decades. Esophagus repairs in animal models and even clinical tests are being attempted and the techniques are being improved. Materials with appropriate physical and chemical properties are still being developed. Optimizing scaffolds and cells for epithelium regeneration or/and muscle constitution, and their combination have been in progress. Some crucial problems, such as complications from stricture to dilation, angiogenesis and innervation consideration, little or no muscle regeneration in the implants, etc., need to be issued before the tissue-engineered esophagus can be a viable conduit for surgical replacement in clinic. And, graft-to-host integration and remodeling of the organ functions like peristalsis and nerve guide would be the important gauge for the success in tissue engineering of esophagus. With the development of material science and engineering, stem cell biology, and other related theories and technologies, tissue-engineered esophagus is able to foresee the promising employment in clinic in near future.

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