International Journal of Bioprinting

RESEARCH ARTICLE

3D bioprinting as a prospective therapeutic strategy for corneal limbal epithelial stem cell deficiency

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(This article belongs to the *Special Issue: Advances in the Application of Bioprinted Biomaterials in Tissue Engineering*)

Abstract

Limbal epithelial stem cells (LESCs) are responsible for the maintenance and repair of the corneal surface. Injuries and diseases of the eye may result in a vision condition called limbal stem cell deficiency (LSCD). Without limbal stem cells, the cornea becomes opaque, vascularized, and inflamed. Cultured LESC therapy as a treatment method was first described in 1997, and LESCs cultured from either patients or donors have been used to treat LSCD successfully. However, the main source of cornea for LSCD treatment is from donors, which are too few to meet the demand (less than 1:70 of cases). The global shortage of donor cornea promotes the need for studies exploring corneal limbus alternatives. Many problems still remain unresolved, such as original geometry reconstruction, corneal epithelial regeneration, and ocular optical function restoration. 3D bioprinting has garnered tremendous attention in recent years, and significant advances have been made in fabricating cell–laden scaffolds. These advancements could lead to a promising treatment for LSCD. It is possible that alternative limbus stem cells can be constructed using 3D printing, which, in corneal limbus regeneration, enables personalized corneal implants and fabrication of single- or multilayer corneal limbus equivalents with corneal limbal stem cells. In this review, the progress, applications, and limitations of the most influential works regarding current treatments of LESC deficiency are discussed. The advantages of 3D bioprinting are illustrated, and some first promising steps toward the creation of a functional cornea limbus with 3D bioprinting are discussed. Finally, insights into the prospects and technical challenges facing the future research of 3D bioprinting of corneal limbus alternatives *in vivo* and *in vitro* are provided.

Keywords: 3D bioprinting; Corneal limbus; Regenerative medicine; Limbal stem cell deficiency

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Citation: Xue Q, Ma L, Hu H, *et al*., 2023, 3D bioprinting as a prospective therapeutic strategy for corneal limbal epithelial stem cell deficiency. *Int J Bioprint*, 9(3): 710. https://doi.org/10.18063/ijb.710

Received: October 21, 2022
Accepted: November 23, 2022
Published Online: March 15, 2023

1. Introduction

The cornea is one of the optical systems in the front part of the eye. The edge of the cornea, the limbus, is continuous with the sclera and located at the margin between the neocortex and the subcortical structures. As the first surface of the eye that interacts with the environment, the cornea serves as an ocular biodefense system. When in a healthy condition, the corneal epithelium can rapidly repair and renew itself through corneal limbal stem cells. A smooth, uninterrupted, healthy, and intact corneal epithelium helps maintain the normal physiological health of the eye, which is essential for clear vision.

Diseases occurring in the cornea limbus may lead to vision loss and even permanent blindness. The cornea of patients with limbal stem cell deficiency (LSCD) is thinner than normal corneal epithelium, usually causing new vessels to grow into the cornea and affect vision. Symptoms of corneal limbal epithelial stem cell (LESC) deficiency may include reduced vision, persistent photophobia, tearing, and blepharospasm. Repeated and persistent epithelial breakdown and recurrent episodes of pain in the eye will cause invasion of conjunctival epithelium onto the corneal surface (conjunctivalization), and even lead to chronic inflammation with redness and corneal blindness. A variety of ocular surface disorders and congenital or acquired diseases will cause corneal stem cell deficiency, such as Stevens–Johnson syndrome, aniridia keratopathy, chronic contact lens-associated epitheliopathy, chemical and burned injury (exposure to injurious agents), and corneal intraepithelial dysplasia.

Usually, patients with corneal stem cell deficiency can only be treated with conventional corneal transplantation, which is a surgical technique for ocular surface epithelial regeneration. Although corneal transplantation is generally successful in the short term, it still has a high failure rate due to rejection in patients with autoimmune diseases. The severe shortage of donor sources and the potential risk of infection further limit the application of allograft corneal transplantations. The trend of an aging population suggests that there will be an increasing imbalance between the supply and demand for high-quality donor cornea. The widespread use of laser-assisted *in situ* keratomileusis (LASIK) also leads to a reduction in the number of complete corneal donors without laser cutting. Lamellar or penetrating keratoplasty can only temporarily replace the corneal epithelium of the host because of the lack of limbal stem cells, as well as the limited proliferative capacity and lifespan of the grafted epithelial cells.

Griffith *et al.* first reported the construction of three-layer corneal substitutes *in vitro* with human corneal cell lines[1]. However, there are safety concerns with this method because of the immortalization properties of the cells. Corneal substitutes without limbal stem cells can only be used for those patients whose limbus is still capable of epithelium regeneration. The advantage of synthetic materials is that they can effectively prevent the spread of potentially infectious diseases, especially viral infections, compared with decellularized corneal stroma and other natural materials. Most of the current artificial cornea equivalents are constructed with immortalized corneal epithelial cells without any renewable epithelial organization. Artificial cornea with limbal stem cell tissue will be a significant development toward corneal regeneration. Cornea equivalents constructed by tissue engineering are slow to manufacture and limited to only thin, flat, and stacked constructs. The novel method for artificial limbus with limbal stem–cell–laden synthetic materials and a geometric-controllable shape remains to be studied. Artificial structures for corneal limbus reconstruction are required to integrate with the host tissue and should be functionally transparent and mechanically stable. Cornea is an optimal target for three-dimensional (3D) bioprinting technology due to its curved-thin shape, which is difficult to build with conventional tissue engineering process in cornea regeneration. 3D bioprinting is currently a hot topic in many fields of regenerative medicine like cartilage, vessels, and others. It can provide precise control of the shape and realize the optical function of lens-like structure and precise positioning of cells. The effective method of limbal stem cells achieves the construction of a structure similar to the natural cornea. Therefore, 3D bioprinting maybe a promising technique for fast production of full-thickness artificial cornea with stem cells. In this paper, we provide a review of current treatment methods of LSCD and recent 3D printing progress toward constructing stem cell–laden structures. The purpose of this article is to help elucidate 3D bioprinting as an exciting treatment for corneal LESC deficiency. We also suggest steps that will be required if 3D-printed artificial corneal limbus equivalents are to be successfully used.

3D bioprinting has garnered tremendous attention in recent years, and significant advances have been made in fabricating cell–laden scaffolds[1]. Charles W. Hull proposed stereolithography as the first 3D printing method in 1986, which constructed solid 3D structures with ultraviolet light in layers. This process was later developed into other forms, such as extrusion-based 3D printing (E-3DP)[2], digital-light-processing 3D printing (DLP), laser-assisted 3D printing (L-3DP)[3], and computed axial lithography (CAL)[4,5], which enables printing with diverse materials from polymer to biomaterials (Figure 1).

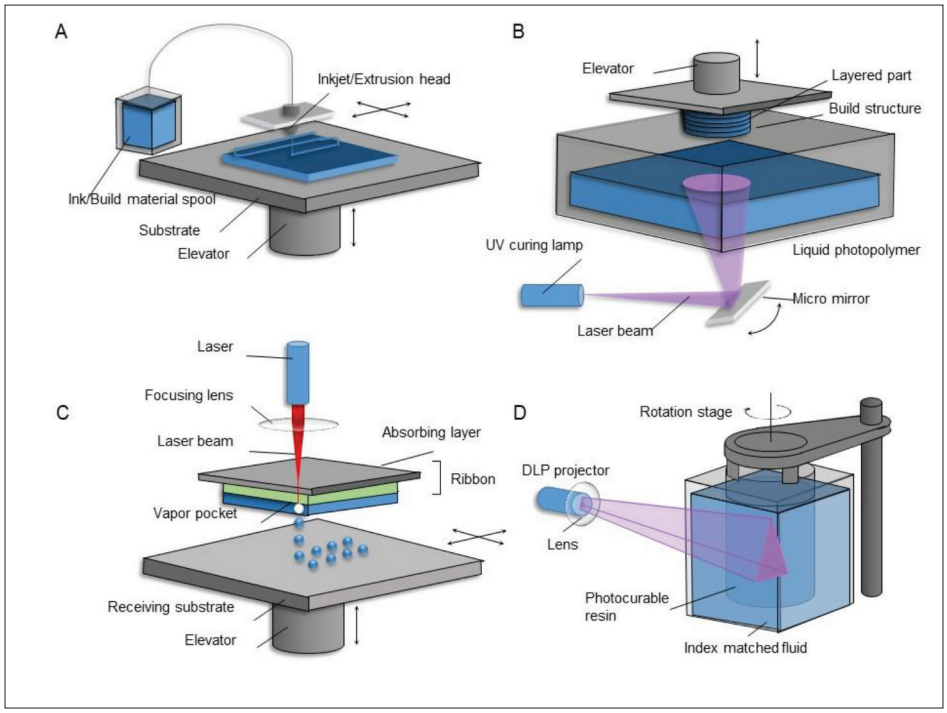

Figure 1. Schematic overview of E-3DP, L-3DP, DLP, and CAL modalities. (A) E-3DP: Schematic of an extrusion 3D-printer, where droplets/filaments/tubes are forced by air-pressure/piezoelectric or ultrasound pressure/vapor bubble/mechanical (piston or screw) dispensing systems, and extruded through a nozzle. (B) DLP: Schematic of a traditional digital-light-processing 3D printer, which constructs multilayer structures cured with a 2D cross-section of light. (C) L-3DP: Schematic of a laser-assisted 3D printer, which generates pressure on absorbing substrate to propel cell–laden materials onto a collector substrate. (D) CAL: Schematic of the CAL system, where the resin in the rotating volume is cured frame by frame[5].

2. Anatomy and physiology of corneal limbus

The ocular surface of the eye refers to the entire mucosal epithelium that borders the skin at the superior and inferior eyelid margins[6]. The epithelial surface is separated histologically and physiologically by the limbus between two major areas: the cornea and the conjunctiva. The cornea can be divided into five layers, i.e., three cell layers and two border layers. The corneal epithelium is composed of five or six layers of epithelial cells and the thickness is approximately 50–60 μm[7]. Flattened cells with microvilli are located at the most outermost layer of the epithelium. The microvilli interact with the tear film, which provides a smooth and moist surface for the cornea[8]. The tear film is also an important source of factors that provide maintenance and repair of the corneal epithelium. Two or three layers of polyhedral wing cells lie beneath the flattened cells. The internal layer is the basal layer with a single layer of cylindrical cells on the basement membrane[9].

LESCs, as tissue stem cells, have long life span, low degree of differentiation, and rapid stress-induced proliferation. Research by Cotsarelis *et al.*[10] showed that stem cells in the region between the cornea and the sclera have a low degree of differentiation and mitosis, and have the characteristics of slow cycling. When the corneal epithelium is damaged, LESCs are activated and begin to proliferate and repair the epithelium. The stratified, nonkeratinized squamous corneal epithelium gradually transits from the stratified, nonkeratinized columnar conjunctival epithelium at the cornea-scleral limbus of cornea, which has 7–10 layers of cells[11-13].

The "X, Y, Z hypothesis of corneal epithelial maintenance" was proposed by Thoft and Friend[14]. This hypothesis indicates that stem cells will divide according to the needs of tissue regeneration as shown in Figure 2. When a stem cell divides, part of the daughter cells continues to serve as mother cells to supplement the stem cell pool, and another part of the daughter cells divides and differentiates to form corneal epithelial cells. These epithelial cells then form the basal cells (X), and the cells that migrate to the central cornea (Y) replace the desquamated cells (Z). The characteristic of cell migration is centripetal and circumferential, characterized by migration from the periphery to the centripetal and circumferential direction, and by vertical movement from the basal layer up to the surface of the cornea[15]. This hypothesis has also been proven in animal models of corneal epithelial wounding[16,17].

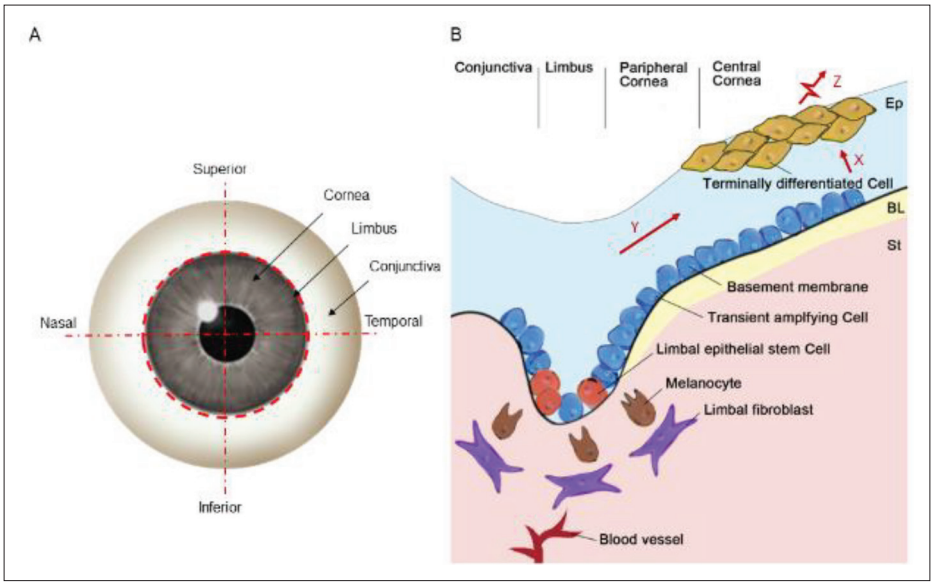

Figure 2. (A) Location of the limbus showing the superior, inferior, nasal, and temporal regions of the limbus. The limbus is continuous with the cornea, and the red circle is where the limbal niche and limbal epithelial stem cells (LESCs) are located. (B) Illustration of the limbus and surrounding epithelium region. Five percent to 15% of the cells in the limbus are stem cells. Limbal epithelial stem cells (LESCs) divide into transit-amplifying cells (TACs). LESC-derived TACs migrate from the peripheral cornea to the central cornea in the Y component. TACs become mature and give rise to terminally differentiated cells (TDCs) in the X component, which progressively replace the desquamated corneal differentiated epithelial cells in the Z component. Ep: basal layer of the epithelium; BL: Bowman's layer; St: corneal stroma. Corneal epithelial maintenance can be defined by XYZ equation. X: proliferation of basal cells, Y: centripetal movement of cells, Z: cell loss from the surface[14].

Dua *et al.* proposed that stem cells exist in the limbal epithelial crypts. These corneal epithelial crypts gather nondirectionally along the limbus, with the highest density in the nasal region[18].

3. Current therapy of LESC deficiency

3.1. Conjunctival limbal autograft

Conjunctival limbal autograft (CLAU) was described for the management of symptomatic partial or total unilateral limbal deficiency by Holland and Schwartz in 1996[19]. In many cases of unilateral limbal deficiency, the autograft harvested from the other healthy eyes can be used in the treatment of unilateral injury through CLAU[20]. CLAU eliminates the need for systemic immunosuppression because there is no risk of tissue rejection in autograft. This therapy is affected by the state of donor eye and the extent of LSCD in recipient eye. Several important criteria for selecting donor tissues for CLAU include: (i) free from long-term use of contact lenses and (ii) never had ocular surface surgery or other conditions that may predispose the patient to LSCD. A major problem is the potential risk of donor eyes. Some studies have shown that partial excision of full-thickness corneal limbus will damage the donor ocular surface and even ocular function[21]. More than 90% of the patients' eye surfaces were restored with overwhelmingly (greater than 80%) successful visual outcomes when using large grafts (donor limbus tissue area: 120°–240°)[22,23]. In order to avoid endangering the donor's eyes, some studies attempted to use smaller grafts (donor limbus tissue area <90°), which resulted in a 60% reduction in visual rehabilitation as well as several complications in donor eye[24,25]. Although complications are rare and re-epithelialization of the donor site usually occurs, the risk associated with taking two large limbal biopsies from the healthy donor eye is a concern associated with CLAU.

3.2. Cultivated limbal epithelial transplantation

Cultivated limbal epithelial transplantation (CLET) carries stem cells through artificial substrate tissues such as a limbus alternative. The cells source of CLET comes from autologous and allogenic tissues. Autologous grafts for CLET were approved by the European Medicines Agency in 2015. The substrate of CLET can be divided into natural and artificial tissues. For natural tissue, amniotic membrane (AM) is used as a substrate to expand LESCs from allogenic eyes for CLET[26,27]. Holoclar is the first commercial and artificial product approved by the European Medicines Agency, and it is made up of expanded LESCs grown on an artificial fibrin membrane and used in patients with chemical burns in one eye[28-31]. The limitation of this approach is that it is only appropriate for a very specific cohort of patients. Human and porcine decellularized corneal stroma has received extensive attention as a scaffold material for culturing LESCs. Thai's introduced epithelial basement membranes of

human decellularized cornea as a substrate for ESC to support potential applications of corneal epithelium tissue engineering[32]. Collagen is also used to build a scaffold that could overcome the inherent variability in tissue samples such as AM[33]. Real Architecture for 3D Tissue (RAFT) technology was introduced to construct a thickness-controllable scaffold to support LESCs[34-37].

3.3. Simple limbal epithelial transplantation

Simple limbal epithelial transplantation (SLET) was first reported in 2012[38]. The tiny limbal fragments from the undamaged eye are transplanted onto the damaged cornea in SLET without needing *ex vivo* expansion[39]. The amniotic membrane is used as the carrier of small limbal pieces as the graft. This therapy combines the benefits of CLAU and CLET. It also prevents damaging the donor's eye as a result of large limbal tissue removal or avoids the high cost of stem cell transplantation. This single procedure offers several benefits compared with the other options[40,41]:

(i) Risk of iatrogenic damage to the donor eye is reduced.

(ii) A small biopsy means that the procedure can be repeated if necessary.

(iii) SLET does not require expensive specialized culture facilities.

(iv) The SLET procedure can be performed in one operation, thereby streamlining patient care, resource management, and reducing costs.

(v) The large donor graft demanded in CLAU can be avoided in SLET, which decreases the risk to the donor eye of losing a significant amount of limbal tissue. However, the risk of symblepharon and inflammation is higher with SLET than with CLAU.

3.4. Keratolimbal allograft

In the situation of bilateral LSCD, it is possible to utilize conjunctival-limbal allografts from a living related relative (lr-CLAL) or limbal tissue attached to a corneoscleral carrier from a cadaveric donor. In eyes with complete bilateral total stem cell deficiency, a high rate of immune reactions needs to be considered in limbal allografts transplants because of the existence of Langerhans cells and HLA-DR antigens. The treatment of bilateral LSCD is to remove the host's altered corneal epithelium and pannus, and to permanently cover the host cornea with epithelium from the donor limbus. A living relative with an HLA-matched tissue is a potential donor that usually gives a better tissue match than an unrelated donor. Fresh cadaver donor tissue as limbal allograft is also a source of healthy limbal stem cells. Tseng *et al.* described a method to obtain a 360° cadaveric donor limbal ring graft surrounding conjunctival edge with improved stabilization[42]. Kenyon and Tseng[23] proposed that limbal auto- or allo-transplantation combined with or followed by keratoplasty can be used for corneal surface reconstruction.

Carrier tissue is needed in corneal stem cell transplants to ensure that the stem cell tissue is fixed to the eye surface steadily and proliferation and differentiation are achieved. Keratolimbal allograft (KLAL) is a proper treatment of LSCD when there is no living relative or autograft tissue available. There have been many reports of modified SLET using allogeneic limbal tissue (alloSLET) to treat bilateral LSCD[43-45]. However, systemic immunosuppression and vigilant postoperative management are necessary to prevent immunologic graft rejection after KLAL. When non-HLA-matched limbal allografts are used in allogeneic transplantation, the immune rejection response needs to be closely monitored for at least 12 months. Immune rejection may cause inflammation, acute or chronic rejection episodes, epithelial defects, and even graft failure[46-48]. In some cases, even permanent administration of systemic immunosuppression drugs may be required to avoid serious consequences of the immune rejection response, such as with oral cyclosporine A, FK 506 (tacrolimus, Fujisawa Ltd), or topical cyclosporine intravenous dexamethasone[46,49-52]. The role of immunosuppression of KLAL is still under evaluation in thousands of clinical cases. The imbalance of supply and demand is also significant in conjunctival-limbal allografts from lr-CLAL and fresh cadaver donor tissue. Thus, new research is more focused on exploring novel tissue materials and construction methods for artificial carriers. One example is the development of cultured limbal epithelial transplantation to restore vision following ocular surface injury or disease caused by LSCD.

As mentioned above, autologous undamaged limbal tissue is transplanted into the damaged contralateral eye in most of existing treatments for corneal LESC deficiency, which solves the inability of epithelial layer regeneration in limbal defect area. However, the size of limbal tissue transplanted to contralateral eye is limited, the number of limbal stem cells is insufficient, and the distribution of limbal stem cells and cover area of regenerated epithelial cells after transplantation is still uncontrollable. In those cases with limited limbal biopsy tissue, only the area of donor biopsy is the epithelial cell source in recipient eye. However, due to the migration capacity of epithelial cells, when a certain volume of limbal biopsy covers a limited area, regenerative epithelial cells from biopsy may not cover the whole cornea. Moreover, in tissue engineering treatment, amniotic membrane is used as a carrier of donor biopsy tissue for suturing. Amniotic membrane appears translucent after transplantation, which has negative impact on visual ability of recipient. Therefore, there is

an urgent need for a new process to solve the problems in tissue engineering treatment of LESC deficiency.

4. 3D bioprinting: A new tool for LESC deficiency

At present, many 3D-printed medical devices have been commercialized, including surgical instruments, implants (such as hip joints, maxillofacial bones, etc.), and external prostheses and exoskeletons. Nowadays, some scientists are investigating how to use 3D bioprinting to make living organs such as the heart, kidney, and liver, but this research is still in the early stage of development. 3D printing can utilize a variety of materials, including synthetic or natural materials, on demand to mimic nature cornea. We systematically analyze potential, advantages and development direction of 3D printing as a novel tool in the future treatment of LESC deficiency.

Corneal limbus and its surrounding tissues have the characteristics of high density as well as multicellular, multilayer, circular curved structure, which requires shape control and function simulation in artificial corneal limbus fabrication. 3D printing has the theoretical advantage of precise shape control and functional simulation. The core of its application in therapy for LSCD is bioactive ink and target structure of the printing process: 3D printing overcomes the limitations of traditional tissue engineering methods, such as single material, complex manual operations, and poor repeatability. The integrated construction of bioactive materials such as cells, growth factors, full-thickness multicellular limbus, and microstructure construction is realizable by 3D printing. 3D printing supports controllable geometric parameters and mechanical properties of printed structures. Based on individual corneal geometric data, a corneal limbal graft model for injured eyes could be constructed and modified easily using computer-aided design (CAD). The target structure could be precisely controlled by adjusting the printing scheme and process. Through the adjustment of biological ink and the improvement of printing scheme, artificial corneal limbus can be manufactured to meet the special needs of transplantation and patients.

4.1. Bioactive ink

The artificial limbus should be equivalent to the natural cornea in terms of structure, function, and shape; limbal stem cells are necessary to replace the function of the natural limbus. High repeatability, size, and dimensions are also indispensable requirements in manufacturing. The artificial limbus should be biocompatible and support the growth, proliferation, and migration of limbal stem cells and could be combined with autologous tissue without any autoimmune rejection. The artificial limbus should also have suitable biomechanical properties and be able to withstand surgical sutures. A neutral material that has high transparency and micro-porosity, and thereby supports the diffusion of oxygen, carbon dioxide, and nutrients is required. The shape and structure affect the refractive power of the cornea and a shape consistent with the natural cornea is conducive to laying the artificial limbus on the surface of the eye and tightly combining it with self-organized tissues.

Research on different stem cells has also made great progress in recent years. 3D printing of stem cells is being widely studied in regenerative medicine. 3D-printed stem cell structures have higher capabilities to fulfill different medical requirements. For example, due to high self-renewal and easier application, mesenchymal stem cells (MSCs) were printed with a laser printing-based 3D printer by Koch *et al.*[53]. Induced pluripotent stem cells (iPSCs) are the most recent advancement made in the area of cell biology, which has been used in 3D skin equivalents[54] and cardiac patches[55]. 3D printing also allows precise control of embryonic stem cells (ESC) to construct neural tubes[56]. Adipose stem cells were printed into mesh structures for hepatogenic differentiation[57]. Advances in the fields of 3D printing processes with stem cells have provided a tremendous leap for research on medical regenerations. The impact of the 3D printing process on the activity, differentiation, and proliferation of stem cells is gradually reducing, which further promotes the development of 3D printing in the construction of artificial corneal limbus. Currently, in the field of limbal reconstruction tissue engineering, it has been used as a cell source to replace limbal stem cells, as presented in Table 1.

The desired qualities of stem cells for limbal substitutes are as follows:

(i) The stem cells can be cultured in bioink both *in vivo* and *in vitro* without affecting the protein and gene expression.

(ii) Ability to control the growth of stem cells through artificial intervention is necessary to ensure that they will differentiate into the specific cells.

(iii) The light transmittance of differentiated epithelial cell layers needs to be fulfilled for the visual function after cornea reconstruction.

(iv) The stem cells can survive for a long term or even permanently in the recipient tissue.

(v) The stem cells will not cause any potential harm to the natural tissues and the health of the recipient.

The corneal epithelium is renewed by limbal stem cells in graft after transplantation. However, the natural cornea limbus also includes the basement membrane and other

Table 1. Cell sources for the treatment of LSCD

Cell types	Substrate materials	Manufacturing method	Clinical status	Follow-up result	Ref.
Conjunctival epithelium	Amniotic membrane	Tissue engineering	*In vivo* rabbit	2 weeks, layers of stratified squamous epithelium generation	[83]
Limbal epithelial stem cells and stromal keratocytes	Acid soluble rat tail type I collagen/proteoglycan hydrogels	Horizontal magnetic field (7 T)	*In vivo* rabbit	2 months, re-epithelialization and recovery of ultrastructural organization	[84]
Limbal epithelial stem cells and keratocytes	Amniotic membrane + laminin-coated compressed collagen gel (rat-tail type I collagen)	Compression and dehydration	*In vitro*		[85]
Corneal epithelial stem cells and stromal keratocytes	Feeder layers of lethally irradiated 3T3-J2 cells	Tissue engineering and molding	*In vitro*		[86]
Corneal epithelial stem cells	Collagen	Tissue engineering and plastically compression	*In vitro*		[87]
Human immature dental pulp stem cells (hIDPSC)	Tissue-engineered cell sheet made from undifferentiated hIDPSC	Tissue engineering	*In vivo* rabbit	3 months, transplantation was improved in chemical burn treatment	[88]
Bone marrow-derived human mesenchymal stem cells	Human amniotic membrane	Tissue engineering	*In vivo* rat	4 weeks, reconstruction of the damaged rat corneal surface	[89]
Bone marrow-derived human mesenchymal stem cells	α-minimum essential medium (α-MEM)	Injections	*In vivo* rabbit	1 months, wound healing improvement with clearer healed tissue	[90]
Mesenchymal stem cell	Lyophilized MSC secretome (MSC-S) including a viscoelastic gel composed of hyaluronic acid (HA) and chondroitin sulfate (CS)	Tissue engineering	*In vivo* rat	2 weeks, wound healing improvement	[91]
Human induced pluripotent stem cells (H-iPSCs)	Cell sheet of mouse-derived feeder cells	Tissue engineering	*In vitro*		[92]
Mouse induced pluripotent stem cells (M-iPSCs)	Cell sheet of mouse-derived feeder cells	Tissue engineering	*In vitro*		[93]
Human embryonic stem cells (ESCs)	Cell sheet of mouse embryonic fibroblasts	Tissue engineering	*In vitro*		[94]
Autologous limbus-derived stem cells	Fibrin	Tissue engineering	Clinical research: 112 patients		[95]
Autologous oral mucosal epithelial cell	Cell sheets of 3T3 feeder cells	Tissue engineering	Clinical research: 4 patients	14 months, corneal surfaces reconstruction and transparency restoration	[96]

various cell populations, such as limbal fibroblasts and melanocytes, which are also necessary for maintenance of limbal stem cells and corneal epithelium regeneration. The local signals generated in the limbal cellular niche will affect the corresponding daughter cells generated by stem cell division to follow different cell fates. In some studies, these signals are simulated by adding drugs, small molecules, cytokines, growth factors, etc., as the modulation strategies to affect limbal stem cells in artificial graft. Some studies have shown that limbal basement membrane may promote the stability of limbal cellular niche and provide key factors for cell regeneration[58,59]. Simulating and high-precision positioning the components of limbal basement membrane, including collagen (IV, XVI), laminin (α1, γ3), tenascin C, and other components[60] is achievable through 3D printing. At present, the complexity and heterogeneousness of different components and activities in limbal cellular niche remains enigmatic. 3D printing with multicomponent may enable further researches on the physiological activities of corneal limbus.

4.2. Target structure

The artificial limbus has the characteristics of a high cell density as well as a multiunit, multilayer, and curved surface. Some studies have shown that the position of cells affects proliferation ability. Limbal basal cells located at the limbus perform better in culture[61,62]. The regeneration of the limbus requires shape control and the restoration of corneal epithelial function. Compared with 2D structure, biomaterials in 3D structure can better simulate the characteristics of cornea, so that researchers and clinicians can better recognize the proliferation, differentiation, and diffusion of corneal cells. 3D bioprinting can accurately locate the cell position, simulate the natural corneal limbus structure, distribute limbal stem cells and primary epithelial cells according to needs, and provide carrier tissue for limbal stem cells and epithelial cells. 3D printing has been used in corneal tissue regeneration engineering[63-66], with these studies proving that 3D printing has extremely high potential in the printing of limbal stem cells. As shown in Figure 3, additive manufacturing has the ability to change the distribution of donor biopsy from single point to multipoint and from single layer to multilayer, so as to realize multiple epithelial cell sources and cover a wider area on surface of recipient cornea. If the distribution of limbal cells of cornea can be simulated as ring-like arrangement in natural cornea, the least amount of limbus stem cells, which means the minimum volume of donor biopsy, is able to support a larger epithelial coverage.

Conventional tissue engineering largely depends on manual operation, which has many problems, such as manual error, low repeatability, high requirements for operators, difficulty in high-throughput manufacturing, and low efficiency. 3D printing technology supports mass production of target structure with less time and cost, and can directly modify the required model through computers according to specific needs, greatly reducing requirements for operators since there are no manual operations involved. The problem of manual error in manufacturing process is avoided. With the development of 3D printing technology, it may achieve high-throughput manufacturing and customization of cornea limbal substitutes. In addition, 3D printing can create microstructure such as micropores in complex structures, thus controlling the physical and chemical properties of printed scaffold at microlevel.

3D printing supports the on-demand distribution of a variety of materials. Many studies focus on 3D printing with growth factors, drugs, and bioactive materials. For example, sustained drug release and other functions can be achieved by combining bioink/structure as a carrier with specific drugs at target location. On the one hand, it may promote rapid recovery and reduce inflammation, eliminating the need to administer medications after surgery. On the other hand, 3D-printed cornea-on-chip could change the type and location of materials according to research needs, providing a more realistic model for limbal medical research.

As a carrier attached to bioactive materials such as cells and factors, inks for 3D-printed structure shall meet the following conditions:

(i) Meet the requirements of 3D printing principles and processes on physical properties

(ii) Stable forming units can be printed and quickly solidified

(iii) Rapid adhesion between forming units with high fidelity

(iv) Suitable swelling characteristics and shape stability in osmotic pressure balance state

(v) Maintain high transparency in recipient eye

(vi) Support the basic physiological and chemical functions of cells and other bioactive substances

(vii) Physical and chemical changes during printing process and transplantation will not damage cells and other bioactive substances

(viii) Promote tissue regeneration after implantation, or it can be stably retained in the body for a long time

Several important factors should be considered when selecting bioink for corneal limbal regeneration, including mechanical properties (viscoelasticity, stiffness, strength, elasticity, surface quality, and integrity), microstructure

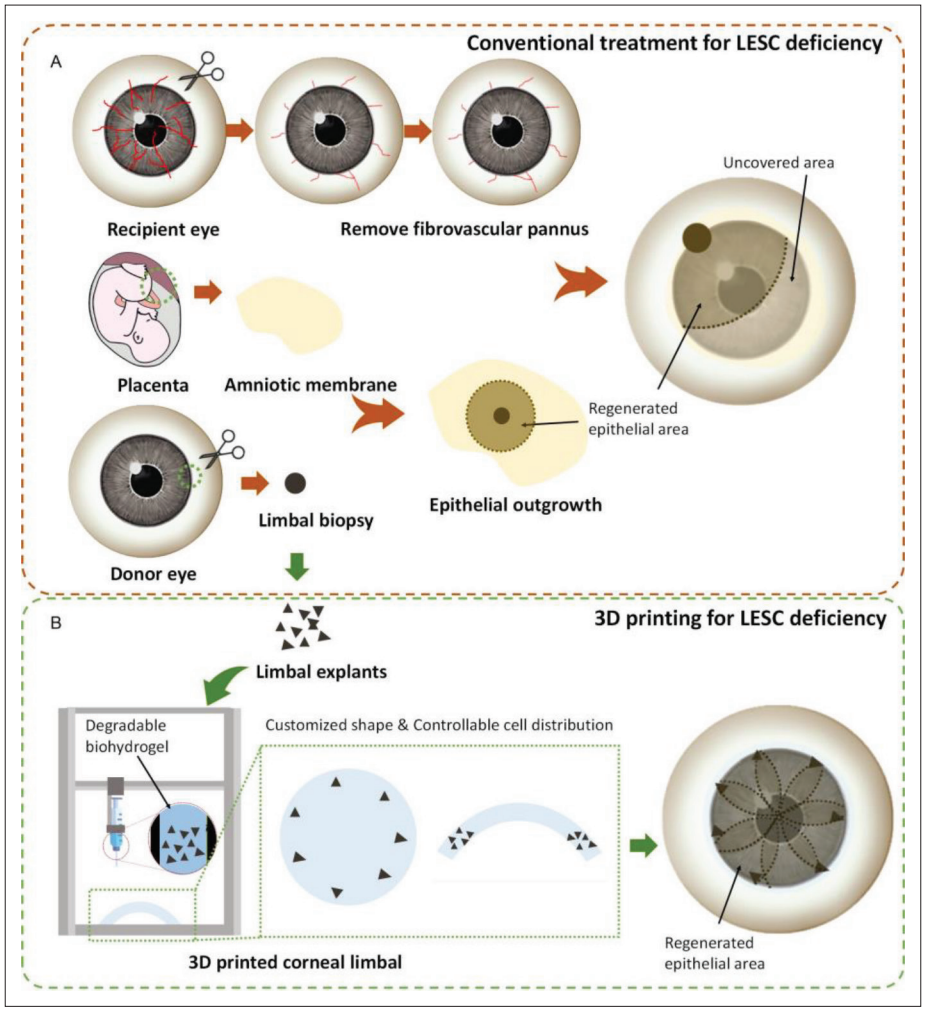

Figure 3. (A) In conventional treatment for LESC deficiency, amniotic membrane as the carrier of donor limbal is transplanted to recipient's ocular surface. Corneal epithelium generates around the donor limbal biopsy on recipient's eye. This method is restrained by insufficient number of donors, poor transparency, and limited outgrowth area of cells. (B) 3D printing can distribute limbal explants, cells and other biomaterials on demand with transparent ink as substrate, and customize the shape as natural cornea to fit recipient better. Limbal explants in 3D-printed cornea limbus could be distributed according to the natural structure, creating possibility for corneal epithelium outgrowth centered on multiple sites, so as to achieve greater coverage of regenerated epithelium. The problem of donor storage or bilateral absence can also be solved by stem cell differentiation in 3D-printed limbus.

(porosity, number, and size of pores), water content, hydrophilicity, oxygen permeability, nutrient permeability, transmittance (central zone), and biocompatibility (Figure 4).

In some studies, naturally derived biomaterials were used to improve the biocompatibility of ink. For example, collagen[67], gelatin[68], fibrin[69], agarose[70], chitosan, hyaluronic acid[71,72], fibroin[73,74], and decellularized stromal tissue[75] have been mixed into materials for artificial cornea fabrication. A variety of synthetic substances, including polyethylene glycol (PEG)[76], poly (lactic acid glycolic acid) copolymer (PLGA)[77,78], polyvinyl alcohol (PVA)[77,79,80], and poly (2-hydroxyethyl methacrylate) (PHEMA)[81,82], are used as carriers of bioactive materials in corneal tissue engineering as well. However, for inks used in 3D-printed corneal limbus, proper mechanical properties and quick polymerization/phase change are required to ensure printability. Therefore, specialized ink for corneal 3D printing is obtained through physical and chemical modification. The bioink widely used in corneal 3D printing research is shown in Table 2. In different 3D printing methods, bioinks with various characteristics can be selected and improved according to specific application needs in the study.

To sum up, it is of high research value to further develop 3D artificial limbus cornea based on the treatment needs of LCSD. Compared with traditional tissue engineering manufacturing methods, 3D printing technology can flexibly realize the accurate construction of

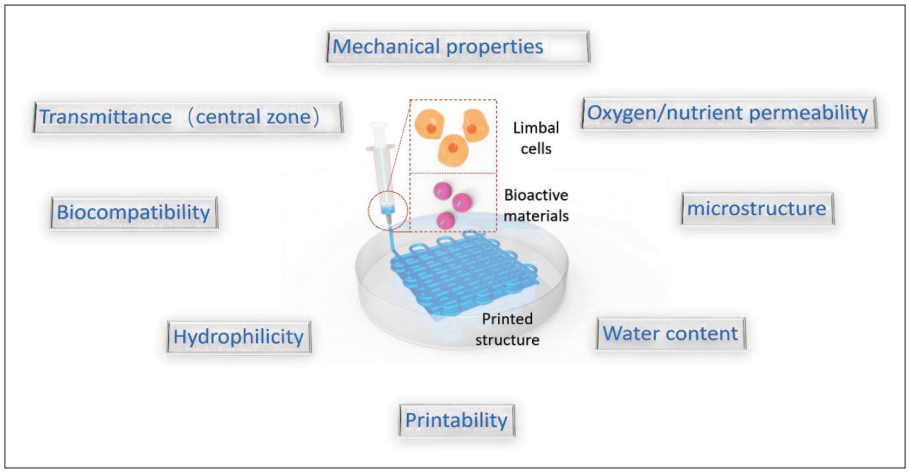

Figure 4. Desired properties of bioinks for cornea limbal regeneration.

Table 2. Bioink in corneal regeneration

Materials	3D printing method	Cells type	Clinical status	Follow-up result	Ref.
Gelatin-methacryloyl (GelMA)	SLA	Human corneal stromal cells	*In vitro*		[97]
Corneal stroma-derived decellularized extracellular matrix	Extrusion printing	Human turbinate derived mesenchymal stem cells (HTMSCs)	*In vivo* rabbit	4 weeks, lattice pattern generation with printed collagen fibrils	[98]
Bioink containing human recombinant laminin-521 & Human collagen I	Laser-assisted bioprinting	Human embryonic stem cell derived limbal epithelial stem cells (HESC-LESC), human adipose tissue derived stem cells (HASCs)	*In vitro*		[64]
Poly (ε-caprolactone)-poly (ethylene glycol) & GelMa	Direct writing	Limbal stromal stem cells (LSSCs)	*In vivo* rat	3 months, stromal tissue regeneration	[99]
Type I collagen-based bioinks	Drop-on-demand (dod) bioprinting	Corneal stromal keratocytes (CSK)	*In vitro*		[100]
Gelatin-methacryloyl (GelMA)	Extrusion printing	Human corneal keratocytes (HKs)	*In vitro*		[101]
Chitosan and polyvinyl-alcohol	Extrusion printing	Human adipose tissue-derived mesenchymal stem cells (HASCs)	*In vitro*		[102]
Sodium alginate and methacrylated type I collagen	Extrusion printing	Corneal keratocytes	*In vitro*		[65]
Polyvinyl acetate/collagen	Electrospun	Human keratocytes (HKs) and human corneal epithelial cells (HCECs)	*In vitro*		[103]
Gelatin & Sodium alginate	Extrusion printing	human corneal epithelial cells (HCECs)	*In vitro*		[63]
Cornea-derived decellularized extracellular matrix (dECM)	Extrusion printing	Human turbinate derived mesenchymal stem cells (hTMSCs)	*In vitro*		[104]

complex structures with multimaterials and multicells in customized arrangement. It greatly shortens the time for model modification and optimization with simple and fast manufacturing process. However, there is a contradiction between forming accuracy and material compatibility in existing 3D printing methods. Biomaterials suitable for simulating precise structure of the limbus and the transparent property of cornea are still limited. There are three main challenges for 3D printing technology in artificial corneal limbus manufacturing: (i) How to realize controllable forming of highly biocompatible materials? (ii) How to realize the multimaterial printing at the

Table 3. Advantages of 3D bioprinting for limbal stem cell deficiency

1	Stem–cell–laden bioink could be accurately divided into target positions with 3D printing, thereby greatly reducing the required number of stem cells and donor tissue. Since the donor tissue required is less than that of conventional tissue engineering, the range of autologous transplantation could be expanded. Therefore, problems such as immune rejection caused by allograft could be avoided.
2	Engineered printers supports mass production of structures in required shape and size in lesser time and lower cost compared with traditional manual processes.
3	With the integration of a variety of cells, the epithelial layer and the tear film layer with 3D bioprinting, the normal ocular surface could be restored rapidly, and the acute ocular surface inflammation can be inhibited.
4	The technology of 3D printing multi-layer structures can fulfill the demand for stem cells, which can survive and work properly in complex structures. Functionalized artificial limbus can benefit from the specific spatial arrangements of different cells and factors in the 3D printing process for further clinical needs.
5	Personalized artificial limbus together with whole corneal epithelium is available by rapid prototyping. With precise control of the 3D printer and 3D computer-aided design (CAD) model based on the geometric data of individual eye, it is possible to reconstruct the damaged limbus and restore optical functions.
6	3D bioprinting allows the integration of multiple materials and print tissue by using multi-bioink within a single process without culturing cells separately. Such a development can break through the limitations of single materials in conventional tissue engineering.
7	This process is feasible without the artificial processes in tissue engineering, which achieves programmed construction and ensures stable fabrication quality without being affected by different batches. The biological control of non-toxic, sterile products with a high-quality level and stability can be ensured with good production management and a mechanized manufacturing system. The potential viral, religious, cultural, policy, and immune rejection problems associated with allogenic donor corneas can be effectively avoided.

micro/nanoscale as limbal niche? (iii) How to balance the degradation of printed structure and the regeneration of recipients' tissue? In the actual researches of 3D-printed limbus, it is necessary to select ink and other components with suitable physical and chemical properties. The corresponding processes based on research needs and the structural characteristics of natural cornea also remain to be studied according to properties of ink.

4.3. Future applications

High-fidelity corneal models will also play an important role in clinical treatment and medical education. For the curved, thin structure such as cornea, conventional manufacturing processes such as casting for preparing the mold are time-wasting, and it is also difficult to fabricate the structure with multiple materials. The individual differences between patients are always neglected as well. 3D-printed personalized corneal limbal model can provide communication tool for doctors, engineers, patients, and medical students. Mass-produced 3D-printed medical models also play an important role in research planning, prosthesis testing, preoperative diagnosis, and surgery.

The main advantages and potential of 3D bioprinting in the reconstruction of artificial limbus *in vitro* are presented in Table 3.

5. Conclusions and future perspectives

It is becoming increasingly urgent to develop treatment for corneal epithelial shedding and LESC deficiency. The development of artificial limbus is an effective solution to addressing this issue. With the emergence of 3D bioprinting in the field of personalized medicine and the emerging research on tissue and organ implantation and regeneration, 3D bioprinting holds great potential in the development of artificial limbus. This article summarizes and compares the materials for artificial limbus and manufacturing methods, and proposes the unique technological advantages and characteristics of 3D bioprinting, which should be taken into consideration in the construction of key features of limbus. Existing animal and clinical experiments have shown that cultured limbal stem cells are a safe and effective choice for treating LSCD. 3D-printed limbal substitutes and even fully functional corneal substitutes are the innovations based on the deep understanding of alternative stem cells in the human body. However, in-depth research is still needed in many aspects, such as the selection and cultivation of stem cells, the improvement of the 3D printing process accuracy, and the realization of high surface quality of the substitute and growth dynamics of stem cells in the recipient. In summary, 3D bioprinting has great potential and development prospects in the treatment of LSCD.

Acknowledgments

None.

Funding

The authors would like to thank the support by the National Key Research and Development Program of China (Grant No. 2018YFA0703000), the Key Research and Development Projects of Zhejiang Province (Grant No. 2017C01054), the National Natural Science Foundation of China (Grant

No. 51875518), and the Fundamental Research Funds for the Central Universities (Grant Nos. 2019XZZX003-02 and 2019FZA4002).

Conflict of interest

The authors declare no conflict of interests.

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Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Availability of data

Not applicable.

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