

3D bioprinting for artificial cornea : challenges and perspectives

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Abstract

Corneal disease is one of the most important causes of blindness worldwide. Currently, the dominating treatment of corneal blindness is corneal transplantation. However, the main source of cornea for transplantation is based on donations which is far from enough to meet the requirement (less than 1:70 of cases). The severe shortage of donor cornea promotes the studies of effective corneal alternatives. However, many problems remain and can't be solved in current researches, such as original geometry reconstruction and ocular optical function restoring.

3D bioprinting can be a promising approach for corneal substitution. The advantages of this technology in corneal regeneration enable personalized corneal implant and single or multi-layer corneal equivalents with controllable structure and designed refractive ability.

In this review, the progress, applications and limitations of most influential works among current keratoprosthesis and tissue-engineering cornea researches are discussed. Then the applications of 3D bioprinting in manufacturing multi-layered structures and surface are mentioned. Further, the potential, advantages in current research of 3D bioprinting single or multi-layer corneal equivalents and alternatives are discussed. Finally, an insight into the technical challenges and prospective facing the future research of 3D bioprinting corneal alternatives *in vivo* and *in vitro* is provided.

Keywords Biofabrication, 3D bioprinting, Cornea regeneration, Tissue engineering

1 Introduction

The cornea is the key component of eye and plays the critical role in the optical system. Cornea diseases is one of the main causes of blindness, 10 million of the patients having bilateral corneal blindness[1].Owing to modern advances in surgical techniques and surgeon expertise, keratoplasty, as known as the whole cornea transplantation, is the world's most frequent form of solid tissue transplantation and considered the only widely accepted

treatment for cornea blindness[2,3].

Donation is the dominant way to obtain fresh cornea with high quality required for successful keratoplasty[4]. However, there is an overall mismatch between the supply and demand of donated cornea with a percentage of 1.4% due to religiosity, aging, policy, etc.[5,6]. An estimated 12.7 million patients are placed on a long waiting list and the number is still growing[7]. This prolongs of transplantation can potentially cause more serious diseases. It is essential and urgent to develop feasible long-term alternatives to alleviate the shortage of donor tissue for transplantation, such as corneal substitutes which has been an important research subject to future progress of corneal regeneration.

Nowadays, patient- specific treatment becomes a fast-growing global trend in modern medicine. In this condition, conventional manufacturing methods of artificial cornea are questioned in terms of restoring integrated corneal functions according to individual needs in a cost-effective and direct manner. The cornea is one of the complex tissues with refractive power. It is difficult to achieve individual functional restoring of many tissues and organs due to the specific functionalities and particularities of structure and materials. The potential of additive manufacturing, specifically 3D printing, is investigated to serve this purpose[8].

In the last few decades, with the development of 3D bioprinting, aiming to the reconstruction of tissues and organs with designed complex geometries, demonstrated the immense potential and promising results in regenerative medicine[9,10]. It overcomes the restriction of conventional tissue engineering fabrication methods—reconstructing the anisotropic physical properties, specific cell arrangement, heterogeneous components and heterotypic architectures of natural tissue[11]. The special advantages of 3D printing provide a new tool to solve the major problems of personalized cornea and donor shortages.

Here, we provide a review of recent progress toward the latest research on artificial cornea. In this framework, we suggest the challenges and value of 3D printed artificial cornea, the steps that will be required for the development direction as well as the guideline for cornea regeneration.

2 The anatomy and physiology of cornea

As an important component of the optical system, the cornea is highly organized, dense, avascular, transparent, relatively immunological privileged connective tissue (80% water, 13.6% collagen, 0.9% glycosaminoglycan) that protect the eye from external environment. Additionally, the cornea acts as an optical interface between anterior chamber and air to contributes 73.5% of the total refractive power in Gullstrand Number 1 relaxed schematic model eye[12,13]. The average diameter of the adult cornea is 11.04 to 12.50 mm for males and 10.70 to 12.58 mm for females [14]. The human cornea comprised of five layers including three distinct cellular layers separated by two interface layers (Figure 1). The corneal anterior curvature is 7.8 mm and posterior curvature is about 1.3 mm larger than the posterior curvature. The refractive index of cornea is 1.376. Rays penetrate through the cornea and enters the anterior chamber with the refraction index of 1.336[15,16]. The components of the corneal stroma are cells and extracellular matrix which is mainly composed of aligned collagen parallels. The cornea stroma acts as a scaffold to stabilize the physical structure and

regulate the balance of the cornea. The thickness of the stroma accounts for 90% of the total corneal thickness (~500 μ m) and the surrounding area is tens of micrometers more than the center zone[17–19].

Biomechanical properties

The cornea acts as an air-liquid interface on the surface of the eyeball, withstand the pressure of the aqueous humor and the extraocular muscle tension. As an anisotropic soft tissue, the cornea contains a large amount of water, and its response to stress sharing dual characteristics of combining solid and viscous liquids. The highly organized collagen fibrils in corneal stroma are the main component of the entire corneal structure so that the biomechanical properties of the cornea is determined primarily by corneal stroma [20], including viscoelastic, viscous (stress relaxation, creep and anisotropy), structural strength, elasticity, integrity, stability, etc. Microstructural changes in the cornea can cause significant changes in biomechanical properties of the eye. When external shearing forces are applied to the cornea, the resistance to deformation occurs inside the cornea in the form of internal friction. Therefore, in the research and development of artificial cornea for clinical purposes, the study of the corneal mechanical properties is indispensable.

Optical function

The optical functions of the cornea include light transmission, refractive power, and the filtering of some harmful rays. The forward transmittance of human cornea is more than 80 percent at visible wavelengths while attenuates slightly at the spectrum close to ultraviolet[21]. It is essential to take the scattering, absorption effect on the tissues and the interference of light among these aligned collagen fibrils into consideration to analysis the transparent nature of the cornea. Therefore, a proper understanding of corneal ultrastructure is essential to reveal the optical properties of the native cornea and create artificial corneal[22–24]. By reason that proteoglycans in corneal stroma has strong hydrophilicity[25], endothelial-based corneal diseases[26]and corneal damage can cause corneal swelling, which leads to disorder of collagen fibers and parallels, stromal deposits, decreased refractive index of the cornea, corneal dysfunction, reduction of transparency[24,27–29], disorder scattering of light[27], etc., also associated with the change on average corneal center thickness[30,31]. Therefore, the artificial cornea has the characteristics of homogeneity, and limited swellability, which can provide uniform refractive power, and the transparency is not affected by the function of the endothelial layer and thus not share the problems mentioned above as native cornea. The corneal epithelium is covered by the tear film that can form an integrated and smooth optical interface on the surface of the eye to refract light regularly and consistently. Most of the refraction of the eye is at the cornea-air interface. The cornea can be divided into four parts: the central zone, the paracentral zone, the peripheral zone, the limbal zone (Fig.1), where the center zone is approximately spherical and relatively thinner than surrounding area so that the cornea can be regarded as a negative meniscus which plays a pivotal role in the refraction of light. Therefore, the shape and structure in aspect of the theoretical understanding and the control methods practically are crucial in artificial corneal research.

3 Categories of corneal replacements: past and current trends

Cornea alternatives currently in clinical or pre-clinical development that have been reported can be divided into two main categories: Keratoprosthesis and artificial biosynthetic cornea. Artificial cornea has several significant advantages over donor corneas as show in the Table.1.

3.1 Keratoprosthesis

In the mid-20th century, with the development of new materials with transparent, non-toxic and well mechanical properties along with the introduction of antibiotics, topical corticosteroids and immunosuppressive agents[32] enabling better postoperative management, some keratoprostheses were reported to be used as artificial replacements for the opaque or damaged corneas[33–38]. Up to now, there are six well-developed keratoprosthesis(Table.2) : the modified osteo-odonto-keratoprosthesis (OOKP) (Falcinelli, 1987; Liu et al., 2005; Mannis et al., 1999), the Boston type I keratoprosthesis (B-KPro Type I)(Aldave et al., 2012; Zerbe et al., 2006), AlphaCor™ keratoprosthesis(Hicks et al., 2002, 2006; Jirásková et al., 2011), the Boston type II keratoprosthesis (B-KPro Type II)(Dohlman et al., 2009; Pujari et al., 2011), Seoul-type keratoprosthesis(Kim et al., 2002, 2007, 2015; Lee et al., 2000), KeraKlear®-Keratoprothese (Keramed Inc.)(Pineda, 2015). Keratoprostheses can be structurally divided into two parts, optical cylinder with skirt[39,40] or collar button[41–44]. As materials continue to evolve, a wide range of inorganic, organic materials, and metals are used for keratoprosthesis designs. The central optical column requires good optical performance, is usually made of a transparent inorganic material with the diameter of the real cornea, which provide an optical channel and visual acuity. The skirt or collar button surrounds the optic cylinder and engages with the recipient tissue so as to play a role in support and fixation is made of titanium, tooth, polyester etc. In order to improve the stability in vivo and maintain the function, keratoprosthesis is usually made of non-degradable and bioinert materials. These materials can usually be prepared by conventional mechanical processing and are capable of achieving certain optical functions. However, due to the special characteristics of materials and structure, the limitations of current keratoprosthesis are as follows:

- (1) As nonbiological prosthetic devices, keratoprosthesis are usually hard that is not comfortable to wear, then even cause wear and tear on the autologous tissue that may lead to complications.
- (2) Due to the characteristics of external prosthesis, the transplantation process is more complex than that of corneal allograft. For example, the osteo-odonto-keratoprosthesis reported by Strampelli et al. [15], the optical cylinder of which supported with the patient's own tooth. It requires two phases of surgery to obtain autologous tissue before the keratoplasty. The production of prosthesis is likely to cause secondary infections. AlphaCor also requires two stages of surgery.
- (3) The visual field is limited after keratoplasty. Artificial cornea has limited field of view, such as the vision field of the patient using the Boston type I keratoprosthesis was reported to be 60°[45]. Because the combination between the cylinder and scaffold is similar to the screw thread or button structures, the prosthesis is biological incompatible with the autologous tissue. Small cracks in

these structures are inevitable, which easily leads to postoperative complications.

(4) The appearance is not satisfactory, by reason that only the central optical cylinder is transparent, while the color and structure of the skirt will affect the appearance of the cornea. Although several different devices are available, keratoprosthesis is with certain limitations in application that is only suitable for patients with irreversible end-stage corneal damage [46–49].

3.2 Artificial biosynthetic cornea

In order to address the complications, biocompatible and transplant failure in Keratoprosthesis, the concept of tissue- engineered cornea using various autologous cultured cells came up first in 1999[50], and different types of scaffolds have been evaluated with the development of materials and manufacturing techniques.

Biosynthetic corneas are usually made of natural and synthetic polymers which are biocompatible, transparent, soft and similar to the natural cornea so that they can be joined with autologous tissues. However, due to the limitations of the technology, the structure of the membrane is usually uncontrollable. The development of artificial biosynthetic cornea is still in a mid-stage study and seldom of them are in the clinical stage. The ideal cornea would be the full-thickness structure with host endothelial cells, keratocytes and epithelium of receptors as well as the full-functional acellular stromal scaffold with customizable structure based on individual eyeball. This artificial biosynthetic cornea with long-term biological and optical properties will certainly be the future research direction of development (Fig.2). The studies reported on artificial biosynthetic cornea can be mainly divided into four categories(Table.3): Decellularized matrix as corneal alternatives, Materials and manufacturing methods of biofilm for artificial cornea, Extracellular Matrix (ECM) Deposited by Cell, Corneal stromal Ultrastructure/Microstructure reconstruction.

1. Decellularized matrix as corneal alternatives

Decellularized xenogeneic tissue utilized as a natural scaffold for artificial biosynthetic cornea, through removing donor cells and antigens before recellularization of the matrix with recipient cells[51]. Clinical trials using decellularized matrix of heterogeneous cornea is currently underway. For example, Lin et al. developed organic acid-decellularized porcine cornea as scaffolds that were prepared by organic acid[52]. The preparation process is complicated with rather long manufacturing cycle. Decellularized matrix has similar anatomy and physiology to those of human corneas. There still remain some differences between the structures of donors and recipients lead to refractive problems. Besides, the existing preparation process is difficult to remove toxic agents in detergents completely. Incomplete removal of cells may lead to immune rejection. As reported by Shuji Sasaki, porcine cornea was decellularized as a tissue-engineered scaffold using ultrahigh hydrostatic pressure (UHP) which could not remove all the cells from the corneas[53]. Other physical methods, such as osmotic pressure, freeze–thaw cycles, and supercritical fluid extraction may lead to

microstructure damage such as collagen permutation disorder[54]. Besides, the risk of cross-species diseases and other indeterminate threats, for example unknown virus, should be seriously concerned[55].

II. Materials and manufacturing methods of biofilm for artificial cornea

Various processes of preparation biofilms with natural and polymeric materials as scaffolds for biomedical applications have been reported. However, most of these processes can only support the manufacture of flat membranes that only provide flat artificial corneas with a certain transmittance rather than required refractive power. For example, Lee et al.[56]proposed a centrifugal casting method to make flat silk fibroin films with designed thickness by controlling the rotational speed[56]. In some studies, the curved film was constructed using a mold, such as contact lens, but the surface morphology and thickness of the film with 3D multilayer structure were difficult to precisely control so that the optical function of artificial cornea could not be achieved[57]. Without customized the structure individually, the artificial cornea produced by these reported manufacturing methods could only provide light to the patient other than a clear vision in both theoretical and practical clinical applications. The problems and limitations of these approaches are summarized as the following: The preparation of multi-layer tissue by conventional manufacturing methods for tissue engineering requires complicated, long-term and staged procedures.

III. Extracellular Matrix (ECM) Deposited by Cells

The extracellular matrix (ECM) is a network of extracellular macromolecules to regulate cell proliferation, differentiation and migration[58]. The intrinsic ability of cells to form extracellular matrix is utilized for obtaining basement membrane as scaffold of tissue engineering. The interaction of the natural scaffold with cells play a significant role in several physiological and pathological processes, such as cell growth, survival and maturation/differentiation. For example, the fibroblast is cultured for several days to deposit integrated matrix to provide structural and biochemical support of cells.

The studies on forming natural membranes as corneal tissue engineering scaffolds include several specific aspects: monolayer membrane construction[59], corneal multi-layer reconstruction[60], functionalized and modified membrane with microstructure construction[61], corneal cell localization and interlaminar adhesion methods[62], etc. However, there are still many limitations on the construction of corneal substitutes by cell deposition: 1. The appropriate cells are limited, and the ability of cells to deposit extracellular matrix is inconstant and uncontrollable. 2. The thickness of film is unpredictable. 3. The mechanical properties of the membrane cannot be regulated. 4. The specific optical property of the deposited ECM is unfulfillable. 5. The manufacturing process is long with low repeatability.

IV. Ultrastructure/Microstructure

The cornea has distinct ultrastructural features over other tissues. As highly oriented tissue, the micro pattern of the cornea has important influence on its mechanical and optical properties. The transparent cornea is the main refractive element of the eye. The special microstructure formed by collagen fibers in the corneal stroma is the key factor in achieving

the optical properties of the cornea. At the same time, the arrangement of collagen fibers guarantees excellent biomechanical properties of the natural cornea. Therefore, the microstructure of cornea is critical to the function and the stability to maintain the shape of the cornea. It has been reported that the shape of corneal is affected by microstructure[24,63]. Many studies on the microstructure of the cornea are in progress to mimic and reconstruct the structure and function of the cornea at the microscopic level. Due to the primary research on the relationship between the corneal microstructure and its functions currently and the limitations of existing manufacturing techniques, research in this area is still in its infancy[22,23,58].

4 3D bioprinting: A new tool for corneal substitutes

The corneal substitute should be equivalent to the natural cornea in terms of structure, function, shape, etc. which can replace the function of donor cornea. Besides, high repeatability, low cost and high quality are also indispensable requirements for manufacturing. The corneal tissue equivalent should be biocompatible and could engage with autologous tissue without any autoimmune rejection. The substitute should have suitable biomechanical properties and able to withstand suturing, clipping and other probable operations in transplant surgery. In addition, materials for corneal substitute should have high transparency and microporous, which support the diffusion of oxygen, carbon dioxide and nutrients. The shape and structure are the key factors affecting the refractive power and other optical properties of the cornea, so that the spatial structure and shape should be similar to that of natural tissue. The shape consistent with the natural cornea facilitates the corneal substitute to be tiled on the surface of the eye. And it can be closely attached to the autologous tissue. The current method of manufacturing corneal substitutes focuses on the research content of the 1, 2, 3 stage shown in the Figure 2. In order to realize the various characters to meet the various requirements of corneal equivalents, further development on the manufacturing process of corneal substitutes is needed.

4.1 Theoretical advantages and expected impact of 3D bioprinted artificial cornea

With the characteristic of high density, multi-cell, multi-layer, and curved surfaces, the regeneration of cornea requires the shape control and function restoration, where 3D bioprinting is capable of providing an effective method for precisely controlling the shape and realizing optical functions of real cornea. The main advantages and potential of 3D bioprinting for corneal tissue engineering are:

1) Precise control of shape and properties: The key optical and physical properties of the cornea for corneal stromal substitute are transparency, refraction, shape and biomechanical properties. The key influential factors of corneal refractive power are the thickness of cornea and curvature of corneal optical interface curvature. Basing on the individual corneal

geometric data, personalized artificial cornea is available by rapid prototyping using three-dimensional computer aided design (CAD) model. 3D bioprinting also enables precise control of the artificial corneal structure, allowing the restoration of optical functions both theoretically and practically.

2) 3D bioprinting supports surface quality and mechanical strength control: In order to ensure the functions of the artificial cornea, for example refractive power, and meet the conditions required for transplantation, it is required to guarantee the surface quality and integrity of the optical interface and film, as well as a certain degree of biomechanical strength in the manufacturing process of artificial cornea. 3D bioprinting makes it possible to construct a multi-material integration, and it is enable to fabricate high-strength scaffold with complex structures by researching and development of bio-inks, which can break through the limitations of single material in conventional methods. Moreover, through the adjustment of the printing scheme and the process, the control of the surface quality can be achieved during the manufacturing process, and the effect of the demolding on the surface quality in the conventional manufacturing method can be avoided.

3) 3D bioprinting helps to achieve full-layer multi-cell corneal *in vitro* models, corneal microstructure construction and corneal regeneration

The flexibility of 3D printing can realize the integral structure of the artificial cornea with multi-layered, multi-cell, and some cell-specific arrangement of curved surface structures. In the *in vitro* corneal models construction and ultrastructure studies, 3D printing has the potential and advantages to co-build complex structures such as corneal epithelium, stroma, endothelium, and limbal stem cells together with the tissue environment which is of great significance for microarchitecture studies, accurately fabrication of three-dimensional model of the cornea as well as a morphological and functional equivalent of the human cornea. 3D bioprinting offers a new strategy of *in vitro* drug screening and toxicological studies for corneal tissue engineering, corneal micro/nanoscale structure reconstruction and the corneal regeneration.

4.2 Potential applications of 3D bioprinting on biosynthetic corneal equivalent manufacturing

Existing bio 3D printing methods can be divided into five categories: inkjet printing, extrusion printing, Laser-assisted printing, Micro electrical scanning and Micro electrical writing (Figure.3).

Single cell positioning is available with inkjet printing by spraying micro/nano-sized cell-laden droplets. The heating units at the nozzles of inkjet printing device lead to formation of bubbles, and the bubble expands to form a driving force to push the bioink out. The droplets are ejected one by one to form an entity[64]. The extrusion printing is based on mechanical extrusion or air pressure to squeeze the liquid material in the chamber from the nozzle. By controlling the movement of the nozzle in the space, the extruded material is ordered to form a designed structure. Laser-assisted bioprinting is to controls cell density and builds 3D tissue structures at a cellular level with laser-induced methods[65]. Micro electrical scanning device includes extrusion printing unit, except that on the basis of extrusion printing,

a finer nozzle is used in this method, and a high voltage electrostatic field is constructed between the nozzle and the receiving board. The introduction of an electrostatic field lead to the formation of a typical Taylor cone at the nozzle, which is due to the electrostatic force helping gravity to counteract the surface tension of the liquid material, resulting in a finer nanoscale filament. Micro electrical writing is a method based on electrospinning. Oriented and controllable extruded filaments can be produced by controlling parameters such as extrusion pressure, electrostatic field voltage and plate distance. The nozzle can move along the designed path to print micron-sized 3D solids.

These 3D bioprinting methods have been applied to many fields such as skin, cardiac muscle, and oral and maxillofacial tissue engineering, and have great potential and feasibility in the construction of biosynthetic artificial cornea. The corneal limbal stem cells are distributed in the niche at the junction of the cornea and sclera. The advantages of inkjet printing and laser-assisted printing are the ability to accurately locate single cells and construct micro-nanoscale structures[11], which is of great value in the study of limbal stem cells and their niche reconstruction. Extrusion printing can control the thickness and geometrical morphology of the printed structure. Variable materials with good bioperformance can be used in extrusion printing. For example, Isaacson et al.[66] applied extrusion printing to the area of corneal tissue engineering and fabricated a corneal like cell-laden structure. In addition, micro electrical scanning and micro electrical writing can control fiber orientation at the micro-nano scale, which are excellent approaches that can be applied in corneal ultrastructure reconstruction. The 3D printing method can precisely control the geometrical morphology and is suitable for constructing multi-layer multi-material structures, and its research in the field of biosynthetic corneal manufacturing is meaningful.

5 Conclusions and future directions

Faced with the global shortage of donated corneas, the demand and urgency of transplantable corneal substitutes are increasing. At the same time, there is growing interest in the development of full-structure corneal *in vitro* models for drug screening and toxicological testing. The development of artificial corneas is an effective solution for this world issue. With the rise of biomaterials and new manufacturing technologies, 3D bioprinting has emerged in the field of personalized medicine, and a number of researches on the tissues and organs construction and regeneration have emerged. This review compares the existing artificial cornea materials and manufacturing methods, proposes the unique process advantages and manufacturing features of biological 3D printing, and can achieve the construction of the key features of the cornea and the functional reconstruction. It has great potential and development prospects in the field of corneal tissue engineering.

3D bioprinting is still in the early stage of research and development. Many development opportunities still need to be explored. For example, the use of nanoparticles to optimize the performance of bio-ink improves the biomechanical properties and transparency of artificial corneas[67]; the use of composite printing technology to precisely control the shape of the cornea, in order to achieve the control and reconstruction of corneal shape-related functions, to achieve personalized customization of corneal substitutes; research and development of

micro-nanoscale printing to achieve the study and reconstruction of the corneal microstructure, in principle to explore the corneal microstructure of corneal cells , metabolism, migration, and the effects and significance of corneal function to better understand the anatomy and physiology of the cornea; 3D bioprinting provides an all-in-one construction scheme that enables rapid construction of a full-thickness, full-structure cornea model for the cornea *in vitro*, provides solutions other than animal experiments.

Work continues across the world on the international standardization of processes and materials, industrializing the systems, and preparing the supply chain for potential demand of products using 3D bioprinted corneal processes. As for the adoption of any new health care technology or intervention, the new procedures involving alternatives to eye bank native tissue for corneal stromal replacement that are currently under development, will not only need to be effective, but also need to be cost-effective. Nowadays, many countries have their own economic criteria for the adoption of a new health care intervention. If the 3D bioprinting corneal procedure ends up being more effective and less costly than existing procedures, it will be easy to convince health care decision makers to adopt this new procedure. Therefore, the economic evaluation is essential to document the economic impact of 3D bioprinted artificial cornea.

In short, the development direction of the artificial cornea must be from experimental to clinical, from four individual studies (heterogeneous corneal alternatives, acellular biomaterials film, Single or Multi-Layer Corneal Equivalents, and ultrastructure) to full-functional corneal substitute development and research. In order to promote the studies of corneal substitutes, a detailed and systematic international standard that can describe the future R&D level and requirements of 3D printed corneal prosthesis are urgent to be introduced. This standard should include comprehensive assessment requirements for 3D bioprinting equipment and processes, *in vitro* and *in vivo* biological, optical and mechanical properties of artificial corneas built with 3D printing. Among them, 3D biometric printing devices constructed for artificial corneas should be classified based on printed principles categories, and correspond to different structures of the cornea, features and functions, and other research directions to improve and develop printing equipment and processes. In terms of assessment, biological properties include material properties and stability before and after transplantation, biocompatibility, penetration of glucose, albumin, and other related molecular size, oxygen permeability, portability, and the assessment of autologous tissue impact after transplantation (postoperative complications such as inflammation, corneal nebulization, autologous tissue degradation, graft migration or excretion). Optical properties include transparency, absorption and transmission of light in different wavelength bands, refractive power, refractive stability before and after transplantation, and assessment of visual performance. Mechanical properties include structural dimensions, surface quality, tensile strength, intraocular pressure resistance, creep test, linearity of anti-suture, stability of *in vitro* and *in vivo* artificial corneas and autologous tissue structures. On this basis, a systematic report was made on the *in vitro* structure and performance of animal and human subject assessments. In short, 3D printing has great research value in the future research direction of artificial cornea construction. It is still necessary to continuously improve existing

technologies and materials, develop new printing equipment, hope to realize the construction of a full-functional artificial cornea.

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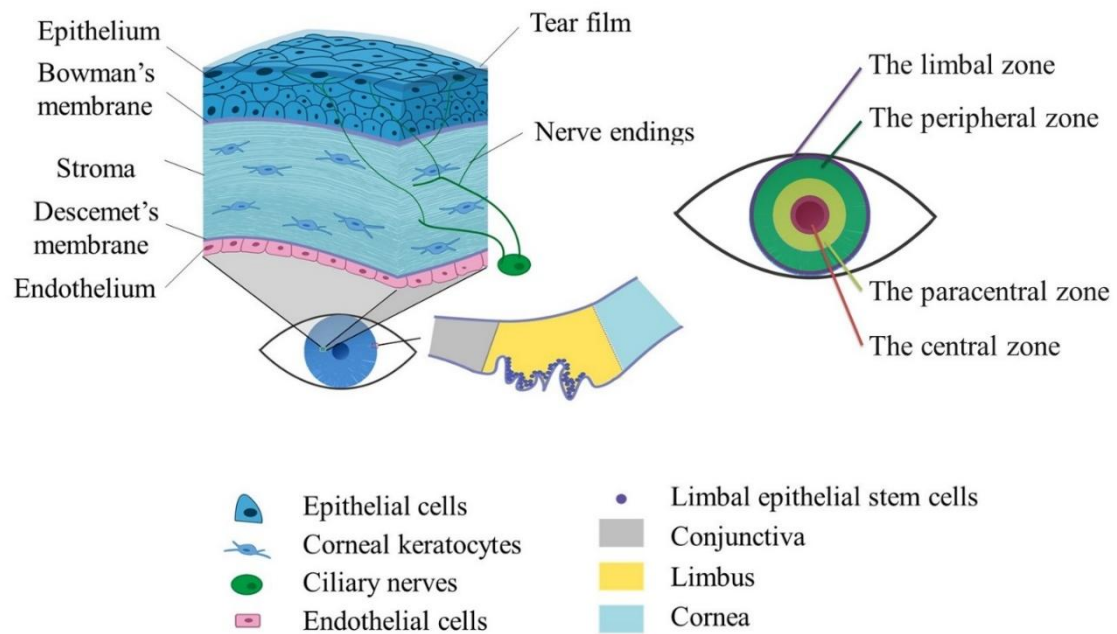
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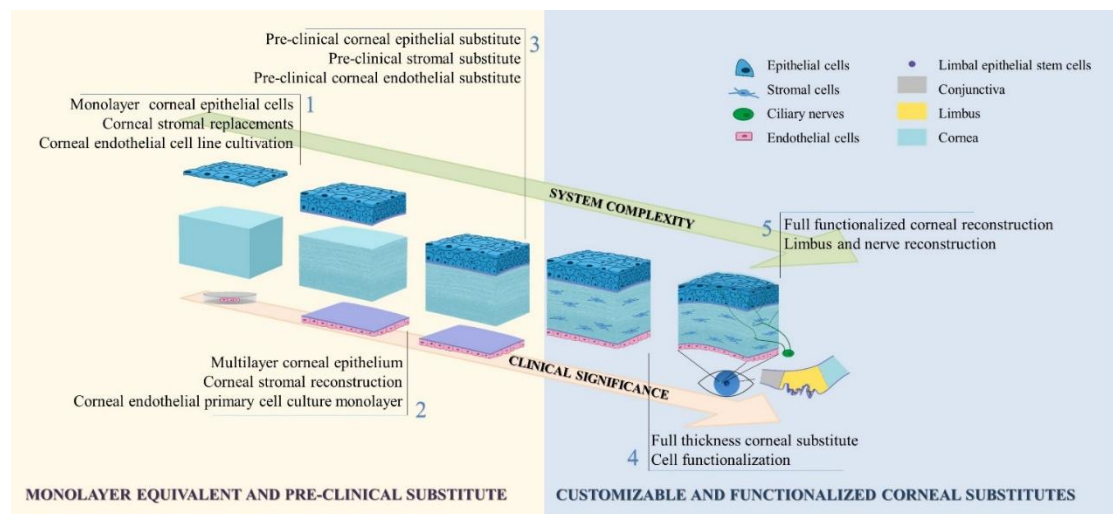
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Figure 1 The anatomy and physiology of cornea

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Figure 2 The clinical significance increase with the system complexity of artificial cornea from a surgical viewpoint. The corneal substitutes would develop from monolayer equivalent and pre-clinical substitute to multi-layer customizable and functional curved corneal substitute. The development of corneal substitutes with controllable shape for refractive and other optical purposes as well as good biological performance is essential and urgent.

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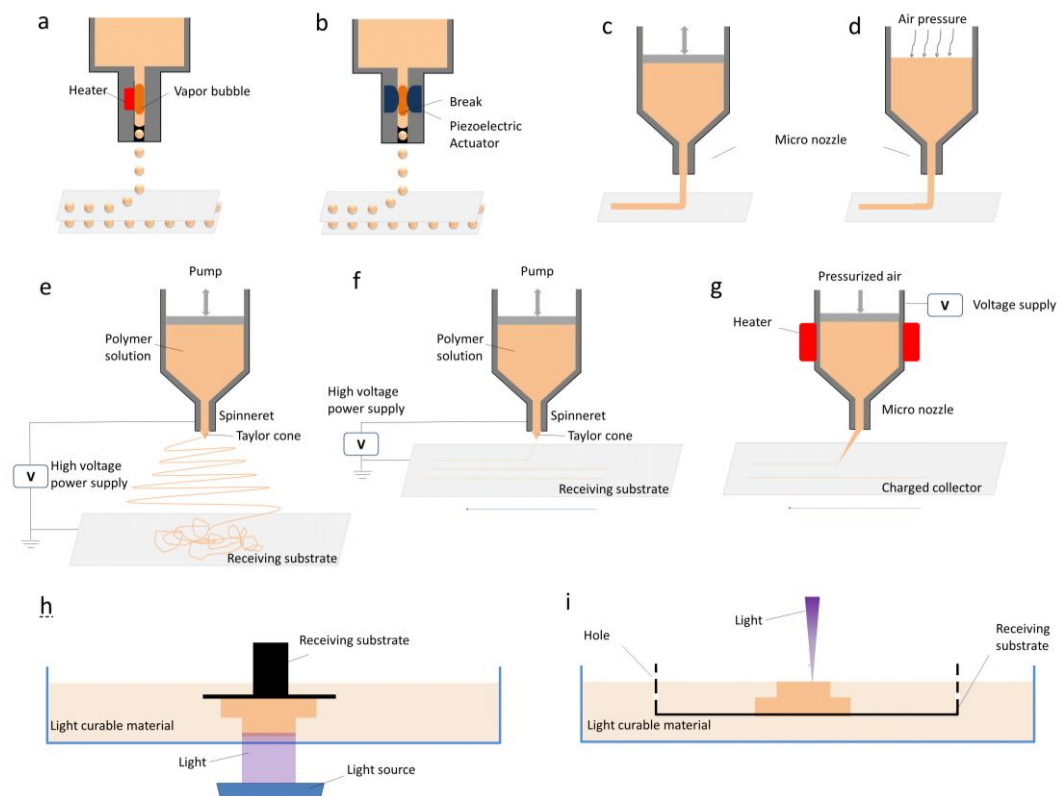


Figure 3 Schematic of major technologies of 3D bioprinting

a, b Inkjet printing[68]; a Thermal inkjet printing; b Piezo electric inkjet printing[69];
c, d Extrusion printing[70]; c Piston extrusion printing; d Pneumatic extrusion printing;
e Electrostatic spinning [71]; f Near-Field Electrospinning[72,73]; g Melt electrowriting[74]
h Digital light processor[75] i Stereolithographic[76]

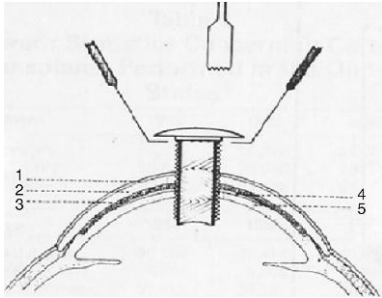
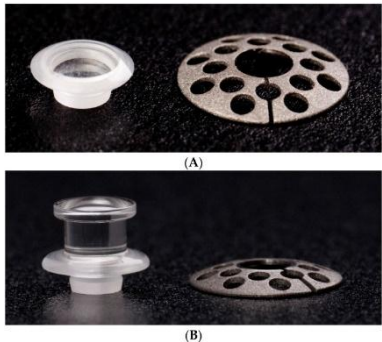

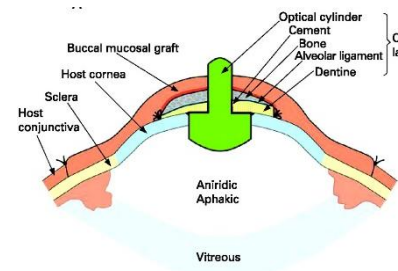
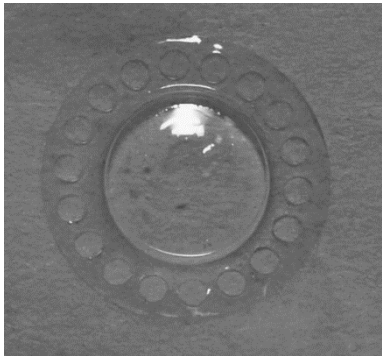
Table 1 Advantages of Artificial Cornea

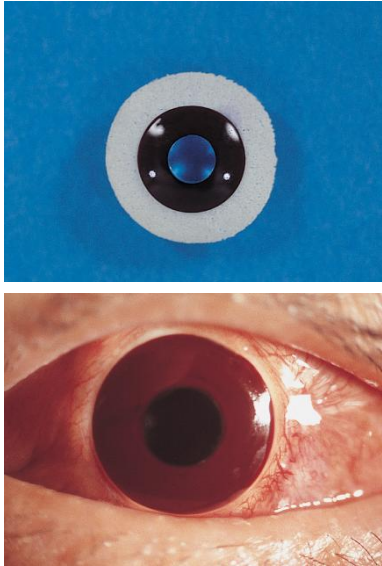
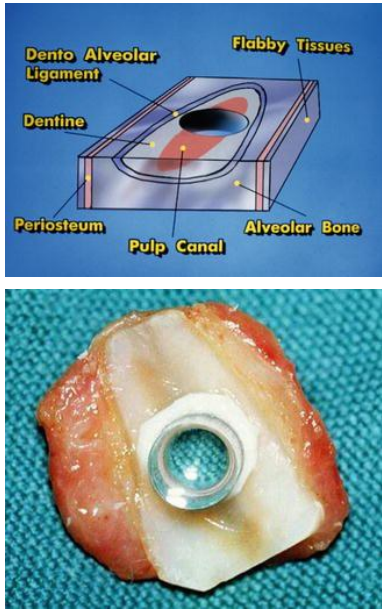
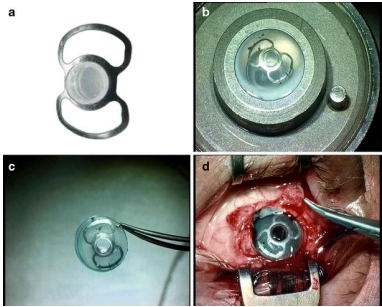
- Artificial cornea offers a potential solution of the global shortage of human donor corneas.
- The well production management and mechanized manufacturing system ensure the biological control of non-toxic, sterile as well as the products quality level and stability, which can effectively avoid the potential virus, religion, culture and policy problems associated with natural corneas.
- The acellular corneal substitute can overcome the obstacles of donor corneas, such as immune rejection induced by allogeneic cells from recipients who face high immune graft risk, ocular surface disease and the corneal graft dysfunctional.
- Artificial cornea can benefit from new advances in biomaterial science, such as artificial synthetic material, surface coating technique, functional materials, nanoparticles and other micro-nano scale materials science.
- The single-component artificial cornea, in contrast to native cornea, has the characteristics of homogeneity, and limited swellability so that free from the water accumulation or scattering of light.


Table 2. Category of Current Keratoprosthesis

	Materials	Schematic	Reference
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- For further clinical needs of full-functionalized artificial cornea, because of the specific spatial arrangements of the tissue structure, some optical, biomechanical and other relevant properties of the cornea can only be restored using 3D fabrication method.

Cardona keratoprosthesis	Poly (methyl methacrylate)PMMA		[34]
Boston Keratoprosthesis ((A) type I and (B) type II)	Poly (methyl methacrylate)PMMA + Titanium		[77–79]
Alphacor keratoprosthesis	(Polyhydroxyethyl methacrylate)PHEMA		[80–82]
The osteo-odonto-keratoprosthesis (OOKP)	Autologous tooth root and alveolar bone + poly (methyl methacrylate)PMMA		[83–85]
Keraklear Artificial Cornea	Poly (methyl methacrylate)PMMA + (polyethylene glycol) PEG		[86]

Korea Seoul-Type keratoprosthesis	Poly (methyl methacrylate)PMMA + PEG		[87–89]
The modified osteo-odonto-keratoprosthesis (MOOKP)	The surface of the osteodental lamina + poly (methyl methacrylate)PMMA		[90]
Fyodorov–Zuev keratoprosthesis	Poly (methyl methacrylate)PMMA + Titanium		[91,92]

MIRO® CORNEA UR keratoprosthesis	Hydrophobic acrylic polymer + Genetically engineered fibronectin		[93]
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Table 3. Current Approaches for Artificial Biosynthetic Corneal Equivalents

Catalog	Cell types	Scaffold materials	Manufacturing method	Clinical status	Ref.
I	Amniotic epithelial cells	Acellular porcine cornea	Decellularization	<i>In vitro</i>	[94]
I	Limbal corneal epithelial cells	Acellular porcine cornea	Decellularization	<i>In vitro</i>	[51]
I	Human corneal epithelial cells	Acellular porcine cornea	Decellularization	<i>In vivo</i> : Rabbit	[52]
I	—	Acellular porcine cornea	High-hydrostatic pressurization decellularization	<i>In vivo</i> : Rabbit	[54]
II	Limbal epithelial stem cells and stromal keratocytes	Acid soluble rat tail type I collagen/proteoglycan hydrogels	Horizontal magnetic field (7 T)	<i>In vitro</i>	[95]
II	Rabbit corneal endothelial, stromal, and epithelial cells	Fibrin-agarose hydrogels	Multi-layered construction	<i>In vitro</i>	[96]
II	Human corneal cell lines	Collagen–chondroitin sulfate substrate cross-linked with glutaraldehyde with scleral rim	Multi-layered construction	<i>In vivo</i>	[97]
II	Human corneal endothelial cells	Dense collagen hydrogel	Confined flow compression method + Seeding	<i>In vitro</i>	[98]
II	Human corneal epithelial cells (hcecs) and dorsal root ganglia (DRG) from chick embryos	Collagen(type-I atelo-collagen) -chitosan hydrogels	Polypropylene contact lens molds (cooper vision, Pleasanton, CA)	<i>In vivo</i> : rat, pig	[99]

II	Corneal epithelial cell	The dendrimer cross-linked collagen gels	—	<i>In vitro</i>	[100, 101]
II		1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC) cross-linked recombinant human collagen	Curved polypropylene molds	Phase I clinical trial: human	[102]
II	Immortalized corneal epithelial cells, Dorsal root ganglia from 8-d-old chick embryos	Collagen–Copolymer	Multi-layered construction	<i>In vitro</i>	[103]
II	1-ethyl-3-[3-dimethylaminopropyl]carbodiimide hydrochloride (EDC) and N-hydroxysuccinimide (NHS) crosslinked recombinant human collagen type III (RHCIII)	Cross-linked recombinant human collagen type III	Curved polypropylene contact lens molds	4-year follow-up	[104]
II	Precursors (precursor/gelatin group) , with fibroblasts (fibroblast /gelatin group)	Porous gelatin-glutaraldehyde	Trephined with a 5.0 mm diameter trephine + seeding	<i>In vivo</i> : rabbit	[105]
II	Stromal fibroblasts	Bovine collagen	Dry casting + seeding	<i>In vitro</i>	[106]
II	Corneal Limbal Epithelial Cells +Keratocytes	Amniotic membrane + laminin-coated compressed collagen gel (rat-tail type I collagen)	Compression and dehydration	<i>In vitro</i>	[107]
II	Corneal epithelial cell line HCE-T	Keratin film	Dry casting on hydrophobic coated PET sheets	<i>In vitro</i> study	[108]
II	Human corneal endothelial cells	Human corneal stromal discs	Seeding	<i>In vivo</i> : rabbit	[109]
II	Corneal endothelial cells	Gelatin hydrogel	Dry casting	<i>In vitro</i>	[110]

II	Human corneal endothelial cells	Hydroxyethyl chitosan, gelatin, and chondroitin sulfate	Dry casting	<i>In vitro</i>	[111]
II	Sheep corneal endothelial cells	Novel ultrathin chitosan–poly(ethylene glycol) (PEG) hydrogel films	Dry casting	<i>In vitro</i>	[112]
II	Human donor-derived corneal endothelial cells	Decellularized thin-layer corneal stroma	Microtome	<i>In vitro</i>	[113]
III	Cells from human oral mucosal tissue +3T3 feeder cells	—	Cell culture	Clinical trial: human	[114]
III	NIH/3T3 feeder cells	—	Cell culture	<i>In vivo</i> : rabbit	[115]
IV	Rabbit Primary stromal keratocytes	Silk fibroin films	Surface patterned by PDMS	<i>In vitro</i>	[116] [117]
IV		Magnetically aligned rat-tail type I	Horizontal magnetic field (7 T)	<i>In vitro</i>	[118]
IV	Rabbit stromal cells	Polyglycolic acid fibers		<i>In vivo</i> : rabbit	[119]
IV	Adult human derived corneal stromal (AHDCS) cells	Poly(l,d lactic acid)	Ordered electrospun	<i>In vitro</i>	[120]
IV	Immortalized human corneal keratocytes	Silk fibroin films	Porous surface patterned with PDMS by dry casting	<i>In vitro</i>	[121]
IV	Human corneal stromal stem cells	Poly(ester urethane) urea	Electrospun	<i>In vitro</i>	[122]

766 Note: I. Decellularized matrix as corneal alternatives, II. Materials and manufacturing methods of biofilm for
767 artificial cornea, III. Extracellular Matrix (ECM) Deposited by Cell, IV. Corneal stromal
768 Ultrastructure/Microstructure reconstruction.
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3D bioprinting for artificial cornea : challenges and perspectives

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Abstract

Corneal disease is one of the most important causes of blindness worldwide. Currently, the dominating treatment of corneal blindness is corneal transplantation. However, the main source of cornea for transplantation is based on donations which is far from enough to meet the requirement (less than 1:70 of cases). The severe shortage of donor cornea promotes the studies of effective corneal alternatives. However, many problems remain and can't be solved in current researches, such as original geometry reconstruction and ocular optical function restoring.

3D bioprinting can be a promising approach for corneal substitution. The advantages of this technology in corneal regeneration enable personalized corneal implant and single or multi-layer corneal equivalents with controllable structure and designed refractive ability.

In this review, the progress, applications and limitations of most influential works among current keratoprosthesis and tissue-engineering cornea researches are discussed. Then the applications of 3D bioprinting in manufacturing multi-layered structures and surface are mentioned. Further, the potential, advantages in current research of 3D bioprinting single or multi-layer corneal equivalents and alternatives are discussed. Finally, an insight into the technical challenges and prospective facing the future research of 3D bioprinting corneal alternatives *in vivo* and *in vitro* is provided.

Keywords Biofabrication, 3D bioprinting, Cornea regeneration, Tissue engineering

1 Introduction

The cornea is the key component of eye and plays the critical role in the optical system. Cornea diseases is one of the main causes of blindness, 10 million of the patients having bilateral corneal blindness[1].Owing to modern advances in surgical techniques and surgeon expertise, keratoplasty, as known as the whole cornea transplantation, is the world's most frequent form of solid tissue transplantation and considered the only widely accepted

treatment for cornea blindness[2,3].

Donation is the dominant way to obtain fresh cornea with high quality required for successful keratoplasty[4]. However, there is an overall mismatch between the supply and demand of donated cornea with a percentage of 1.4% due to religiosity, aging, policy, etc.[5,6]. An estimated 12.7 million patients are placed on a long waiting list and the number is still growing[7]. This prolongs of transplantation can potentially cause more serious diseases. It is essential and urgent to develop feasible long-term alternatives to alleviate the shortage of donor tissue for transplantation, such as corneal substitutes which has been an important research subject to future progress of corneal regeneration.

Nowadays, patient- specific treatment becomes a fast-growing global trend in modern medicine. In this condition, conventional manufacturing methods of artificial cornea are questioned in terms of restoring integrated corneal functions according to individual needs in a cost-effective and direct manner. The cornea is one of the complex tissues with refractive power. It is difficult to achieve individual functional restoring of many tissues and organs due to the specific functionalities and particularities of structure and materials. The potential of additive manufacturing, specifically 3D printing, is investigated to serve this purpose[8].

In the last few decades, with the development of 3D bioprinting, aiming to the reconstruction of tissues and organs with designed complex geometries, demonstrated the immense potential and promising results in regenerative medicine[9,10]. It overcomes the restriction of conventional tissue engineering fabrication methods—reconstructing the anisotropic physical properties, specific cell arrangement, heterogeneous components and heterotypic architectures of natural tissue[11]. The special advantages of 3D printing provide a new tool to solve the major problems of personalized cornea and donor shortages.

Here, we provide a review of recent progress toward the latest research on artificial cornea. In this framework, we suggest the challenges and value of 3D printed artificial cornea, the steps that will be required for the development direction as well as the guideline for cornea regeneration.

2 The anatomy and physiology of cornea

As an important component of the optical system, the cornea is highly organized, dense, avascular, transparent, relatively immunological privileged connective tissue (80% water, 13.6% collagen, 0.9% glycosaminoglycan) that protect the eye from external environment. Additionally, the cornea acts as an optical interface between anterior chamber and air to contributes 73.5% of the total refractive power in Gullstrand Number 1 relaxed schematic model eye[12,13].The average diameter of the adult cornea is 11.04 to 12.50 mm for males and 10.70 to 12.58 mm for females [14]. The human cornea comprised of five layers including three distinct cellular layers separated by two interface layers (Figure 1). The corneal anterior curvature is 7.8 mm and posterior curvature is about 1.3 mm larger than the posterior curvature. The refractive index of cornea is 1.376. Rays penetrate through the cornea and enters the anterior chamber with the refraction index of 1.336[15,16].The components of the corneal stroma are cells and extracellular matrix which is mainly composed of aligned collagen parallels. The cornea stroma acts as a scaffold to stabilize the physical structure and

regulate the balance of the cornea. The thickness of the stroma accounts for 90% of the total corneal thickness (~500 μ m) and the surrounding area is tens of micrometers more than the center zone[17–19].

Biomechanical properties

The cornea acts as an air-liquid interface on the surface of the eyeball, withstand the pressure of the aqueous humor and the extraocular muscle tension. As an anisotropic soft tissue, the cornea contains a large amount of water, and its response to stress sharing dual characteristics of combining solid and viscous liquids. The highly organized collagen fibrils in corneal stroma are the main component of the entire corneal structure so that the biomechanical properties of the cornea is determined primarily by corneal stroma [20], including viscoelastic, viscous (stress relaxation, creep and anisotropy), structural strength, elasticity, integrity, stability, etc. Microstructural changes in the cornea can cause significant changes in biomechanical properties of the eye. When external shearing forces are applied to the cornea, the resistance to deformation occurs inside the cornea in the form of internal friction. Therefore, in the research and development of artificial cornea for clinical purposes, the study of the corneal mechanical properties is indispensable.

Optical function

The optical functions of the cornea include light transmission, refractive power, and the filtering of some harmful rays. The forward transmittance of human cornea is more than 80 percent at visible wavelengths while attenuates slightly at the spectrum close to ultraviolet[21]. It is essential to take the scattering, absorption effect on the tissues and the interference of light among these aligned collagen fibrils into consideration to analysis the transparent nature of the cornea. Therefore, a proper understanding of corneal ultrastructure is essential to reveal the optical properties of the native cornea and create artificial corneal[22–24]. By reason that proteoglycans in corneal stroma has strong hydrophilicity[25], endothelial-based corneal diseases[26]and corneal damage can cause corneal swelling, which leads to disorder of collagen fibers and parallels, stromal deposits, decreased refractive index of the cornea, corneal dysfunction, reduction of transparency[24,27–29], disorder scattering of light[27], etc., also associated with the change on average corneal center thickness[30,31]. Therefore, the artificial cornea has the characteristics of homogeneity, and limited swellability, which can provide uniform refractive power, and the transparency is not affected by the function of the endothelial layer and thus not share the problems mentioned above as native cornea. The corneal epithelium is covered by the tear film that can form an integrated and smooth optical interface on the surface of the eye to refract light regularly and consistently. Most of the refraction of the eye is at the cornea-air interface. The cornea can be divided into four parts: the central zone, the paracentral zone, the peripheral zone, the limbal zone (Fig.1), where the center zone is approximately spherical and relatively thinner than surrounding area so that the cornea can be regarded as a negative meniscus which plays a pivotal role in the refraction of light. Therefore, the shape and structure in aspect of the theoretical understanding and the control methods practically are crucial in artificial corneal research.

3 Categories of corneal replacements: past and current trends

Cornea alternatives currently in clinical or pre-clinical development that have been reported can be divided into two main categories: Keratoprosthesis and artificial biosynthetic cornea. Artificial cornea has several significant advantages over donor corneas as show in the Table.1.

3.1 Keratoprosthesis

In the mid-20th century, with the development of new materials with transparent, non-toxic and well mechanical properties along with the introduction of antibiotics, topical corticosteroids and immunosuppressive agents[32] enabling better postoperative management, some keratoprostheses were reported to be used as artificial replacements for the opaque or damaged corneas[33–38]. Up to now, there are six well-developed keratoprosthesis(Table.2) : the modified osteo-odonto-keratoprosthesis (OOKP) (Falcinelli, 1987; Liu et al., 2005; Mannis et al., 1999), the Boston type I keratoprosthesis (B-KPro Type I)(Aldave et al., 2012; Zerbe et al., 2006), AlphaCor™ keratoprosthesis(Hicks et al., 2002, 2006; Jirásková et al., 2011), the Boston type II keratoprosthesis (B-KPro Type II)(Dohlman et al., 2009; Pujari et al., 2011), Seoul-type keratoprosthesis(Kim et al., 2002, 2007, 2015; Lee et al., 2000), KeraKlear®-Keratoprothese (Keramed Inc.)(Pineda, 2015). Keratoprostheses can be structurally divided into two parts, optical cylinder with skirt[39,40] or collar button[41–44]. As materials continue to evolve, a wide range of inorganic, organic materials, and metals are used for keratoprosthesis designs. The central optical column requires good optical performance, is usually made of a transparent inorganic material with the diameter of the real cornea, which provide an optical channel and visual acuity. The skirt or collar button surrounds the optic cylinder and engages with the recipient tissue so as to play a role in support and fixation is made of titanium, tooth, polyester etc. In order to improve the stability in vivo and maintain the function, keratoprosthesis is usually made of non-degradable and bioinert materials. These materials can usually be prepared by conventional mechanical processing and are capable of achieving certain optical functions. However, due to the special characteristics of materials and structure, the limitations of current keratoprosthesis are as follows:

- (1) As nonbiological prosthetic devices, keratoprosthesis are usually hard that is not comfortable to wear, then even cause wear and tear on the autologous tissue that may lead to complications.
- (2) Due to the characteristics of external prosthesis, the transplantation process is more complex than that of corneal allograft. For example, the osteo-odonto-keratoprosthesis reported by Strampelli et al. [15], the optical cylinder of which supported with the patient's own tooth. It requires two phases of surgery to obtain autologous tissue before the keratoplasty. The production of prosthesis is likely to cause secondary infections. AlphaCor also requires two stages of surgery.
- (3) The visual field is limited after keratoplasty. Artificial cornea has limited field of view, such as the vision field of the patient using the Boston type I keratoprosthesis was reported to be 60°[45]. Because the combination between the cylinder and scaffold is similar to the screw thread or button structures, the prosthesis is biological incompatible with the autologous tissue. Small cracks in

these structures are inevitable, which easily leads to postoperative complications.
(4) The appearance is not satisfactory, by reason that only the central optical cylinder is transparent, while the color and structure of the skirt will affect the appearance of the cornea. Although several different devices are available, keratoprosthesis is with certain limitations in application that is only suitable for patients with irreversible end-stage corneal damage [46–49].

3.2 Artificial biosynthetic cornea

In order to address the complications, biocompatible and transplant failure in Keratoprosthesis, the concept of tissue- engineered cornea using various autologous cultured cells came up first in 1999[50], and different types of scaffolds have been evaluated with the development of materials and manufacturing techniques.

Biosynthetic corneas are usually made of natural and synthetic polymers which are biocompatible, transparent, soft and similar to the natural cornea so that they can be joined with autologous tissues. However, due to the limitations of the technology, the structure of the membrane is usually uncontrollable. The development of artificial biosynthetic cornea is still in a mid-stage study and seldom of them are in the clinical stage. The ideal cornea would be the full-thickness structure with host endothelial cells, keratocytes and epithelium of receptors as well as the full-functional acellular stromal scaffold with customizable structure based on individual eyeball. This artificial biosynthetic cornea with long-term biological and optical properties will certainly be the future research direction of development (Fig.2). The studies reported on artificial biosynthetic cornea can be mainly divided into four categories(Table.3): Decellularized matrix as corneal alternatives, Materials and manufacturing methods of biofilm for artificial cornea, Extracellular Matrix (ECM) Deposited by Cell, Corneal stromal Ultrastructure/Microstructure reconstruction.

1. Decellularized matrix as corneal alternatives

Decellularized xenogeneic tissue utilized as a natural scaffold for artificial biosynthetic cornea, through removing donor cells and antigens before recellularization of the matrix with recipient cells[51]. Clinical trials using decellularized matrix of heterogeneous cornea is currently underway. For example, Lin et al. developed organic acid-decellularized porcine cornea as scaffolds that were prepared by organic acid[52]. The preparation process is complicated with rather long manufacturing cycle. Decellularized matrix has similar anatomy and physiology to those of human corneas. There still remain some differences between the structures of donors and recipients lead to refractive problems. Besides, the existing preparation process is difficult to remove toxic agents in detergents completely. Incomplete removal of cells may lead to immune rejection. As reported by Shuji Sasaki, porcine cornea was decellularized as a tissue-engineered scaffold using ultrahigh hydrostatic pressure (UHP) which could not remove all the cells from the corneas[53]. Other physical methods, such as osmotic pressure, freeze–thaw cycles, and supercritical fluid extraction may lead to

microstructure damage such as collagen permutation disorder[54]. Besides, the risk of cross-species diseases and other indeterminate threats, for example unknown virus, should be seriously concerned[55].

II. Materials and manufacturing methods of biofilm for artificial cornea

Various processes of preparation biofilms with natural and polymeric materials as scaffolds for biomedical applications have been reported. However, most of these processes can only support the manufacture of flat membranes that only provide flat artificial corneas with a certain transmittance rather than required refractive power. For example, Lee et al.[56]proposed a centrifugal casting method to make flat silk fibroin films with designed thickness by controlling the rotational speed[56]. In some studies, the curved film was constructed using a mold, such as contact lens, but the surface morphology and thickness of the film with 3D multilayer structure were difficult to precisely control so that the optical function of artificial cornea could not be achieved[57]. Without customized the structure individually, the artificial cornea produced by these reported manufacturing methods could only provide light to the patient other than a clear vision in both theoretical and practical clinical applications. The problems and limitations of these approaches are summarized as the following: The preparation of multi-layer tissue by conventional manufacturing methods for tissue engineering requires complicated, long-term and staged procedures.

III. Extracellular Matrix (ECM) Deposited by Cells

The extracellular matrix (ECM) is a network of extracellular macromolecules to regulate cell proliferation, differentiation and migration[58]. The intrinsic ability of cells to form extracellular matrix is utilized for obtaining basement membrane as scaffold of tissue engineering. The interaction of the natural scaffold with cells play a significant role in several physiological and pathological processes, such as cell growth, survival and maturation/differentiation. For example, the fibroblast is cultured for several days to deposit integrated matrix to provide structural and biochemical support of cells.

The studies on forming natural membranes as corneal tissue engineering scaffolds include several specific aspects: monolayer membrane construction[59], corneal multi-layer reconstruction[60], functionalized and modified membrane with microstructure construction[61], corneal cell localization and interlaminar adhesion methods[62], etc. However, there are still many limitations on the construction of corneal substitutes by cell deposition: 1. The appropriate cells are limited, and the ability of cells to deposit extracellular matrix is inconstant and uncontrollable. 2. The thickness of film is unpredictable. 3. The mechanical properties of the membrane cannot be regulated. 4. The specific optical property of the deposited ECM is unfulfillable. 5. The manufacturing process is long with low repeatability.

IV. Ultrastructure/Microstructure

The cornea has distinct ultrastructural features over other tissues. As highly oriented tissue, the micro pattern of the cornea has important influence on its mechanical and optical properties. The transparent cornea is the main refractive element of the eye. The special microstructure formed by collagen fibers in the corneal stroma is the key factor in achieving

the optical properties of the cornea. At the same time, the arrangement of collagen fibers guarantees excellent biomechanical properties of the natural cornea. Therefore, the microstructure of cornea is critical to the function and the stability to maintain the shape of the cornea. It has been reported that the shape of corneal is affected by microstructure[24,63]. Many studies on the microstructure of the cornea are in progress to mimic and reconstruct the structure and function of the cornea at the microscopic level. Due to the primary research on the relationship between the corneal microstructure and its functions currently and the limitations of existing manufacturing techniques, research in this area is still in its infancy[22,23,58].

4 3D bioprinting: A new tool for corneal substitutes

The corneal substitute should be equivalent to the natural cornea in terms of structure, function, shape, etc. which can replace the function of donor cornea. Besides, high repeatability, low cost and high quality are also indispensable requirements for manufacturing. The corneal tissue equivalent should be biocompatible and could engage with autologous tissue without any autoimmune rejection. The substitute should have suitable biomechanical properties and able to withstand suturing, clipping and other probable operations in transplant surgery. In addition, materials for corneal substitute should have high transparency and microporous, which support the diffusion of oxygen, carbon dioxide and nutrients. The shape and structure are the key factors affecting the refractive power and other optical properties of the cornea, so that the spatial structure and shape should be similar to that of natural tissue. The shape consistent with the natural cornea facilitates the corneal substitute to be tiled on the surface of the eye. And it can be closely attached to the autologous tissue. The current method of manufacturing corneal substitutes focuses on the research content of the 1, 2, 3 stage shown in the Figure 2. In order to realize the various characters to meet the various requirements of corneal equivalents, further development on the manufacturing process of corneal substitutes is needed.

4.1 Theoretical advantages and expected impact of 3D bioprinted artificial cornea

With the characteristic of high density, multi-cell, multi-layer, and curved surfaces, the regeneration of cornea requires the shape control and function restoration, where 3D bioprinting is capable of providing an effective method for precisely controlling the shape and realizing optical functions of real cornea. The main advantages and potential of 3D bioprinting for corneal tissue engineering are:

1) Precise control of shape and properties: The key optical and physical properties of the cornea for corneal stromal substitute are transparency, refraction, shape and biomechanical properties. The key influential factors of corneal refractive power are the thickness of cornea and curvature of corneal optical interface curvature. Basing on the individual corneal

geometric data, personalized artificial cornea is available by rapid prototyping using three-dimensional computer aided design (CAD) model. 3D bioprinting also enables precise control of the artificial corneal structure, allowing the restoration of optical functions both theoretically and practically.

2) 3D bioprinting supports surface quality and mechanical strength control: In order to ensure the functions of the artificial cornea, for example refractive power, and meet the conditions required for transplantation, it is required to guarantee the surface quality and integrity of the optical interface and film, as well as a certain degree of biomechanical strength in the manufacturing process of artificial cornea. 3D bioprinting makes it possible to construct a multi-material integration, and it is enable to fabricate high-strength scaffold with complex structures by researching and development of bio-inks, which can break through the limitations of single material in conventional methods. Moreover, through the adjustment of the printing scheme and the process, the control of the surface quality can be achieved during the manufacturing process, and the effect of the demolding on the surface quality in the conventional manufacturing method can be avoided.

3) 3D bioprinting helps to achieve full-layer multi-cell corneal *in vitro* models, corneal microstructure construction and corneal regeneration

The flexibility of 3D printing can realize the integral structure of the artificial cornea with multi-layered, multi-cell, and some cell-specific arrangement of curved surface structures. In the *in vitro* corneal models construction and ultrastructure studies, 3D printing has the potential and advantages to co-build complex structures such as corneal epithelium, stroma, endothelium, and limbal stem cells together with the tissue environment which is of great significance for microarchitecture studies, accurately fabrication of three-dimensional model of the cornea as well as a morphological and functional equivalent of the human cornea. 3D bioprinting offers a new strategy of *in vitro* drug screening and toxicological studies for corneal tissue engineering, corneal micro/nanoscale structure reconstruction and the corneal regeneration.

4.2 Potential applications of 3D bioprinting on biosynthetic corneal equivalent manufacturing

Existing bio 3D printing methods can be divided into five categories: inkjet printing, extrusion printing, Laser-assisted printing, Micro electrical scanning and Micro electrical writing (Figure.3).

Single cell positioning is available with inkjet printing by spraying micro/nano-sized cell-laden droplets. The heating units at the nozzles of inkjet printing device lead to formation of bubbles, and the bubble expands to form a driving force to push the bioink out. The droplets are ejected one by one to form an entity[64]. The extrusion printing is based on mechanical extrusion or air pressure to squeeze the liquid material in the chamber from the nozzle. By controlling the movement of the nozzle in the space, the extruded material is ordered to form a designed structure. Laser-assisted bioprinting is to controls cell density and builds 3D tissue structures at a cellular level with laser-induced methods[65]. Micro electrical scanning device includes extrusion printing unit, except that on the basis of extrusion printing,

a finer nozzle is used in this method, and a high voltage electrostatic field is constructed between the nozzle and the receiving board. The introduction of an electrostatic field lead to the formation of a typical Taylor cone at the nozzle, which is due to the electrostatic force helping gravity to counteract the surface tension of the liquid material, resulting in a finer nanoscale filament. Micro electrical writing is a method based on electrospinning. Oriented and controllable extruded filaments can be produced by controlling parameters such as extrusion pressure, electrostatic field voltage and plate distance. The nozzle can move along the designed path to print micron-sized 3D solids.

These 3D bioprinting methods have been applied to many fields such as skin, cardiac muscle, and oral and maxillofacial tissue engineering, and have great potential and feasibility in the construction of biosynthetic artificial cornea. The corneal limbal stem cells are distributed in the niche at the junction of the cornea and sclera. The advantages of inkjet printing and laser-assisted printing are the ability to accurately locate single cells and construct micro-nanoscale structures[11], which is of great value in the study of limbal stem cells and their niche reconstruction. Extrusion printing can control the thickness and geometrical morphology of the printed structure. Variable materials with good bioperformance can be used in extrusion printing. For example, Isaacson et al.[66] applied extrusion printing to the area of corneal tissue engineering and fabricated a corneal like cell-laden structure. In addition, micro electrical scanning and micro electrical writing can control fiber orientation at the micro-nano scale, which are excellent approaches that can be applied in corneal ultrastructure reconstruction. The 3D printing method can precisely control the geometrical morphology and is suitable for constructing multi-layer multi-material structures, and its research in the field of biosynthetic corneal manufacturing is meaningful.

5 Conclusions and future directions

Faced with the global shortage of donated corneas, the demand and urgency of transplantable corneal substitutes are increasing. At the same time, there is growing interest in the development of full-structure corneal *in vitro* models for drug screening and toxicological testing. The development of artificial corneas is an effective solution for this world issue. With the rise of biomaterials and new manufacturing technologies, 3D bioprinting has emerged in the field of personalized medicine, and a number of researches on the tissues and organs construction and regeneration have emerged. This review compares the existing artificial cornea materials and manufacturing methods, proposes the unique process advantages and manufacturing features of biological 3D printing, and can achieve the construction of the key features of the cornea and the functional reconstruction. It has great potential and development prospects in the field of corneal tissue engineering.

3D bioprinting is still in the early stage of research and development. Many development opportunities still need to be explored. For example, the use of nanoparticles to optimize the performance of bio-ink improves the biomechanical properties and transparency of artificial corneas[67]; the use of composite printing technology to precisely control the shape of the cornea, in order to achieve the control and reconstruction of corneal shape-related functions, to achieve personalized customization of corneal substitutes; research and development of

micro-nanoscale printing to achieve the study and reconstruction of the corneal microstructure, in principle to explore the corneal microstructure of corneal cells , metabolism, migration, and the effects and significance of corneal function to better understand the anatomy and physiology of the cornea; 3D bioprinting provides an all-in-one construction scheme that enables rapid construction of a full-thickness, full-structure cornea model for the cornea *in vitro*, provides solutions other than animal experiments.

Work continues across the world on the international standardization of processes and materials, industrializing the systems, and preparing the supply chain for potential demand of products using 3D bioprinted corneal processes. As for the adoption of any new health care technology or intervention, the new procedures involving alternatives to eye bank native tissue for corneal stromal replacement that are currently under development, will not only need to be effective, but also need to be cost-effective. Nowadays, many countries have their own economic criteria for the adoption of a new health care intervention. If the 3D bioprinting corneal procedure ends up being more effective and less costly than existing procedures, it will be easy to convince health care decision makers to adopt this new procedure. Therefore, the economic evaluation is essential to document the economic impact of 3D bioprinted artificial cornea.

In short, the development direction of the artificial cornea must be from experimental to clinical, from four individual studies (heterogeneous corneal alternatives, acellular biomaterials film, Single or Multi-Layer Corneal Equivalents, and ultrastructure) to full-functional corneal substitute development and research. In order to promote the studies of corneal substitutes, a detailed and systematic international standard that can describe the future R&D level and requirements of 3D printed corneal prosthesis are urgent to be introduced. This standard should include comprehensive assessment requirements for 3D bioprinting equipment and processes, *in vitro* and *in vivo* biological, optical and mechanical properties of artificial corneas built with 3D printing. Among them, 3D biometric printing devices constructed for artificial corneas should be classified based on printed principles categories, and correspond to different structures of the cornea, features and functions, and other research directions to improve and develop printing equipment and processes. In terms of assessment, biological properties include material properties and stability before and after transplantation, biocompatibility, penetration of glucose, albumin, and other related molecular size, oxygen permeability, portability, and the assessment of autologous tissue impact after transplantation (postoperative complications such as inflammation, corneal nebulization, autologous tissue degradation, graft migration or excretion). Optical properties include transparency, absorption and transmission of light in different wavelength bands, refractive power, refractive stability before and after transplantation, and assessment of visual performance. Mechanical properties include structural dimensions, surface quality, tensile strength, intraocular pressure resistance, creep test, linearity of anti-suture, stability of *in vitro* and *in vivo* artificial corneas and autologous tissue structures. On this basis, a systematic report was made on the *in vitro* structure and performance of animal and human subject assessments. In short, 3D printing has great research value in the future research direction of artificial cornea construction. It is still necessary to continuously improve existing

technologies and materials, develop new printing equipment, hope to realize the construction of a full-functional artificial cornea.

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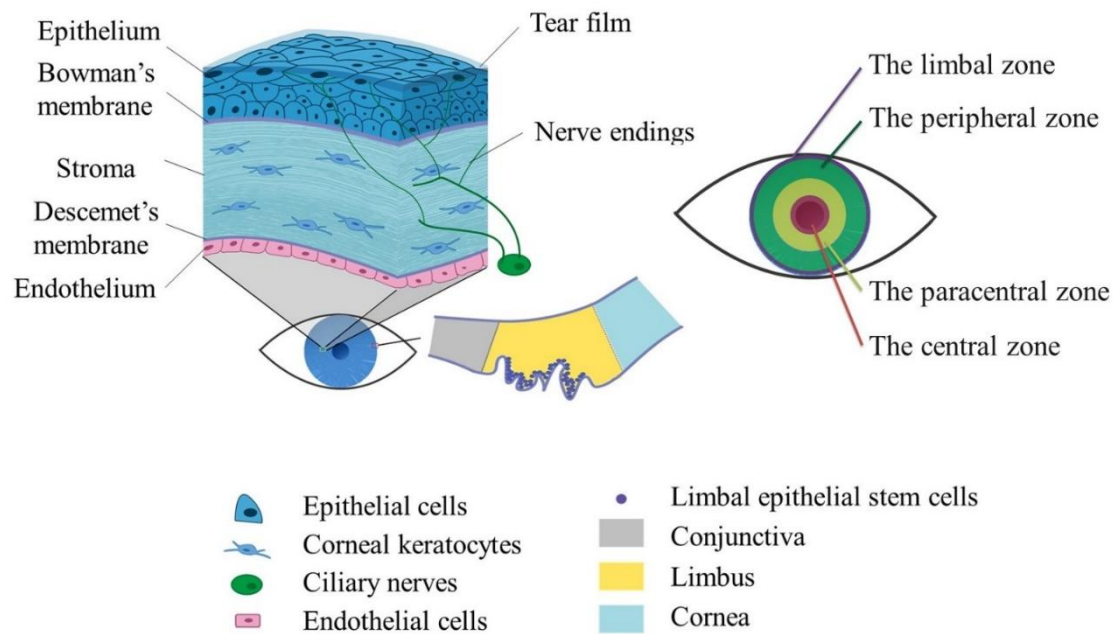
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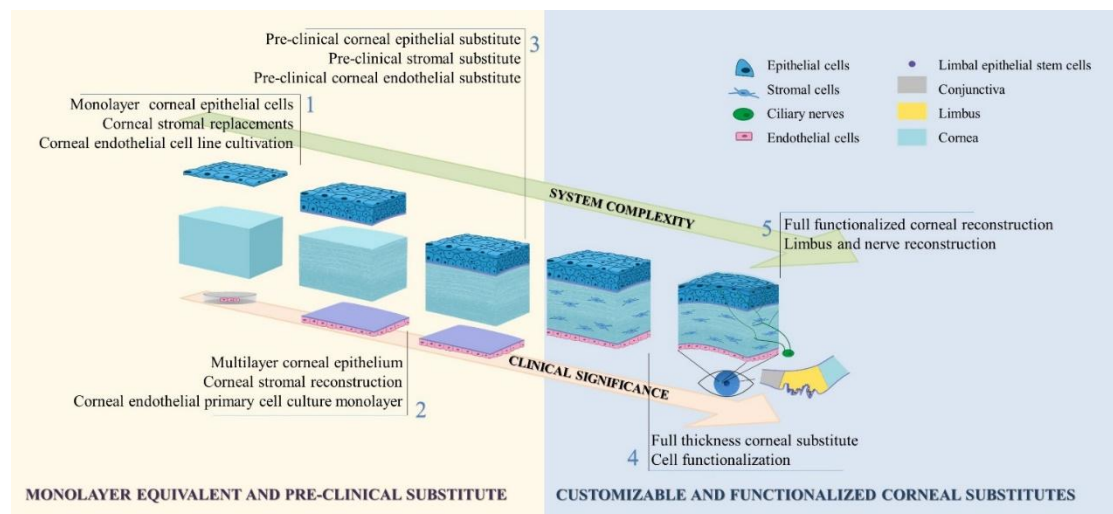
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Figure 1 The anatomy and physiology of cornea

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Figure 2 The clinical significance increase with the system complexity of artificial cornea from a surgical viewpoint. The corneal substitutes would develop from monolayer equivalent and pre-clinical substitute to multi-layer customizable and functional curved corneal substitute. The development of corneal substitutes with controllable shape for refractive and other optical purposes as well as good biological performance is essential and urgent.

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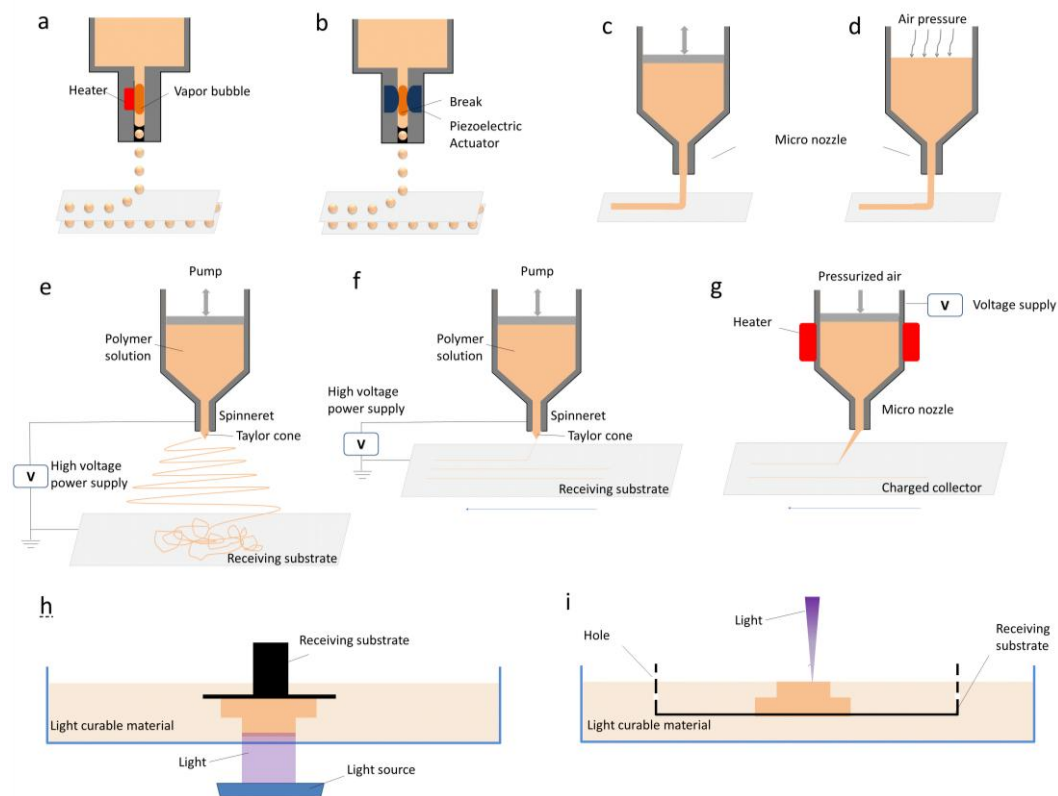


Figure 3 Schematic of major technologies of 3D bioprinting

a, b Inkjet printing[68]; a Thermal inkjet printing; b Piezo electric inkjet printing[69];
c, d Extrusion printing[70]; c Piston extrusion printing; d Pneumatic extrusion printing;
e Electrostatic spinning [71]; f Near-Field Electrosinining[72,73]; g Melt electrowriting[74]
h Digital light processor[75] i Stereolithographic[76]

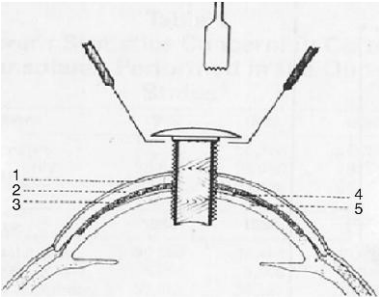
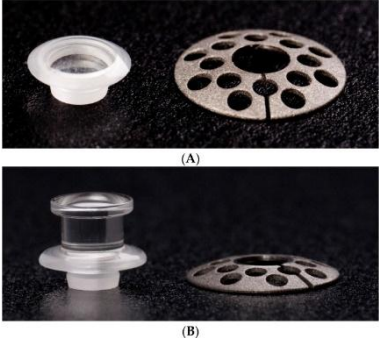

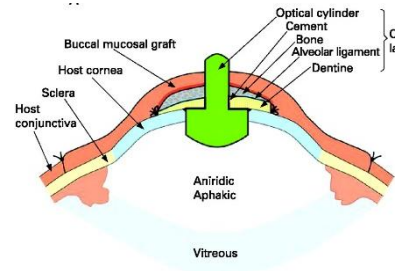
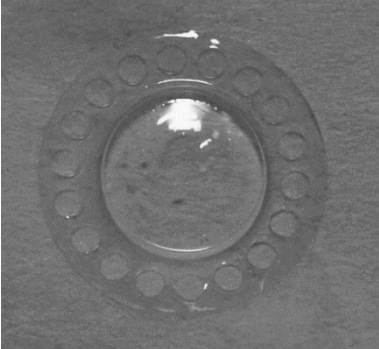
Table 1 Advantages of Artificial Cornea

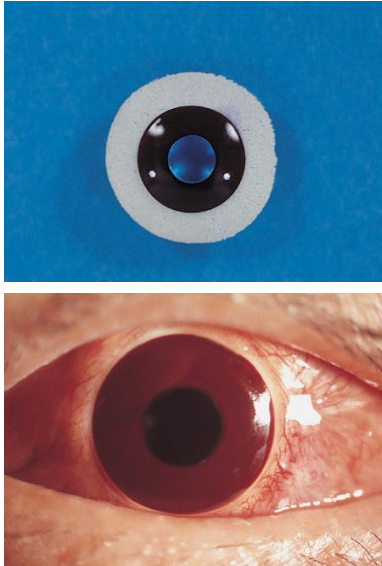
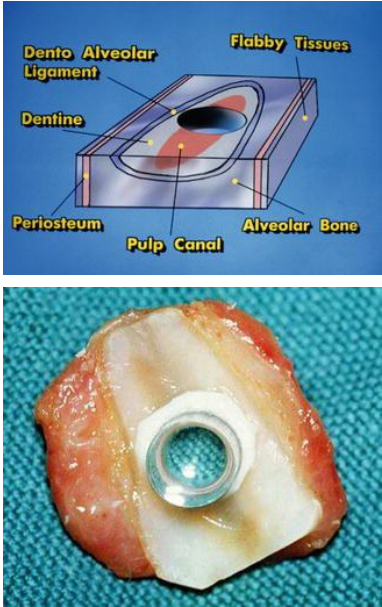
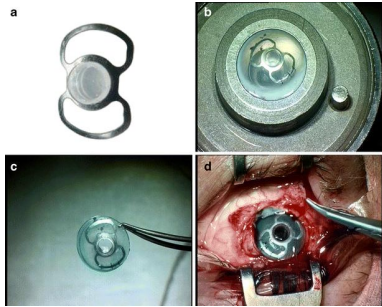
- Artificial cornea offers a potential solution of the global shortage of human donor corneas.
- The well production management and mechanized manufacturing system ensure the biological control of non-toxic, sterile as well as the products quality level and stability, which can effectively avoid the potential virus, religion, culture and policy problems associated with natural corneas.
- The acellular corneal substitute can overcome the obstacles of donor corneas, such as immune rejection induced by allogeneic cells from recipients who face high immune graft risk, ocular surface disease and the corneal graft dysfunctional.
- Artificial cornea can benefit from new advances in biomaterial science, such as artificial synthetic material, surface coating technique, functional materials, nanoparticles and other micro-nano scale materials science.
- The single-component artificial cornea, in contrast to native cornea, has the characteristics of homogeneity, and limited swellability so that free from the water accumulation or scattering of light.


Table 2. Category of Current Keratoprosthesis

	Materials	Schematic	Reference
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- For further clinical needs of full-functionalized artificial cornea, because of the specific spatial arrangements of the tissue structure, some optical, biomechanical and other relevant properties of the cornea can only be restored using 3D fabrication method.

Cardona keratoprosthesis	Poly (methyl methacrylate)PMMA		[34]
Boston Keratoprosthesis ((A) type I and (B) type II)	Poly (methyl methacrylate)PMMA + Titanium		[77–79]
Alphacor keratoprosthesis	(Polyhydroxyethyl methacrylate)PHEMA		[80–82]
The osteo-odonto-keratoprosthesis (OOKP)	Autologous tooth root and alveolar bone + poly (methyl methacrylate)PMMA		[83–85]
Keraklear Artificial Cornea	Poly (methyl methacrylate)PMMA + (polyethylene glycol) PEG		[86]

Korea Seoul-Type keratoprosthesis	Poly (methyl methacrylate)PMMA + PEG		[87–89]
The modified osteo-odonto-keratoprosthesis (MOOKP)	The surface of the osteodental lamina + poly (methyl methacrylate)PMMA		[90]
Fyodorov–Zuev keratoprosthesis	Poly (methyl methacrylate)PMMA + Titanium		[91,92]

MIRO® CORNEA UR keratoprosthesis	Hydrophobic acrylic polymer + Genetically engineered fibronectin		[93]
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Table 3. Current Approaches for Artificial Biosynthetic Corneal Equivalents

Catalog	Cell types	Scaffold materials	Manufacturing method	Clinical status	Ref.
I	Amniotic epithelial cells	Acellular porcine cornea	Decellularization	<i>In vitro</i>	[94]
I	Limbal corneal epithelial cells	Acellular porcine cornea	Decellularization	<i>In vitro</i>	[51]
I	Human corneal epithelial cells	Acellular porcine cornea	Decellularization	<i>In vivo</i> : Rabbit	[52]
I	—	Acellular porcine cornea	High-hydrostatic pressurization decellularization	<i>In vivo</i> : Rabbit	[54]
II	Limbal epithelial stem cells and stromal keratocytes	Acid soluble rat tail type I collagen/proteoglycan hydrogels	Horizontal magnetic field (7 T)	<i>In vitro</i>	[95]
II	Rabbit corneal endothelial, stromal, and epithelial cells	Fibrin-agarose hydrogels	Multi-layered construction	<i>In vitro</i>	[96]
II	Human corneal cell lines	Collagen–chondroitin sulfate substrate cross-linked with glutaraldehyde with scleral rim	Multi-layered construction	<i>In vivo</i>	[97]
II	Human corneal endothelial cells	Dense collagen hydrogel	Confined flow compression method + Seeding	<i>In vitro</i>	[98]
II	Human corneal epithelial cells (hcecs) and dorsal root ganglia (DRG) from chick embryos	Collagen(type-I atelo-collagen) -chitosan hydrogels	Polypropylene contact lens molds (cooper vision, Pleasanton, CA)	<i>In vivo</i> : rat, pig	[99]

II	Corneal epithelial cell	The dendrimer cross-linked collagen gels	—	<i>In vitro</i>	[100, 101]
II		1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC) cross-linked recombinant human collagen	Curved polypropylene molds	Phase I clinical trial: human	[102]
II	Immortalized corneal epithelial cells, Dorsal root ganglia from 8-d-old chick embryos	Collagen–Copolymer	Multi-layered construction	<i>In vitro</i>	[103]
II	1-ethyl-3-[3-dimethylaminopropyl]carbodiimide hydrochloride (EDC) and N-hydroxysuccinimide (NHS) crosslinked recombinant human collagen type III (RHCIII)	Cross-linked recombinant human collagen type III	Curved polypropylene contact lens molds	4-year follow-up	[104]
II	Precursors (precursor/gelatin group) , with fibroblasts (fibroblast /gelatin group)	Porous gelatin-glutaraldehyde	Trephined with a 5.0 mm diameter trephine + seeding	<i>In vivo</i> : rabbit	[105]
II	Stromal fibroblasts	Bovine collagen	Dry casting + seeding	<i>In vitro</i>	[106]
II	Corneal Limbal Epithelial Cells +Keratocytes	Amniotic membrane + laminin-coated compressed collagen gel (rat-tail type I collagen)	Compression and dehydration	<i>In vitro</i>	[107]
II	Corneal epithelial cell line HCE-T	Keratin film	Dry casting on hydrophobic coated PET sheets	<i>In vitro</i> study	[108]
II	Human corneal endothelial cells	Human corneal stromal discs	Seeding	<i>In vivo</i> : rabbit	[109]
II	Corneal endothelial cells	Gelatin hydrogel	Dry casting	<i>In vitro</i>	[110]

II	Human corneal endothelial cells	Hydroxyethyl chitosan, gelatin, and chondroitin sulfate	Dry casting	<i>In vitro</i>	[111]
II	Sheep corneal endothelial cells	Novel ultrathin chitosan–poly(ethylene glycol) (PEG) hydrogel films	Dry casting	<i>In vitro</i>	[112]
II	Human donor-derived corneal endothelial cells	Decellularized thin-layer corneal stroma	Microtome	<i>In vitro</i>	[113]
III	Cells from human oral mucosal tissue +3T3 feeder cells	—	Cell culture	Clinical trial: human	[114]
III	NIH/3T3 feeder cells	—	Cell culture	<i>In vivo</i> : rabbit	[115]
IV	Rabbit Primary stromal keratocytes	Silk fibroin films	Surface patterned by PDMS	<i>In vitro</i>	[116] [117]
IV		Magnetically aligned rat-tail type I	Horizontal magnetic field (7 T)	<i>In vitro</i>	[118]
IV	Rabbit stromal cells	Polyglycolic acid fibers		<i>In vivo</i> : rabbit	[119]
IV	Adult human derived corneal stromal (AHDCS) cells	Poly(l,d lactic acid)	Ordered electrospun	<i>In vitro</i>	[120]
IV	Immortalized human corneal keratocytes	Silk fibroin films	Porous surface patterned with PDMS by dry casting	<i>In vitro</i>	[121]
IV	Human corneal stromal stem cells	Poly(ester urethane) urea	Electrospun	<i>In vitro</i>	[122]

766 Note: I. Decellularized matrix as corneal alternatives, II. Materials and manufacturing methods of biofilm for
767 artificial cornea, III. Extracellular Matrix (ECM) Deposited by Cell, IV. Corneal stromal
768 Ultrastructure/Microstructure reconstruction.
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