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REVIEW ARTICLE

Advances in tissue engineering and 3D bioprinting for corneal regeneration

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Abstract

Blindness resulting from corneal damage affects millions of people worldwide. The scarcity of corneal donors adds a layer of complexity to patient treatment. Consequently, exploring artificial cornea substitutes has become imperative in the realm of clinical research. Scientific advancements have ushered in a plethora of innovative solutions, including keratoprostheses or decellularized cornea scaffolds. The development of three-dimensional (3D) printing has further expanded the horizons of research in this field, delving into the feasibility of bioprinted corneas and yielding numerous promising outcomes. However, the manufacturing of corneal products via 3D printing poses a substantial challenge, demanding a meticulous selection of materials and techniques to ensure the transparency and preservation of the optical and mechanical properties of the artificial cornea. In the review, we present the artificial cornea substitutes. Additionally, we aim to provide a concise overview of the 3D printing techniques and materials applicable to corneal bioprinting.

Keywords: Cornea; Blindness; Artificial cornea; 3D printing; Stem cells

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1. Introduction

Corneal blindness constitutes a significant global health challenge, with multifaceted etiologies including infections, scarring, and corneal dystrophy. Corneal transplants are undertaken for various reasons, with bullous keratopathy emerging as the predominant indication in developed countries, while infections and scarring are more prevalent causes in developing countries.[1]

As per the World Health Organization (WHO), the global population of blind individuals stands at 45 million, a figure that may escalate rapidly given the rise in life expectancy.[2] A study conducted in the United Kingdom between 2008 and 2011 revealed a breakdown of corneal transplant indications, with keratoconus accounting for 27.4%, Fuchs' dystrophy for 25.8%, cataract-caused endothelial dysfunction for 21%, infections

for 9.5%, ulcerative keratitis for 2.6%, and injuries for 2.4%.[3] Subsequently, a separate study conducted between August 2012 and August 2013 highlighted a substantial global demand for corneal transplants, estimating that 12.7 million individuals across 131 countries awaited this procedure.[4] In stark contrast, the annual incidence of corneal transplants in the United States is limited to 40,000.[5]

The treatment landscape is significantly challenged by the pronounced disjunction between the number of patients awaiting transplantation and the limited availability of cornea donors. This stark demand has prompted intensive research and development efforts in the realm of artificial cornea and cornea replacement products, which must meet stringent criteria.[6] Even when a suitable cornea donor is identified, the healing process may face impediments due to immune response-driven rejection.[5] The evolving landscape of science and medicine has ushered in myriad possibilities in the field of corneal research. The contemporary medical arena increasingly embraces personalized therapies, a trend underscored by the advent of translational biomedicine. A noteworthy surge in possibilities, such as the adoption of three-dimensional (3D) bioprinting, is reshaping the field. Traditional surgical approaches for reconstructing various tissues and organs confront formidable challenges owing to the distinctive functions of these tissues. To overcome these limitations, there has been a discernible escalation in research dedicated to the application of 3D printing techniques.

In corneal tissue engineering, in addition to 3D printing, nanotechnology offers a new avenue thanks to recent physical and chemical breakthroughs. These advancements enable the creation of specialized surfaces that facilitate cell adhesion and proliferation, establishing unique microenvironments to enhance nutrient supply. By incorporating various nanomaterials into hydrogels, it becomes possible to influence the physical and mechanical properties of the gel, including gelation. Nanoliposomes, when combined with stem cells in the gel, can mediate active substances, facilitating cell differentiation, reducing inflammation, and enhancing wound healing. The promising properties and versatile applications of nanomaterials hold potential for the future of corneal tissue engineering and regenerative medicine. However, it is important to note that the application of this technology in this field is still in the early stages of research.[5]

In 3D bioprinting for regenerative medicine, we are already witnessing promising results that allow for the printing of tissues with complex structures. Consequently, 3D bioprinting presents a new opportunity in personalized medicine for corneal replacement, addressing the challenge of donor shortage.[7]

2. The structure of the cornea

The cornea, a thin and transparent membrane, serves two primary functions: protecting the interior of the eye and facilitating light refraction. Optically, it is responsible for two-thirds of light refraction. The structural composition of the cornea involves various cell types, including epithelial cells, keratocytes, stromal cells, and corneal endothelial cells. In addition, extracellular components such as collagen or glycosaminoglycans (GAG) contribute to its composition.

The cornea is anatomically divided into five principal layers: epithelium, Bowman's membrane, stroma, Descemet's membrane, and endothelium (Figure 1). In terms of structure, the epithelial layer has a thickness of 5–7 cells, comprising three cell types: surface epithelial cells, stem cells, and basal cells. These cells collectively form a uniform layer with a thickness of 50 μm. Notably, corneal epithelial cells differentiate from limbal epithelial stem cells (LESCs) and do not undergo keratinization.[8] Following the epithelial layer, Bowman's membrane, characterized by an acellular structure, is constructed from a disordered multitude of collagen fibers. The stroma, which represents the thickest layer and constitutes roughly 90% of the total corneal thickness, plays an essential role in providing mechanical strength and critical optical properties. Structurally, it comprises approximately 200–250 parallel collagen fibers. Similar to Bowman's layer, Descemet's membrane is also acellular, composed of collagen, laminin, and fibronectin. The last layer of the cornea is the endothelial layer, semi-permeable to water and nutrients. Due to this property, the endothelial layer ensures fluid flow for the stroma. However, it is noteworthy that the number of cells forming the endothelial layer decreases with aging, and the proliferation capacity of these cells is significantly lower in the adult cornea.[9,10]

In addition to the well-established five layers, a sixth layer was recently discovered in 2013 by Dua and his colleagues.[11,12] Termed pre-Descemet's or Dua's layer, this membrane is located anterior to Descemet's layer. Dua's layer is a thin membrane primarily composed of type IV collagen, with a thickness ranging from 6 to 15 μm. The collagen fibers within this layer are organized into 5–8 layers.[8,9,11]

3. Cells of the cornea

3.1. Corneal epithelium

The corneal epithelium acts as a physical barrier, consisting of three different cell types: surface squamous

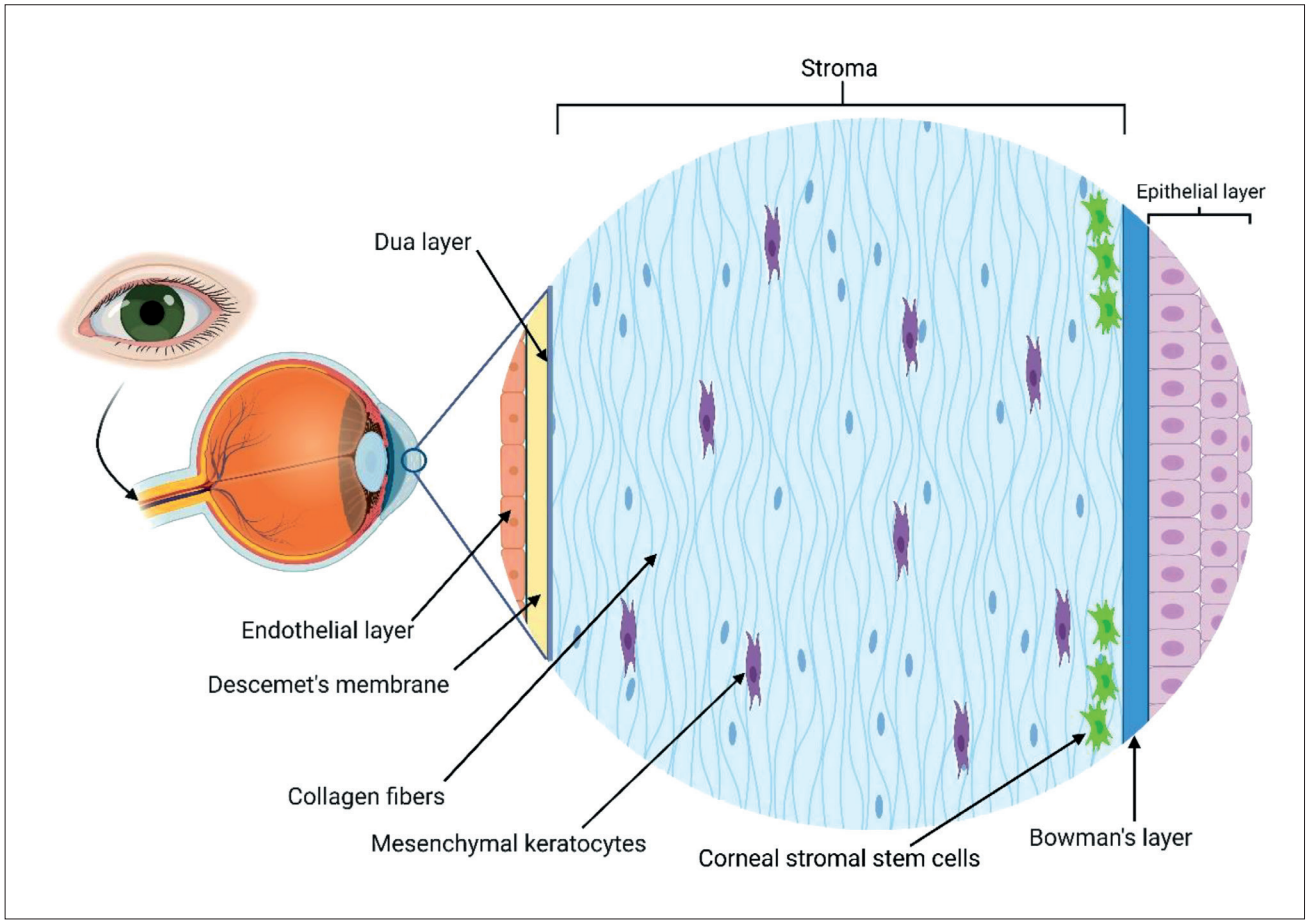

Figure 1. Structure of the cornea.

cells, suprabasal cells, and basal columnar cells located in the lower part of the epithelium. The upper three layers are comprised of differentiated surface squamous cells, covered with microvilli that significantly increase the surface of the squamous cell layer and facilitate contact with the thin tear layer protecting the cornea. The middle layer is composed of suprabasal cells, often referred to as Wing cells due to their wing-like shape.[13,14] Compared to squamous cells, suprabasal cells are flatter and more spread out, exhibiting elongated nuclei, with many vacuoles found in proximity to the squamous cell nucleus.[14] These cells divide relatively rarely, migrate toward the upper layer, and differentiate into squamous cells. The lowest layer of the epithelium is formed by basal cells in a single layer. These cells carry out essential tasks such as replacing suprabasal cells and secreting matrix factors that make up the lamina basalis and stroma. Basal cells are also responsible for the assembly of hemidesmosomes. These functions may play a role in cell migration during wound healing.[13] During eye development, the cornea's epithelium originates from the surface epidermal ectoderm.[4]

3.2. Keratocytes

The keratocytes residing in the cornea are situated amidst the collagen fibers comprising the stoma, originating from mesenchymal cells. Within this collagenous matrix, keratocytes form a unified network, interconnecting with neighboring cells in a dendritic manner. These cells contain crystals and proteins that contribute to the cornea's transparency. Play an important role in corneal wound healing and scarring, keratocytes exhibit two phenotypes. One phenotype is characterized as regenerative or repair-oriented, while the other promotes cell death. During the phenotypic transformation, cells deviate from their resting state. The regenerative phenotype facilitates wound healing through fibrotic scarring and tissue repair. However, it is noteworthy that scarring adversely affects corneal transparency.[13,15]

3.3. Corneal endothelium

The endothelium, constituting the cornea's innermost layer, comprises a single layer of cells located posterior to Descemet's membrane. Comprising squamous cells that

have undergone terminal differentiation, the endothelial layer lacks regenerative capacity. These cells fulfill crucial roles, including maintaining corneal transparency, supplying nutrients to the cornea, and ensuring hydration by permitting the passage of water into the stroma. Additionally, their pivotal function extends to preventing overhydration of the stroma through active transport mechanisms. This dual role is vital in preserving the organized structure of collagen fibers within the stroma, which is essential for facilitating light transmission and upholding corneal transparency. The endothelium and stroma are formed during eye development from the periocular mesenchyme—also known as periocular neural crest cells.[4,16-18]

3.4. Limbal epithelial stem cells

Adult limbal epithelial stem cells (LESCs) are located in the peripheral limbus, playing a pivotal role in the renewal of the corneal epithelium, specifically in the continuous replacement of the upper 4–6 layers, predominantly composed of squamous cells. These LESCs are located in the basal layer of the limbal epithelium, forming distinctive niches. Despite their essential function, it is noteworthy that the proliferation potential of these stem cells is extremely low. The potential loss or damage of these stem cells can occur due to various factors such as physical, chemical, or thermal impact, genetic diseases, or infections. Consequently, limbal stem cell deficiency triggers neovascularization and angiogenesis in the conjunctiva, leading to vision loss. To address this, clinically cultured LESCs are employed as a therapeutic strategy. These cultured cells are transplanted into the patient's cornea, and it is important to note that LESCs can be derived from either autologous or allogeneic sources.[19]

3.5. Corneal stromal stem cells

Corneal stromal stem cells (CSSCs) represent another crucial cell type within the cornea, specifically located in the limbal stoma. Functioning as mesenchymal progenitors of keratocytes, these cells contribute to wound healing and regeneration, essential processes for maintaining corneal transparency. The therapeutic potential of CSSCs extends to applications in artificial tissue replacement.[20] Notably, CSSCs demonstrate the capability to produce matrix components resembling the composition of the collagen matrix present in the stroma. In research endeavors, it was observed that stromal stem cells, when cultured on nanorods arranged in parallel, generated a collagen layer mirroring the structure and composition of the natural stroma.[21] These findings suggest that CSSCs hold promise for *in vitro* production of stroma-like tissue. Such advancements could open avenues for replacing the stroma in transplantation procedures.

4. Extracellular matrix proteins in the cornea

A crucial determinant of achieving optimal visual acuity is the correct composition, structure, and interplay of the extracellular matrix (ECM) situated within the cornea, forming what is known as the corneal stroma (Table 1). An illustrative instance of this is the organized network of keratocytes within the stroma, situated amidst collagen fibers and layers. This arrangement serves as a cornerstone for one of the main functions of the stroma, specifically imparting mechanical strength to the cornea while significantly contributing to its transparency.

The primary constituent of the ECM is collagen, accompanied by various leucine-rich proteoglycans.[22] The collagen matrix forming the corneal stroma incorporates diverse collagen types, including type I, type IV, type V, and type VIII. Unlike collagen layers in other connective tissues, the corneal collagen matrix is notably thinner, a characteristic that contributes to corneal transparency. The collagen fibers within the cornea serve as pivotal load-bearing elements, enduring tensile and compressive forces generated by intraocular pressure and external impacts to safeguard inner ocular tissues. This robust strength of collagen fibers is attributed to their rope-like structure and the different lateral orientations of the layers. Dermatan sulfate-containing proteoglycans in the stroma prevent the adhesion of neighboring collagen fibers.[9] Among the collagens, type I collagen prevails in abundance throughout the human body, with exceptions such as the eye's vitreous body or brain. Conversely, type V collagen is notably more abundant in the cornea. This collagen variant, characterized by its small fibrillar structure, plays an important role in fiber formation. The stroma layers mainly consist of type I and type V collagens, with an estimated count of approximately 250–300 layers enveloping the entire cornea. The distribution of particular collagen types may vary depending on the cornea's structure and condition. Given that type I and type V collagens collectively contribute to corneal transparency, any changes in their distribution or ratio can affect this transparency. For example, structural changes in type I collagen resulting from corneal wounds or scarring may lead to a decrease in transparency.[16]

Type VII collagen assumes a significant role in facilitating adhesion between the epithelial layer and the stroma, as well as in fiber fixation and wound healing. Unlike the previously discussed collagens, type VIII and type XII collagens lack the ability to independently form fibers. Nevertheless, they can engage in interactions with other collagen types, actively participating in fiber formation.[23,24]

Table 1. Proteins in the extracellular matrix of the cornea

Cornea layers	Extracellular matrix component	References
Epithelium	Very scanty or relatively no extracellular matrix	[96-103]
Epitope base membrane	Type I collagen	
	Type IV collagen	
	Laminin	
	Fibronectin	
	Fibrin	
	Proteoglycans	
Bowman's membrane	Type I collagen	
	Type III collagen	
	Type IV collagen	
	Fibronectin	
Stroma	Type I collagen	
	Type III collagen	
	Type V collagen	
	Fibronectin	
	Proteoglycans	
	Elastin	
Dua's layer	Type I collagen	
	Type IV collagen	
	Type V collagen	
	Type VI collagen	
	Fibronectin	
	Proteoglycans	
Descemet's membrane	Type I collagen	
	Type IV collagen	
	Type VIII collagen	
Endothelium	Very scanty or relatively no extracellular matrix	

In the fibrillogenesis process of the cornea, the longitudinal and linear growth of collagen fibers plays an important role in ensuring the formation of the eye's structural integrity. Significantly, inhibiting the lateral growth of fibers, which prevents the formation of thick fibers, is crucial for maintaining corneal transparency.[25] Proteoglycans contribute to this process during fibrillogenesis and matrix formation. Notably, research has underscored the substantial role played by proteoglycans containing keratan sulfate and dermatan sulfate in shaping the cornea's collagen structure and organizing the matrix, thereby contributing to corneal transparency.[16,26]

The predominant portion of proteoglycans present in the corneal stroma belongs to a small family of leucine-rich proteoglycans. These proteoglycans engage in binding with collagen fibrils, profoundly influencing the arrangement of collagen fibers within the stroma. Beyond their structural role, these proteoglycans play a significant regulatory role in cell adhesion, proliferation, and migration within the cornea. Consequently, they exert influence over cellular responses during the wound-healing process.[27]

5. Artificial corneas and corneal regeneration

Artificial cornea replacement products must adhere to a myriad of specifications to closely emulate the native cornea. From a manufacturing standpoint, these artificial corneas should be cost-effective, ensuring high-quality production, and designed for ease of mass production. On the medical front, crucial prerequisites include transparency and a sufficiently porous structure that facilitates the supply of nutrients and oxygen to the surrounding tissues. In

addition, the optical properties of these products must closely align with those of the native cornea while also demonstrating resilience to withstand transplantation and mitigate the risk of eliciting autoimmune reactions from the recipient's body.

5.1. Keratoprostheses

Blindness resulting from disease or corneal damage constitutes a significant global health concern affecting millions of individuals. Corneal transplantation, or keratoplasty, stands as the primary method for addressing corneal blindness. However, the scarcity of suitable donors poses a considerable challenge in caring for affected patients. Consequently, biomimetic substitutes that emulate the cornea have gained increasing prominence.

Artificial corneas, known as keratoprostheses, are laboratory-manufactured products comprising both synthetic and biological materials. These substitutes offer many advantages, including enhanced biocompatibility that mitigates the risk of rejection. Adhering to strict manufacturing regulations ensures the sterility of keratoprostheses, minimizing the risk of infections during implantation, which could potentially lead to further corneal damage. Notably, keratoprostheses exhibit reduced light scattering owing to their unique properties.[28]

The evolution of these products over the years reflects substantial advancements. Early keratoprostheses had a more artificial effect than the current, more advanced artificial corneas. The center of the early versions of these products featured a rigid, poly(methyl methacrylate) (PMMA) optical element attached by synthetic or alternative materials. However, challenges in patient implantation arose due to potential immune responses triggered by certain materials. Research suggests that porous materials such as Teflon, poly(2-hydroxyethyl methacrylate), or Dacron (polyethylene terephthalate [PET]) facilitate the integration of implants into the host body, potentially addressing these challenges.[29]

However, these prostheses come with inherent limitations and disadvantages, partly stemming from the structure and material composition of the implants. The typical hardness and relative rigidity of these materials can induce discomfort upon implantation in the patient's eyes, potentially causing damage to the surrounding healthy tissue. Another disadvantage is the restricted vision often experienced post-keratoplasty, a consequence of the material used for implant fixation. The choice of fixation material may compromise corneal transparency. Given the implantation of artificial materials, the transplantation process becomes more complicated than allografts, with the need for two surgical procedures in many cases, thereby elevating the risk of infections.[7]

5.2. Amniotic membrane

In addition to keratoprostheses, the amniotic membrane (AM) stands out as one of the most commonly used corneal substitutes. Derived from the placenta, the amniotic membrane is typically 0.02–0.5 mm thick and devoid of blood vessels and nerves. Structurally, the AM consists of three layers: the epithelial layer, the vascular stroma, and the basement membrane. Both the basement membrane and the stroma are rich in collagen, fibronectin, and laminin. The membrane serves multifaceted functions, promoting the migration of epithelial cells, orchestrating the organization of collagen fibers, and concurrently inhibiting neovascularization and fibrosis.[30]

Several studies have explored the applicability of the amniotic membrane, including research conducted by Rohaina et al.[31] In their study, the amnion was combined with stem cells for epithelial replacement, revealing enhanced post-operative transparency of the implanted AM attributed to reduced neovascularization. These findings suggest that the AM holds promise as a corneal substitute in corneal reconstruction surgeries.

Efforts have also been directed toward enhancing the stability and durability of the membrane by incorporating additional scaffolds, often involving various nanomaterials and nanofibers. However, the use of such supporting elements introduces uncertainties, including uncontrolled degradation, tissue interaction, and potential cytotoxicity. Despite these challenges, the use of AM is not without disadvantages, encompassing limitations such as the restricted number of available donors, difficulties in isolation from the placenta, and the inherent risks of infections that could be transmitted through transplantation.[32,33]

5.3. Corneal bioscaffolds

The development of artificial tissue production has presented a new opportunity to address the shortage of cornea donors. One approach involves combining real tissue with an artificial scaffold crafted from biomaterial. In comparison to keratoprostheses, these products may offer greater ease in terms of biocompatibility. Careful consideration of the chosen scaffold material and its preparation is crucial during the planning of these substitutes to ensure resulting tissue closely mimics native tissue.[32]

Another alternative involves using decellularized corneal stroma of animal origin, providing a potential remedy for the donor shortage. The effectiveness of using these scaffolds for stroma reconstruction is heightened

when the epithelium and endothelium are well-preserved. However, animal-derived products introduce limitations and disadvantages, including the necessity for extensive donor screening to detect various pathogens.[34] An additional disadvantage lies in the potential immune response triggered by residual cellular elements within the foreign tissue, which may lead to rejection.[7] Moreover, remnants of substances used in the decellularization process, such as Triton X-100, formic acid, sodium dodecyl sulfate, and dispase, may possess toxicity post-implantation.[28] Subsequently, these stroma substitutes undergo recellularization with various cell types using diverse techniques.

The shortage of donors has spurred the development of numerous techniques to meet the demand for artificial corneas. A notable example is the porcine collagen-based cornea pioneered by Xeroudaki et al. This approach utilized highly pure, medical-grade collagen extracted from pig skin, effectively replacing a segment of the stroma in a thin layer. The outcomes of their study reveal the successful survival, proliferation, and migration of cells within this layer. The surgical procedures utilizing this method are characterized by rapid regeneration, resulting in a transparent cornea. Impressively, over the examined 6-month period, the prepared implant retained its original morphology and successfully replaced the surgically affected part of the stroma.[35]

5.4. Tissue bioprinting

3D bioprinting emerges as a potential solution to address the biocompatibility challenges associated with artificial corneas and alleviate the demand stemming from the scarcity of donors. Leveraging 3D design programs and bioprinting technologies facilitates the creation of complex shapes using a variety of materials. A key advantage of 3D bioprinting, distinguishing it from existing methods, lies in its high-quality spatial resolution and the extensive array of available hydrogel materials and compatible cell types. The flexibility of various printing techniques allows for tailored approaches, enabling the selection of the most suitable method for the specific challenge at hand. 3D bioprinting enables the precise recreation of the cornea's different layers and anatomical features. This capability ensures high-fidelity reproducibility, allowing for the creation of corneal replacements with exceptional precision and accuracy for multiple patients.[5]

In the realm of the cornea, 3D bioprinting offers the capacity to create multicellular, multi-layered structures and easily print curved surfaces. This capacity is instrumental in fulfilling the crucial requirement for artificial corneas to resemble the native tissue. Furthermore, these properties significantly contribute to both the optical and mechanical properties of the final product. 3D bioprinting not only enables the modeling of individual corneal components but also paves the way for creating multi-component systems, facilitating the comprehensive recreation of the entire cornea. The potential to generate complex systems holds significant promise for drug development and toxicological studies, offering an alternative to conventional animal models and the less effective two-dimensional cell cultures employed thus far.[7,36]

The matrix, essential for cell adhesion and proliferation during tissue printing, can be provided by either natural materials (gelatin, collagen, laminin, cellulose) or artificial polymers (poly(ethylene glycol) diacrylate [PEGDA], poly(caprolactone) [PCL], poly(ethylene glycol) [PEG]).[34,37,38] Natural polymers possess numerous advantageous properties that can be easily adapted to the specific tissue and cell type to be printed.[39] However, it is crucial during printing to select materials that do not impede the proliferation and migration of cells.[40] In the design of bioprinting, careful consideration of certain properties of the polymer—such as viscosity, gelation time, or concentration—is necessary to establish an environment conducive to the cells in contact with the printed tissue.[38,40]

One disadvantage of these polymers is their mechanical sensitivity in many cases, a limitation that can be mitigated by mixing them with other materials to improve their physical properties.[41] For example, constructs made of alginate may readily disintegrate in a calcium-free environment and dissolve in the surrounding liquid. In contrast, gelatin scaffolds exhibit sensitivity to temperature changes, softening at room temperature and liquefying at around 37°C.

In the case of natural polymers, a cross-linking agent is used to address this issue, fostering bonds between the polymer chains. Cross-linking can be achieved through physical means (UV, blue light), chemical processes (divalent cations, pH change), or biological mechanisms—with the help of enzymes. The resulting cross-linked structure forms a semi-permeable system that facilitates the permeation of metabolites, nutrients, and oxygen. This permeability is essential for sustaining cell viability and the functionality of the 3D-printed tissue.[38]

In addition, the decellularized extracellular matrix holds great potential as a natural polymer for bioprinting. In this realm, the research conducted by Kim et al.[42] provides great novelty and promising results in the development of corneal analogs. The group formulated a decellularized ECM-based hydrogel derived from corneal tissue, cross-linked with a ruthenium and sodium persulfate-containing photoinitiator.

An important property of their hydrogels is the utilization of visible light for cross-linking, thereby safeguarding cells from damage inherent in common UV cross-linking methods. Moreover, the hydrogel can undergo gelation regardless of pH conditions, facilitating easier handling. The resultant scaffold exhibited commendable physio-mechanical properties, maintaining the printed shape of the cornea. After 30 min of saline washing to eliminate the yellow color of ruthenium, the scaffold retained 94% transparency and remained transparent after 10 days. Human trabecular meshwork stem cells (hTMSCs) mixed into the corneal dECM hydrogel demonstrated 90% viability even 48 h after printing. Notably, cornea-specific gene upregulation was observed in the cells, and immunostaining revealed collagen production. Zhang et al.[43] utilized cornea-derived dECM (CECM) and GelMA-based hydrogel for DLP-printed cornea structures. *In vitro* employed human corneal fibroblasts, while *in vivo* testing utilized rabbit models. Rheological tests demonstrated excellent physio-mechanical properties of their cornea scaffold, exhibiting stability and resistance to various forces and near-complete transparency. The CECM/GelMA hydrogel exhibited only 17% water loss after 4 h of air drying compared to pure GelMA (31%). Cell viability gradually increased during the 14-day culture period, indicating cell proliferation within the hydrogel, with observed migration toward inner areas. The CECM/GelMA hydrogel provided an optimal microenvironment to the cells, leading to a gradual increase in collagen, lumican, and ALDH3A1 production, as observed through immunostaining. In an *in vivo* rabbit model, the implanted CECM/GelMA hydrogel did not induce inflammation or rejection, and increased re-epithelialization was observed around the wound, resulting in a healing rate of 93.5% at 28 days after surgery.

Another significant category of polymers applicable to tissue printing comprises synthetic polymers, often preferred over biopolymers due to their mechanical strength and non-immunogenic properties.[38,44] Typically produced through chemical reactions, these materials are transformed into hydrogels using the inverse dispersion technique.[39] However, a disadvantage of synthetic polymers is their reliance on organic solvents and high temperatures for 3D printing, potentially compromising the biological activity of cells and various active substances and factors incorporated into the hydrogels.[38,45,46] Consequently, synthetic polymers find greater utility in constructing the frames of printed structures.

6. Type of printing methods

The primary challenge in constructing complex structures lies in the necessity for scaffolds to incorporate multiple cell types. One solution to this issue is the utilization of 3D fabric printing. Currently, several 3D printing techniques are available in the market, such as methods based on inkjet, extrusion, or light. The selection among these methods hinges on the specific characteristics of the intended sample, considering both their advantages and disadvantages. Depending on the type of printer used, the scaffold or printing mold can have a positive or negative pattern, and it is even possible to print without a mold using materials with special properties, such as poloxamer (Figure 2).

6.1. Material extrusion methods

Material extrusion-based printers can be categorized into two types based on the method used to dispense the material for printing: pneumatic (utilizing compressed air) and mechanical material extrusion. In both methods, printing is executed by one or more fixed print heads positioned above a printing table movable in three dimensions (X, Y, and Z directions). Pneumatic systems may exhibit less direct control over material flow due to the delay introduced by gas volume compression. Conversely, mechanically operated systems employ a screw-controlled piston in the syringes, making them more suitable for printing high-viscosity hydrogels. Continuous material flow must be carefully maintained in both types. Hydrogels used in this technique must undergo cross-linking during or after printing, achieved through physical or chemical methods. This technique is versatile, allowing the printing of various tissues, including the cornea. Material extrusion facilitates fast and cost-effective printing. A range of starting materials, including hydrogel containing different cell types, dECM, and synthetic polymer fibers, can be used for scaffolds. However, material extrusion techniques have drawbacks. Inadequate and excessive pressure application and overly swift movement of the printing table can disrupt the continuity of the print pattern, resulting in lower resolution and slower printing speeds compared to other methods. Attention must be given to the viscosity of the hydrogel, as excessive viscous hydrogels can lead to print head clogging. Moreover, cellular viability during cellular printing using material extrusion may be lower due to the high pressure and shearing forces applied to the cells, in contrast to other techniques.[6,36,47-50]

6.2. Inkjet printing

Within the inkjet printing technique, six methods can be distinguished: piezoelectric, thermal, electrostatic, electrohydrodynamic, microvalve-based, and acoustic. Material jetting offers the advantages of computer-controlled droplet formation with high precision and resolution, enabling control over the placement

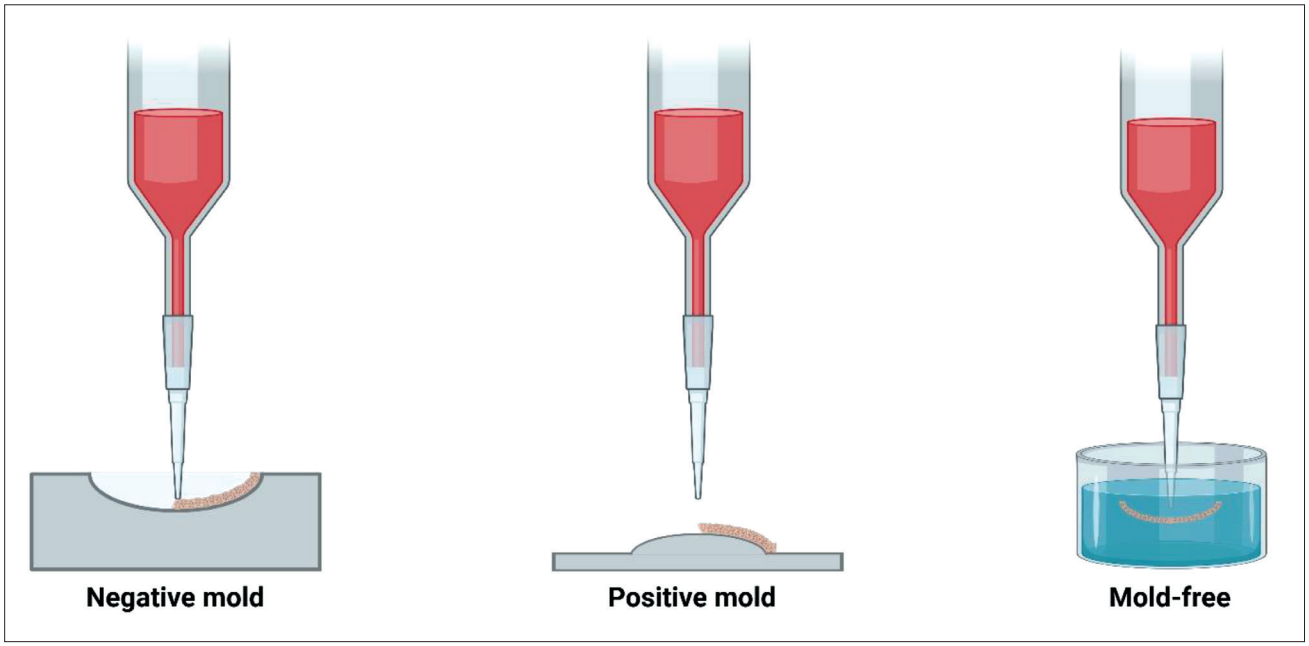

Figure 2. Types of mold used in corneal bioprinting.

of individual droplets in the scaffold. The difference among these techniques lies in the methods used for droplet production, such as a heat actuator in thermal inkjet printing, a pressure plate in electrostatic inkjet printing, and an electric field in electrohydrodynamic printing. Each technique has its drawbacks. In thermal inkjet printing, the hydrogel must be subjected to high temperatures (200–300°C) for droplet formation, which can potentially harm the cells inside the print head and inactivate biologically active proteins in the hydrogel. Maintaining appropriate viscosity is another challenge, as overly viscous materials may not form droplets effectively and can clog the print head, while excessively thin hydrogel may flow out of the print head without proper binding. Certain types of 3D printing methods may be suitable for corneal printing, such as the reactive inkjet method (RIJ) based on inkjet technology. Duffy et al.[51] utilized RIJ to construct a cornea based on poly-ε-lysine and gellan gum hydrogel. This inkjet printing method employs two print heads, each containing different components of the hydrogel. While each component is inactive on its own, their simultaneous printing on a special substrate results in binding through physical or chemical reactions *in situ*.[36,47,51-53]

6.3. Light-based methods

Among the 3D printing methods using light, two categories can be distinguished: laser-induced forward printing (LIFT) and methods based on resin polymerization. LIFT uses laser pulses—collimated, monochromatic light beams—to print individual bioink droplets. The equipment consists of a laser passing through a focusing system, heating a quartz glass plate coated with an absorption metal layer (such as gold) at a specific point. The opposite side of the plate contains a hydrogel coating. The laser induces the formation of droplets at a designated point, which then lands on a surface equipped with a receiving substrate. One advantage of this technique is the absence of print head clogging, allowing for a wide viscosity range during printing. High cell density can be achieved without compromising cell viability. However, the disadvantage is the potential introduction of particles from the metal layer into the sample during printing, and the method incurs a high material cost. This technique is applicable for printing various tissues and organs, including the cornea. Techniques based on the resin polymerization method include stereolithography (SLA), digital light processing (DLP), and two-photon polymerization (2PP). In these methods, a photoactive or light-binding bioresin is used and bound layer by layer with an LED or laser-based light source. SLA and DLP are very similar, with SLA employing a UV laser or visible light source focused on a movable platform to bind the bioresin through laser scanning. In contrast, DLP focuses the image of the pattern to be printed in the resin using a complex mirror system, allowing for the creation of entire layers at once. Two-photon polymerization is a method in which a resin molecule absorbs two photons.

This resin is composed of two special components (a positive and negative tone resin). Printing is accomplished using a femtosecond near-infrared (NIR) laser source and a glass plate situated at the bottom of the resin bath. In contrast to cellular printing, laser techniques are primarily used for scaffolds, given that the unbound resin from the printed sample is dissolved using solvents that can be harmful to cells. However, the advantage lies in the capability of these techniques to swiftly and economically produce substantial quantities of constructs.[36,52,54-57]

In addition to potentially harmful solvent and resin-based techniques, many water-soluble, less, or non-cytotoxic photoinitiators can be used in corneal bioprinting with common hydrogels. For instance, Irgacure and LAP (lithium phenyl-2,4,6-trimethylbenzoylphosphinate) are commonly used in the photocuring of acrylate and methacrylate-based hydrogels. Studies have demonstrated that a low concentration of LAP exhibits enhanced cytocompatibility and improved physio-mechanical properties in printed constructs compared to Irgacure 2959.[58] Cytotoxicity tends to escalate with the initiator concentration and exposure to cross-linking UV light.[59] Barroso et al.[60] also used LAP as a photoinitiator for printing a methacrylated silk fibroin-based bioink (SilkMA) in artificial corneal research. Their study revealed that LAP-cured SilkMA exhibited good viability, and metabolic activity increased over the 14-day observation period. The prepared hydrogel could be cured with neutral pH and low-energy UV light or through lithography-based printing. In another study, He et al.[61] prepared PEGDA-GelMA hydrogel with LAP as a photoinitiator, successfully printed with cells using the DLP method. Cells demonstrated proliferation in the hydrogel 6 days after printing with approximately 90% viability. Additionally, numerous photoinitiators can facilitate cross-linking via visible light, such as Eosin Y, riboflavin, or ruthenium (as mentioned in Zhang et al.[43]). These methods represent viable alternatives in light-based bioprinting with high biocompatibility, as measured using MTT or Live/Dead assay.[58]

7. Hydrogels and scaffolds for corneal bioprinting

7.1. Alginic acid

Alginic acid, commonly known as alginate, is a polysaccharide extracted from brown algae. Its versatile applications extend across industries, including textiles, pharmaceuticals, and food, where it serves as a thickener, gelling agent, and emulsifier marked as E400. Another common form is sodium alginate, the sodium salt of alginic acid. Cross-linking of alginate is typically achieved with divalent cations such as calcium (Ca^{2+}), magnesium (Mg^{2+}), or barium (Ba^{2+}). This binding method allows reversible cross-linking, as the cations are released from the cross-linked alginate in a cross-linker-free environment. In addition to cationic cross-linking, alginic acid exhibits excellent cross-linking through enzymatic and photoactive compounds. While alginate-bound cells can be efficiently recovered, and cells remain viable for weeks in alginate scaffolds, there are disadvantages. Cells enclosed in alginate hydrogel tend to maintain a spherical shape due to encapsulation, and they may exhibit lower proliferation and differentiation rates.[38,44,62]

7.2. Gelatin

Gelatin, derived from collagen through the partial hydrolysis of its tertiary structure, is a protein that originates from various sources, such as pork, calf, or fish, each with slightly different properties. Gelatin-based hydrogels are renowned for their excellent biocompatibility and biodegradability, making them a commonly used hydrogel component in 3D printing. Gelatin-based hydrogels maintain cell viability and differentiation potential, which are important factors when working with stem cells. One disadvantage of using gelatin lies in its thermosensitive property, wherein its cross-linked structure becomes unstable and liquefies under physiological conditions above 20°C, such as the standard human body temperature of 37°C.[38,44] To address this, improving the mechanical properties of gelatin-based hydrogels is necessary and can be achieved by incorporating other polymers, such as chitosan, collagen, or alginate, into the gelatin matrix.[38,63] Alternatively, chemical modifications on gelatin, such as methacrylation, result in the formation of gelatin methacrylamide (GelMA). The addition of a photoinitiator to GelMA allows photopolymerized using UV light.[64]

7.3. Collagen

Collagen, an important component of ECM, is widely used in clinical settings as a tissue replacement and regenerative material, as well as in 3D bioprinting due to its excellent mechanical and degradable properties. This ECM protein is abundantly present in the connective tissues of many organisms, with the primary sources for laboratory uses being animals like calves, pigs (skin and bone), or marine animals.[65]

In the context of tissue printing, collagen stands out as an excellent hydrogel material due to its scaffold structure. The porous structure of collagen facilitates the diffusion

of nutrients and growth factors within the hydrogel.[66] Its degradation characteristics are contingent on chemical and thermal conditions: for instance, it resists dissolution in acidic solvents (e.g., acetic acid) and maintains stability when stored at a low temperature (2–8°C). Under physiological conditions, preventing fibrillogenesis (neutralized pH, 37°C) ensures gel formation occurs within hours. Despite its numerous advantages, collagen does have notable disadvantages, including low stiffness and short duration of maintained stability.[62]

However, the utilization of animal-derived collagens in humans carries the risk of provoking unwanted immune responses, which, in some cases, can be severe. In addition, the use of collagens sourced from various tissues poses a potential risk of transmitting infections and pathogens.[67] In an experiment conducted by Cooperman and Michaeli on volunteers, employing high-purity dermal calf collagen resulted in side effects in 3% of patients (2 out of 61), highlighting the immunogenic nature of animal-derived proteins.[68] To address this, recombinant collagens present a viable solution. Various host organisms, such as mammalian cells, yeasts, and bacteria (e.g., *Escherichia coli*), are suitable for collagen production, each capable of producing collagen in different quantities. Notably, hydroxylated full-length collagen can only be produced with transfected mammalian cells. Nonetheless, it is completely equivalent to tissue collagen.[69]

7.4. Hyaluronic acid

Hyaluronic acid (HA) is a naturally occurring glycosaminoglycan, characterized by the repetition of disaccharide units (glucuronate and N-acetylglucosamine). For applications in tissue printing, HA can undergo chemical modifications, resulting in derivatives such as sodium hyaluronate, tyramine-substituted HA, and thiolated hyaluronic acid.[62] Found in various tissues (e.g., skin, cartilage), HA serves as an excellent scaffold in bioprinting.[32] The development of stable HA hydrogels can be achieved through cross-linking with hydrazone, employing a two-component system that promotes rapid gel formation and embeds cells within the hydrogel matrix. In the context of cornea, HA promotes cell migration and tissue regeneration.[70,71]

7.5. Other materials

Silk fibrin, a high-molecular-weight substance derived from the cocoon of the *Bombyx mori* silkworm, exhibits low immunogenicity, favorable mechanical properties, and a controllable degradation rate. The incorporation of this material into hydrogels improves the mechanical properties of scaffolds, a critical requirement for a corneal scaffold. This augmentation promotes stromal cell proliferation while maintaining the transparency of the printed pattern. The application of silk fibrin in 3D bioprinting and cornea reconstruction has been extensively explored by researchers. Investigations into combining silk fibrin with other materials, such as PEG or RGD peptides, have been conducted to enhance cell adhesion. The outcomes of these studies consistently reveal an enhancement in the adhesion of specific cell types.[30,32,72,73]

Chitosan, an amino polysaccharide derived from chitin found in shellfish, insects, and fungi, stands as a widely used natural biopolymer with numerous positive attributes, such as biocompatibility or biodegradability. These characteristics make it well-suited for applications in wound healing and 3D bioprinting. The stability of chitosan can be enhanced through cross-linking; without this process, chitosan would undergo rapid degradation, particularly under acidic pH conditions.[39,65,74] The incorporation of chitosan into scaffolds promotes cell adhesion, proliferation, and differentiation, and remarkably, only minor rejection incidents were observed post-transplantation.[75]

Fibronectin is a multi-domain glycoprotein capable of binding to numerous ECM components, playing an important role in establishing the cell–ECM connection through cell surface integrins. Synthesized by diverse cell types, fibronectin has significant involvement in various physiological processes, including blood clot formation and the regulation of cell migration. Moreover, its contributions extend to embryonic development and tissue regeneration.[39,76]

A key component of the lamina basalis, laminin, forms a thin, sheet-like structure through a heterotrimer comprising α, β, and γ polypeptide chains. These polypeptides, structurally arranged in a cross-shaped configuration connected by disulfide bonds, contribute to lamini's vital roles in cellular processes such as adhesion, migration, and differentiation.[39,76,77]

7.6. Combined materials for enhanced properties

Beyond various standalone hydrogel and scaffold materials, composite hydrogels offer the unique benefit of combining and enhancing the desirable properties of multiple components. This allows for tailoring physical and mechanical properties such as water retention, flexibility, and optical properties to a greater extent. Notably, chitosan serves as an excellent hydrogel base material due to its biocompatibility, solubility, antimicrobial properties, and biodegradability. However, its standalone mechanical properties often fell short. Tayebi et al.[78] addressed this limitation by incorporating chitosan nanoparticles into

composite membranes alongside chitosan and PCL. The resulting hydrated membranes exhibited increased flexibility and ease of handling, with the 50% nanoparticles and 25% PCL composition demonstrating near transparency comparable to the acellular stroma.

In an effort to improve the properties of chitosan, Ulag et al.[79] incorporated polyvinyl alcohol (PVA), a widely-used synthetic polymer in biomedicine that is known for its utility as a carrier material due to its physio-mechanical properties. Employing an aluminum mold shaped like a cornea, the hydrogel was printed using the extrusion method without the use of a cross-linking material. Although the prepared composite gel was completely transparent, measured transmittance indicated values between 49% and 56%. Importantly, the scaffold perfectly retained the shape of the cornea post-printing. Chen et al.[80] developed a composite hydrogel using type 1 collagen, chitosan, and sodium hyaluronate (NaHA). The study explored the effect of the ratio of individual components on transmittance and water content within the prepared hydrogels. Notably, the hydrogels containing 0.5 and 0.9 (wt)% NaHA exhibited a transmittance of 95%. *In vitro* cytocompatibility studies and *in vivo* rabbit experiments revealed that the hydrogel composed of 20% collagen, 10% chitosan, and 0.5% NaHA proved to be the most efficacious, maintaining transparency even 5 months post-implantation.

8. Stem cells in cornea bioprinting

There are two main categories of stem cells: embryonic stem cells (ESCs) and adult stem cells. In addition, induced pluripotent stem cells (iPSCs) represent another category created through the dedifferentiation of somatic cells.[81] Adult stem cells include mesenchymal stem cells, which can be sourced from diverse tissues such as corneal stroma (CS-MSC), bone marrow (BM-MSC), adipose tissue (AD-MSC), umbilical cord (UC-MSC), placenta (P-MSC), and dental pulp.[82] Mesenchymal stem cells exhibit the ability to differentiate into multiple cell types under *in vitro* conditions, such as adipocytes, chondrocytes, osteocytes, and cardiomyocytes. These cells, characterized by exceptionally high immune tolerance and the capacity to exert an anti-inflammatory effect through their immunomodulation function, find application in allografts. This usage serves to reduce the likelihood of rejection and contributes to expedited wound healing.[83,84]

The use of stem cells in bioprinting stands as a widespread practice in regenerative medicine research, facilitating the production of various implants and tissue models[85] (Figure 3). The 3D printing technique orchestrates the arrangement of cells, multiple factors, and active substances into complex 3D structures. The construction of the scaffolds involves three main steps. Firstly, data pertaining to the organs and tissues slated for printing are collected to facilitate the selection of appropriate models and materials. Secondly, a computer model is generated based on this data, and the corresponding printing code is written. The final step involves the physical construction of the structure through 3D printing.[86]

During the design and material selection phases, an important consideration is ensuring that the bioprinted scaffold effectively provides the appropriate supply of nutrients and oxygen for the diverse cell types encapsulated within the hydrogel.

These bioprinted tissue models serve as valuable tools in elucidating the behavior of immobilized stem cells within different matrix materials. Examining cell functions post-bioprinting yields invaluable insights into the impact of processes during 3D printing on cellular behavior. A comprehensive understanding of these dynamics is essential not only for the success of future bioprinting endeavors but also for their widespread applications.[87]

9. Pre-clinical and clinical studies with bioprinted cornea

Many clinical solutions are currently available to restore the epithelial layer of the cornea, and it is even possible to replace the endothelial layer through endothelial keratoplasty (e.g., Descemet stripping endothelial keratoplasty [DSEK]).[88] However, only three therapies exist for replacing the stroma, which constitutes about 90% of the cornea. These options include the transplantation of the entire cornea from a human donor (penetrating keratoplasty), the partial transplantation of the stroma in a deeper layer (deep anterior lamellar keratoplasty), or the transplantation in a less deep layer (anterior lamellar keratoplasty)[89] (Figure 4). However, these surgical solutions pose a considerable risk of scarring, rejection, and infection.

In recent years, owing to technological advancements, numerous new techniques have been explored in the quest to develop corneal substitutes, among which 3D printing has emerged as a notable contender. The advent of 3D printing introduces possibilities for regenerative medicine and drug testing. Consequently, the growing interest in personalized medicine finds additional avenues, positioning itself as an excellent model for research. The application of this technology in ophthalmic contexts holds promise for advancing clinical practices, enriching medical education, and presenting a cost-effective solution for corneal transplants.[90]

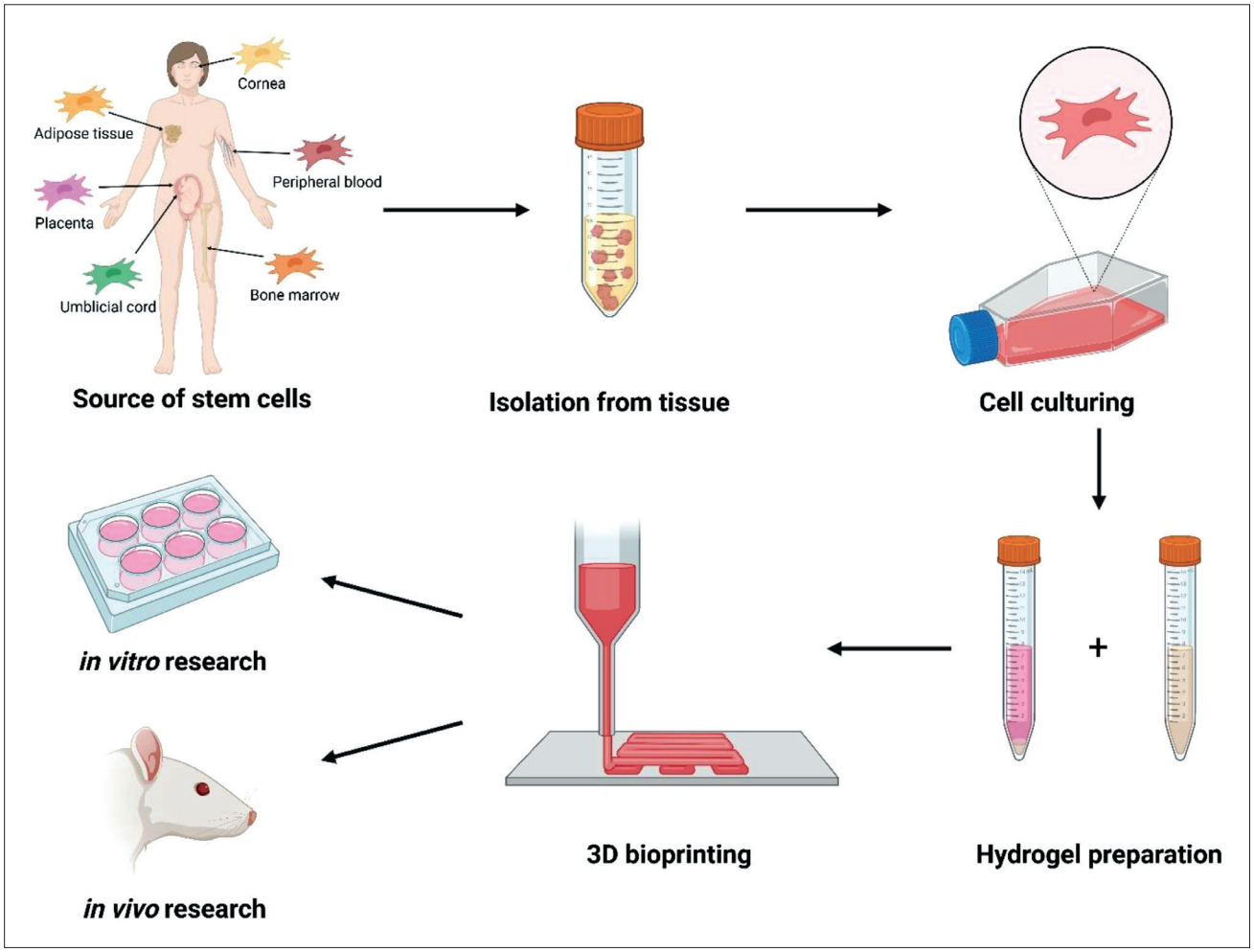

Figure 3. Stem cells in bioprinting: the processes from isolation to research experiments.

In this context, however, a method for the clinical replacement of the stroma with bioprinted artificial cornea substitutes remains elusive. A substantial portion of the current research is situated within the phases of *in vitro* studies and *in vivo* animal experiments, with only a handful of methodologies progressing to the clinical trial phase. Throughout these investigations, the overarching objective is to create a viable cornea capable of replacing either the cornea organ or specific components thereof[91] (Table 2). The integration of 3D bioprinting, coupled with the biotinylation of individual matrix components (such as fibronectin), has gained prominence in research endeavors. These techniques aim to fabricate implants that closely mimic native tissue.[92] Notably, several research groups have achieved success in generating 3D bioprinted constructs, marking a significant stride toward the development of products applicable to corneal reconstruction (Table 3).

For example, Isaacson et al. successfully applied 3D printing to fabricate a stroma with a scaffold composed of biotin-containing sodium alginate and methacrylated type I collagen.[6] In the study, they utilized extrusion-based bioprinting to craft the artificial stroma, embedding human keratocyte cells in an alginate/methacrylated type I collagen hydrogel for their 3D-printed construct.[6] In another study, Sorkio et al. explored the utilization of biotinylated human and recombinant materials for constructing artificial corneas. Employing human embryonic stem cell-derived limbal epithelial stem cells (hESC-LESCs) and human adipose tissue-derived stem cells (hASCs), they utilized laser-assisted bioprinting (LASP) to create an artificial cornea model. The hydrogel matrix for hESC-LESCs comprised human recombinant laminin and type I collagen, while for hASCs, a hydrogel consisting of type 1 collagen, EDTA, thrombin, and plasma was employed. While the results from their artificial cornea

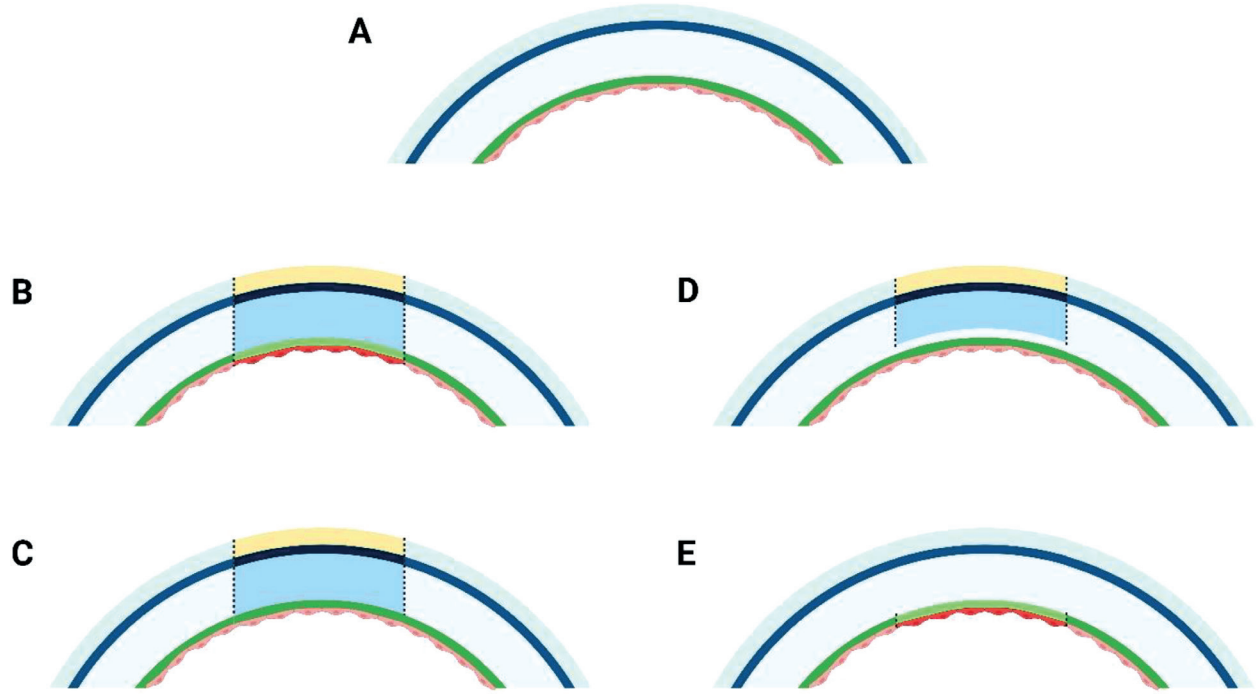

Figure 4. Types of keratoplasty. (A) Structure of healthy cornea, (B) penetrating keratoplasty, (C) deep anterior lamellar keratoplasty, (D) anterior lamellar keratoplasty, and (E) Descemet stripping endothelial keratoplasty.

model are very promising, further functional studies are imperative.[57] Alternatively, Campos et al. utilized inkjet-based bioprinting to create a stroma-like construct from a hydrogel containing type I collagen/agarose and human keratocytes.[93]

Goran et al. employed human BM-MSCs, AD-MSCs, and CS-MSCs in their investigation of corneal replacement. The potential of femtosecond laser-assisted intrastromal keratoplasty using 3D-printed constructs was also explored, using porcine eyes as a model. Alginate-nanocellulose hydrogel, with or without the addition of type 1 collagen, served as the matrix. Individual MSC hydrogels were printed through an extrusion-based method and cultured *in vitro* for 14 days. The viability of cells within the fabricated constructs was assessed through Live/Dead staining, PrestoBlue assay, lactate dehydrogenase (LDH) cytotoxicity test, and immunostaining. Additionally, the physio-mechanical properties of the artificial cornea were examined. Notably, the cells demonstrated resilience during the bioprinting process, and they exhibited the ability to produce ECM and other biomolecules, such as pigment epithelium-derived factor (PEDF). The findings from the study hold significant implications for the advancement of 3D-bioprinted corneas and their potential clinical applications.[94]

10. Future perspectives

Considering the substantial clinical unmet medical need, there exists a high probability that an artificial cornea produced using 3D bioprinting will be among the first to receive approval from regulatory bodies such as the Food and Drug Administration (FDA) or European Medicines Agency (EMA). The technological background is secure, and notably, unlike other tissues and organs, the cornea lacks blood vessels, thereby reducing the engineering and technological challenge. However, achieving optical perfection is paramount for vision improvement, necessitating a tissue that can sustain and regenerate itself over the long term, making cellular components a primary focus. Addressing this focus presents a significant challenge, notably in ensuring sufficient cellular resources for autologous procedures. The scarcity of donor numbers exacerbates this challenge. In regions with inadequate donor availability, 3D printing technology emerges as a viable alternative, even if economically costlier than utilizing a cadaver cornea. Given the trends observed in recent years, it is anticipated that the cost of a 3D tissue-printed cornea will soon align with or even surpass the economic feasibility of traditional alternatives, providing a considerable stimulus to this field. However, it is crucial

Table 2. Tissue engineering approaches for corneal replacement

Corneal replacement	Clinical status	Biopolymer used	Results	References
Full thickness cornea	*In vitro* study and *in vivo* experiment on rabbit	Acellular porcine cornea	Fully transparent; suture retention (5 Newton) near to NPC (6 Newton); tier resistance (3.42 Newton) lower than NPC (5.35 Newton); cell-loaded APC repaired alkali corneal burn on rabbit	[91,104]
Full thickness cornea	Animal model: pig	Collagen-chitosan hydrogels	Fully transparent after transplantation; visible light transmittance (>90%) better than native human cornea (~80%); light scatter lower than the human cornea; 100% suturable	[91,105]
Full thickness cornea	*In vitro* study	Fibrin-agarose hydrogels	Different cells proliferate in hydrogel; epithelial cells formed normal, several-layer epithelia; high expression of vimentin and cytokeratin; similar structure to the native cornea by SEM	[91,106]
Full thickness cornea	Phase I clinical trial: human	Cross-linked recombinant human type III collagen	10 patients (8 male, 2 female); 500 μm thick biosynthetic corneal scaffold; sutures were removed 6.5 weeks after implantation; no pain or discomfort; complete epithelization in about 2.5 months	[91,107]
	4 years of follow-up		Optical clarity was higher (95.1%) than human cornea (>87%); water content 91.5% (cornea: 78%); no significant biodegradation; mechanical strength was lower than the cornea; tensile strength: 0.286 MPa (cornea: 3.81 MPa); modulus: 1.749 MPa (cornea: 3–13 MPa); only 1 rejection was reported	[91,108]
Epithelial tissue	*In vitro* study and Clinical study with 4 human patients	Heat-sensitive cell culture for tissue-engineered oral mucosal epithelial cell sheets	Directly transplantation without suture; re-epithelization within 1 week; transparent during 14-month follow-up; no complication	[91,109]
	Animal model: rabbit	Automated cell culture system for epithelial sheet scaffold preparation	Cell viability 93.6%, growth in multilayer; culturing time: 2 weeks; 10 times medium change with an automated system; fully transparent sheet 1 week after transplantation; no adverse effect	[91,110]
Epithelial tissue	*In vitro* study	Silk foil with pattern	Silk produced by *Bombyx mori* on a patterned silicon layer; HCLEs had better culturing conditions on silk sheets; better vinculin and actin production	[91,111]
Epithelial tissue	*In vitro* study	Rat-tail Type I collagen hydrogel	Cells seeded on cell-loaded hydrogel with crypted surface; cells were able to proliferate; 7 layers produced by cells; different epithelial markers expressed	[91,112]
Epithelial tissue	*In vitro* study	Rat-tail Type I collagen hydrogel	Cell-embedded in compressed collagen hydrogel; morphology similar to the amniotic membrane observed with SEM; the gel was mechanically dense and strong; the peripheral region showed more proliferative capacity than the central; higher cytokeratin and collagen expression than on amniotic membrane	[91,113]
Epithelial tissue	*In vitro* study	Chemically cross-linked collagen hydrogel	80–90% transmittance of dendrimer cross-linked collagen; modulus: 1.4–5.3 MPa (in the range of natural human cornea); cells proliferated in hydrogel and remained viable	[91,114]

(Continued)

Table 2. *(Continued)*

Epithelium and anterior stroma	Animal model: pig	Collagen-copolymer	Robust, suturable scaffold; refractive index: 1.343 (human tier film: 1.336); optically clear; good glucose permeability; intraocular pressure after surgery: 10–16 Hgmm (10–13 Hgmm before surgery)	[91,115]
Epithelium and anterior stroma	Animal model: pig	Cross-linked recombinant human Type I and Type III collagen	Type III collagen gels were more transparent (87–92%) than human cornea (87%) depending on cross-linker concentration; refractive index (1.3451 to 1.3552) lower than human cornea (1.373 to 1.380); fully biocompatible *in vitro*; promoted regeneration of cells and nerves *in vivo*	[91,116]
Stroma	*In vitro* study	Magnetically oriented collagen/proteoglycan hydrogels	Oriented collagen sheets prepared HDFs colonized the gel successfully; collagen concentration (2 mg/mL) was very low compared to native cornea; concentrated scaffold was not transparent but was improved by proteoglycans; cells could align to the orientation of collagen gel	[91,117]
		Magnetically oriented Rat tail type I collagen	Non-cytotoxic; cells could penetrate and remodel scaffold; cells in gel had aligned multilayered structures; cells produce collagen in the matrix but degrade the original scaffold	[91,118]
Stroma	*In vitro* study	Bovine collagen film	Scaffold size showed a 10% decrease during 4 weeks of culture; fibroblast cells flattened after 1 week of culturing on film and formed multilayer; cells penetrated the film itself; surface roughness: 0.987 nm (cornea: 1.197 nm)	[91,119]
Stroma	*In vitro* and *in vivo* study	Gelatin hydrogels	4-week follow-up; *in vivo*, cells expressed more vimentin than *in vitro*; precursor in gel had better results on collagen and vimentin expression; gelatin in gel promotes only ECM production; ECM and vimentin production arise from host species, not from transplanted cells	[91,120]
Stroma	*In vitro* study	Surface patterned silk foil	Silk film was fully transparent; cells aligned to the circular pattern of silk film; proliferation was lower than on TCP; cell density was higher on patterned silk than on flat silk film; high expression of type V collagen and vimentin; films stackable to create 3D structures	[73,91]
Stroma	*In vitro* study	Silk foil with porous and patterned surface	RGD functionalized silk films; cells do not grow to confluent without RGD; cell morphology was more elongated on the patterned surface; cells proliferate more on patterned/porous silk film with RGD RGD content results in stronger expression of ECM; stacked silk films had good transparency	[91,121,122]
Endothelium	*In vitro* and *in vivo* study on cat model	Decellularized amniotic membrane	Cells formed a confluent monolayer on dAC; dAC and cell-loaded dAC were transplanted into cats; inflammation in the cell-loaded group decreased more; cell-loaded dAC group had less or no opaque cornea; cell morphology on dAC was similar to normal culture	[91,123]

(Continued)

Table 2. *(Continued)*

Endothelium	*In vitro* and *in vivo* experiments on rabbit model	Decellularized human Descemet's membrane	HCECs seeded on decellularized membrane; cell morphology and structure were similar to normal cornea; different grafting methods examined in *ex vivo*; transparency and edema healed better in cell-loaded membranes *in vivo*; the preserved sclerocorneal button is not suitable for grafting; younger cell donors are better for cell loading	[91,124]
Endothelium	*In vitro* study	Gelatin hydrogel	The gelatin hydrogel sheet had almost 100% transparency; tensile strength: 2–3.5 MPa (depending on cross-linking time); good diffusion properties for cell carrier application; morphology of HCECs on gelatin sheet similar to *in vivo*	[91,125]
Endothelium	*In vitro* study	Dense collagen hydrogel	Easy handle acellular RAFTs; HCECs formed a monolayer on RAFT; high viability over 14 days; immunostaining showed that cells keep their functional phenotype	[91,126]

Abbreviations: APC: acellular porcine cornea; dAC: decellularized amniotic membrane; ECM: extracellular matrix, HCLEs: human corneal-limbal epithelial cells; HCECs: human corneal endothelial cells; HDF: human dermal fibroblast; NPC: nature porcine cornea; RAFT: Real Architecture For 3D Tissues; RGD: arginyl-glycyl-aspartic acid; SEM: scanning electron microscopy; TCP: tissue culture plastic.

Table 3. Established 3D-bioprinted constructs until 2023

Corneal layer	Bioink	Printing technique	Cell type	**Result**	***In vivo* results**	**Reference**
Epithelial tissue	Methacrylated gelatin bioink; methacrylated gelatin in the form of a dome	Extrusion	Human corneal epithelial cells	Transparent gel	No data	[127,128]
	Sodium alginate, gelatin, and type I collagen	Extrusion	Human corneal epithelial cells	Good transparency, high cell viability after bioprinting, Production of degradation-controllable systems using sodium citrate	No	[50,90]
Stroma	Methacrylate gelatin bioink	Extrusion	Human corneal stromal keratocytes	High mechanical strength, good transparency, low metabolic activity of cells	No	[90,127,129]
	Alginate type I collagen bioink	Extrusion	Human corneal stromal keratocytes	Transparent gel, high cell viability after plucking. Creation of optimal curvature	No	[6,90,127]
	Methacrylated gelatin bioink, reinforced with PEG-PCL fibers	Extrusion	Rat limbal stromal stem cells	Cell viability is good after printing, and the construction is transparent.	No data	[127,130]
	Type I collagen and agarose bioink	Drop-on-demand	Human corneal stromal keratocytes	Transparent gel	No	[90,93,127]
	Matrigel Type I collagen bioink; Laminin-type IV collagen on a carrier base	Laser	Human limbal epithelial cells + adipose-derived stem cells	Highly transparent, high cell viability	No	[57,90,127]

(Continued)

Table 3. *(Continued)*

Stroma	Corneal origin decellularizes ECM bioink	Extrusion	Turbinate-derived mesenchymal stem cells by keratocyte induction	Good transparency, keratocytes activated after transplantation	Rabbit	[90,127,131,132]
	Alginate-nanocellulose-type I collagen hydrogel	Extrusion	Human adipose tissue-, bone marrow-, and corneal stroma-derived mesenchymal stem cells	Good transparency, High viability, ECM production	No	[94]
Endothelium	Gelatin-RGD bioink; amniotic membrane decellularizes ECM	Extrusion	Human corneal endothelial cells	No data	Rabbit	[90,127,133]

Abbreviations: ECM: extracellular matrix; PCL: poly(caprolactone); PEG: poly(ethylene glycol); RGD: arginyl-glycyl-aspartic acid.

to acknowledge the overarching challenge that currently there are no medical devices or Good Manufacturing Practice (GMP)-certified tissue printers capable of mass-producing artificial tissues with the requisite clinical and therapeutic quality. Consequently, only a fraction of the localized and personalized therapeutic needs are presently met. These challenges underscore the need for continued advancements in technology and regulatory frameworks to fully realize the potential of 3D bioprinting in meeting broader clinical demands for corneal regeneration.

11. Conclusion

Studies revealed that 3D bioprinting holds significant promise in advancing regenerative medicine research. Among the myriad printing techniques available, extrusion processes predominate in research owing to their relative cost-effectiveness. However, despite notable progress, tissue printing with 3D bioprinting remains in its nascent phases. While the majority of research focuses on developing stromal substitutes, promising results are also emerging in the reconstruction of the epithelial and endothelial layers.

Materials utilized in bioprinting must meet stringent criteria, encompassing bifunctionality, stability, and the ability to foster appropriate biochemical and physiological interactions with cells. Equally critical is the requirement that these materials do not induce autoimmune reactions in the body. Frequently, 3D-printed cornea analogs are limited to using only one or two cell types and can successfully print in only one or two layers. Consequently, artificial corneas fall short of wholly resembling native tissue. An additional important consideration before clinical application revolves around the challenges of vascularization and the implantation of artificially produced tissue.[95] These issues are pervasive not only in artificial corneas but also in the exploration of other tissue types and organs using 3D printing, such as the skin or liver, underscoring the complexity of the field.[36,39]

To date, stem cells or progenitor cells are the most commonly utilized cell types in research, primarily due to their expansive differentiation potential and wound-healing capabilities. The immunomodulation effect exhibited by these cells further positions them to potentially mitigate inflammation during transplantation.

The future commercialization of 3D-bioprinted tissues necessitates the consideration of additional criteria, including the control of production processes, standardization of protocols, cost-effectiveness, and the logistics of manufactured products. For biological products, challenges related to storage and potential ethical considerations await resolution. However, ongoing research, in parallel with the development of 3D-bioprinted tissues *in vitro* and further investigations into *in vivo* applications, will collectively contribute to the advancement of 3D bioprinting for clinical use.[90]

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Conflict of interest

The authors declare no conflicts of interest.

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