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Review Article

A Review of Bioink Development for 3D Bioprinting: Application in Corneal Tissue Regeneration

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ABSTRACT

This review was to describe the state and progress regarding methods for 3D bioprinting applicable for corneal regeneration; these are identified with developments related to bioink and related bioprinting technologies. This review article has pointed out in detail the mechanism of the 3D printing process; it maintains that bioink ideally consists of biomaterial components deriving both from natural and synthetic ones, cells, and biochemical factors, in such ways which could imitate the properties of a native cornea in several respects, in its mechanic, structural, and ultrastructural features. Biocompatibility, transparency, strength for tension, and corresponding rheology were emphasized as relevant. This review discusses recent modalities of bioprinting, including inkjet, extrusion-based, laser-assisted bioprinting, and stereolithography, for their adequacy in corneal tissue engineering. The integrated use of advanced bioprinting techniques combined with optimized bioinks is a highly promising approach in the fabrication of functional corneal tissues for restored vision and the treatment of ocular disorders.

INTRODUCTION

Corneal blindness is considered the most important global health condition, affecting approximately 10 million people worldwide. The human cornea faces an immense demand due to a shortage of donors: currently, more than 12 million people globally are waiting for corneal transplants, yet only one in seventy patients receives one (Puistola et al., 2024). In Malaysia, corneal blindness is a major contributor to public health challenges. A 2014 national survey found that the leading causes of blindness among Malaysians were untreated cataracts (58.6%), diabetic retinopathy (10.4%), and glaucoma (6.6%) (Chew et al., 2018) Despite Malaysia's advancements in medical technology, corneal blindness remains a significant public health concern, particularly affecting individuals aged 50 and above. Recent estimates show that approximately 160,000 Malaysians in this age group experience visual impairments, with 8% classified as blind (Salowi et al., 2024). This highlights the increasing need for scaffolds to serve as corneal grafts in transplantation procedures. Regenerative medicine and tissue engineering have demonstrated exceptional potential to improve patient treatment outcomes (Z. Li et al., 2023). Efforts to address the shortage have included public awareness campaigns and initiatives to encourage corneal donations. However, the gap between supply and demand remains substantial, necessitating the search for alternative solutions. Regenerative medicine, particularly through the development of bioengineered corneal tissues, offers a promising new approach to addressing the shortage of donor corneas and expanding treatment options (Whitney Stuard et al., 2021).

The cornea is essentially a transparent, dome-shaped tissue that forms the outermost layer of the eye, covering the iris and pupil. Its avascular nature—meaning it lacks blood vessels—is crucial for maintaining transparency and ensuring clear vision. The cornea is composed of three primary layers: the epithelium, stroma, and endothelium, each contributing to its structural integrity and functionality (Jirsova, 2018). Traditionally, corneal blindness has been treated through corneal transplantation, where a damaged cornea is replaced by a

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healthy one from a donor. However, this method presents significant challenges, including a global shortage of donor corneas, leading to long waiting times for patients. Moreover, immune rejection—where the recipient's immune system attacks the transplanted tissue—remains a critical issue that can lead to graft failure and necessitates further surgical procedures. As a result, current efforts in 3D bioprinting for corneal tissue regeneration are focusing on developing bioinks that replicate the native corneal extracellular matrix. This involves combining natural polymers like collagen with synthetic polymers such as polylactic acid to achieve a balance between biocompatibility and mechanical strength. These bioinks are applied through various bioprinting techniques, including extrusion-based, inkjet, and laser-assisted printing, to recreate the cornea's complex structure (Ozbolat & Hospodiuk, 2016). 3D bioprinting is a biofabrication technique that allows for the creation of artificial tissues by printing cells or cell clusters suspended in a natural or synthetic extracellular matrix. By using patient-derived cells, 3D bioprinting can generate custom corneal constructs that replicate the transparency and curvature of native tissue, potentially addressing the donor cornea shortage in transplantation.

This highlights the essential role of bioinks in 3D printing, as each bioprinting method has specific requirements for their formulation (Aghamirsalim et al., 2022; Z. Li et al., 2023). This review explores the latest advancements in bioink development and 3D bioprinting technologies for corneal tissue regeneration, emphasizing their potential to produce functional and transplantable corneal tissues, ultimately improving patient outcomes and restoring vision.

FUNDAMENTALS OF 3D BIOPRINTING

Principles of 3D Bioprinting

Bioprinting is a manufacturing technique used to create functional tissues and organs by arranging biomaterials and bioactive molecules in three dimensions. This method allows for precise control over both acellular and cell-laden constructs, replicating specific configurations and structural properties to guide biological processes (Mirshafiei et al., 2024). The bioprinting process begins with Magnetic resonance imaging (MRI) or Computed tomography (CT) scans to obtain organ and tissue structures modelled using CAD software The functionality of the printed structures depends on the materials used, the bioprinting device, and how the cells interact (Singh et al., 2020).

There are five main approaches to existing 3D bioprinting technologies used for fabricating functional human tissues and organs: (1) extrusion-based, (2) droplet-based, (3) micro-valve bioprinting, (4) laser-induced forward transfer, and (5) stereolithography bioprinting. Each technique has sub-categories based on the process of placing materials and cells.

Extrusion-based bioprinting is one of the most widely used techniques in 3D bioprinting. This method relies on mechanical forces—such as air pressure, a piston, or a screw—to push biomaterial ink through a nozzle, creating structures by layering the bioink (Züger et al., 2023). Pneumatic systems are particularly effective at handling a variety of bioink types and thicknesses by adjusting valve gate time and pressure. Both pneumatic and mechanical systems can manage thick bioinks, but mechanical systems tend to offer greater spatial precision (Boularaoui et al., 2020).

Inkjet-based bioprinting, a form of drop-based bioprinting (DBB), is a contactless technique used to fabricate small 3D structures by layering droplets. This method operates in two modes: continuous inkjet (CIJ) and drop-on-demand (DOD), with DOD being the most commonly applied. DOD methods include piezo and thermal inkjet printing, which use actuators to eject individual droplets of bioink through either thermal or piezoelectric heads (Karvinen & Kellomäki, 2023).

Acoustic-droplet-ejection bioprinting differs from inkjet methods by eliminating stress on the bioink caused by heat, pressure, voltage, or shear forces. Instead, acoustic waves generate droplets from bioink held in an open reservoir. Surface tension at a narrow exit channel keeps the bioink in place. Acoustic droplet bioprinters can utilize single or multiple 2D microfluidic channels (Donderwinkel et al., 2017).

Micro-valve bioprinting another form of drop-on-demand printing, like inkjet and laser-based bioprinting. Unlike continuous bioprinting methods, micro-valve printing enables precise droplet deposition containing cells or biomaterials (Okubo et al., 2019). In this process, droplets are formed by opening and closing a microvalve through pneumatic pressure. The printer typically includes a solenoid coil and a plunger that blocks the nozzle orifice. The droplet size and cell viability depend on several factors, including pneumatic pressure, nozzle design, cell concentration, and bioink composition. Compared to other drop-based bioprinting (DBB) methods, micro-valve bioprinting often produces larger droplets, resulting in lower resolution.

Laser-Induced Forward Transfer (LIFT) bioprinting was originally developed for inorganic materials but has since been adapted for bioprinting applications. This method uses a donor layer coated with an energy-absorbing material, such as gold or titanium, placed above a layer of bioink. When the laser heats a section of the donor layer, a high-pressure bubble forms, propelling the bioink onto the substrate where it crosslinks.

Stereolithography represents an advanced form of bioprinting that utilizes light to crosslink bioinks layer by layer. Unlike LIFT bioprinting, which heats the donor layer, stereolithography employs a laser or digital light projector to photolytically crosslink bioinks, allowing for the formation of successive layers in a single plane.

Components of Bioinks

Bioinks are pivotal in the efficacy of 3D bioprinting, acting as the substance printed layer by layer to form three-dimensional structures. They comprise essential components, each vital in maintaining the printed tissue constructs' viability, functionality, and structural soundness. Key constituents of bioinks encompass biomaterials, cells, and biochemical agents. Bioinks and biomaterial bioinks are sometimes used interchangeably, but bioinks specifically include a cellular component within 3D hydrogels (Groll et al., 2019).

Bioinks are categorized into four different classes based on their roles (Williams et al., 2018):

- Structural bioinks: Support cell functions like adhesion, proliferation, and differentiation, mimicking the natural environment for cell growth and maintaining construct integrity.
- Fugitive or sacrificial bioinks: Temporary materials used to create internal voids or channels within a 3D-printed structure.

- Support bioinks: Typically non-biological, they offer mechanical strength to support softer materials or complex structures during printing.
- Functional bioinks: Provide mechanical, biochemical, and electrical cues post-printing, influencing cellular behavior.

The primary method in tissue engineering through additive manufacturing involves seeding cells onto porous scaffolds after printing with biomaterial inks rather than using cell-laden bioinks. The biomaterials used as the base for bioinks play a crucial role in cell encapsulation and viability, with low-modulus hydrogels being more favourable for cell attachment, viability, and proliferation.

Bioprinting Process Workflow

The fabrication of corneal tissue through extrusion-based bioprinting involves a meticulous workflow that integrates design, material preparation, and optimization of printing parameters to develop functional and biomimetic corneal constructs. The process begins with imaging and 3D modeling of the patient's cornea, creating a digital blueprint that reflects the unique curvature and stromal thickness (Isaacson et al., 2018; Murphy & Atala, 2014). This design guides the layer-by-layer deposition of bioinks containing corneal keratocytes or stromal cells suspended in hydrogels, mimicking the native extracellular matrix (Balters & Reichl, 2023).

Key process parameters are meticulously adjusted to replicate the transparency, mechanical integrity, and smooth surface topography of the natural cornea. The diameter of the extruded strand is critical, as it influences scaffold porosity, mechanical strength, and overall transparency. In corneal bioprinting, achieving a strand diameter between 100–200 µm helps ensure adequate light transmission and structural stability. The applied pressure (typically between 30–70 kPa) and printing speed are optimized to maintain print fidelity and prevent material overflow or under-deposition, which could compromise corneal clarity (Isaacson et al., 2018; Schneider et al., 2012). Printing distances, usually in the range of 0.1–0.3 mm, are adjusted to match corneal layer thickness, allowing for precise vertical stacking of cells and biomaterials (DeBari et al., 2021). Post-printing, constructs undergo UV or ionic crosslinking to stabilize their structure, followed by incubation under controlled conditions to promote cell proliferation and matrix deposition (Zhang et al., 2019). This meticulous workflow enables the generation of corneal grafts with appropriate curvature, biomechanical properties, and optical transparency necessary for clinical application. The process of 3D bioprinting for corneal tissue regeneration is depicted in Fig. 1.

BIOINK DEVELOPMENT STRATEGIES: COMPOSITION AND PROPERTIES FOR CORNEAL TISSUE

Biocompatibility

The development of bioinks is pivotal in corneal tissue engineering, as their composition directly influences cell viability, proliferation, and the structural integrity of bioprinted constructs. Three-dimensional (3D) bioprinting offers a transformative approach for corneal regeneration by enabling the precise fabrication of complex, multi-layered corneal tissue models. The success of this technology hinges on formulating bioinks that not only facilitate accurate printing but also support the physiological environment necessary for corneal cells to thrive. Bioinks designed for corneal applications must exhibit several essential properties, including biocompatibility, transparency, mechanical strength, and appropriate degradation rates. The cornea's optical clarity and structural resilience necessitate bioinks capable of forming transparent constructs that replicate the extracellular matrix (ECM) of the native cornea. The ideal bioink must exhibit high biocompatibility, promoting a suitable host response (Grönroos et al., 2024).

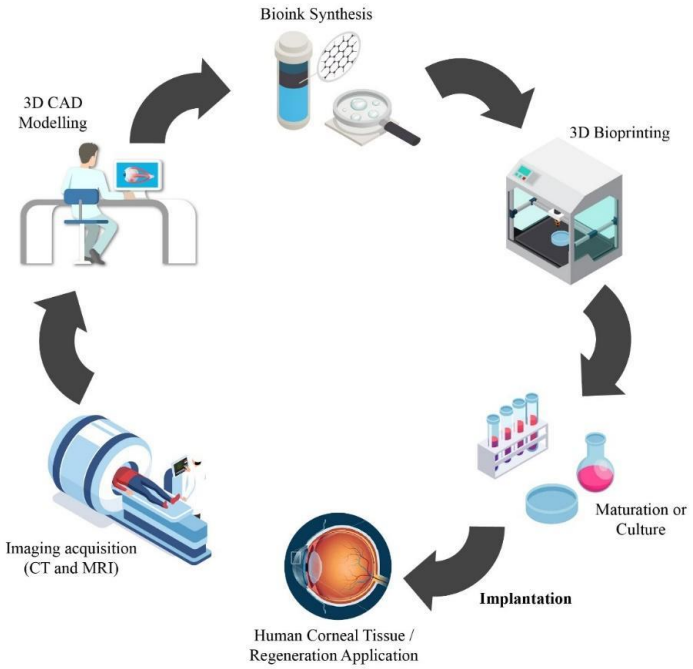

Fig.1 3D Bioprinting Process for Corneal Tissue Regeneration

Natural Polymers

Recent advancements have focused on utilizing natural polymers in bioink formulations due to their inherent biocompatibility and ECM-mimicking properties. For instance, Kim et al. developed a cornea-specific bioink derived from decellularized corneal extracellular matrix (Co-dECM), demonstrating high transparency and improved in vivo safety, which are critical for corneal tissue engineering applications (Kim et al., 2019).

Furthermore, the incorporation of human and recombinant extracellular matrix proteins into bioinks has been explored to enhance the printability and functionality of corneal constructs. Puistola combined these proteins to create a novel bioink composition, which, when used with human pluripotent stem cell-derived corneal epithelial cells, showed promise in developing corneal epithelium-like tissues (Paula Puistola, 2020).

These strategies underscore the importance of tailoring bioink compositions to meet the specific requirements of corneal tissue engineering, thereby advancing the potential for 3D bioprinted corneal grafts that closely mimic native tissue. Such developments hold significant promise in addressing the global shortage of donor corneas by providing scalable and patient-specific alternatives for corneal transplantation.

Synthesize Synthetic Polymers

Synthetic materials from non-biological sources through chemical synthesis offer adaptable mechanical properties like stiffness, toughness, and elasticity in 3D bioprinting. Despite challenges in cell encapsulation, polycaprolactone (PCL) stands out for its biocompatibility and simplicity. These materials enhance structural integrity in extrusion-based bioprinting, where high temperatures and organic solvents are ordinary. They form scaffold frameworks with mechanical solid properties, onto which cell-laden hydrogels are printed or injected to create hybrid scaffolds (Fang et al., 2023). Natural materials like collagen and gelatin offer excellent biocompatibility and biodegradability, supporting cell adhesion and growth, while synthetic materials such as polycaprolactone (PCL) provide the mechanical strength needed to maintain scaffold structure during and after bioprinting. By combining natural polymers with synthetic scaffolds, 3D bioprinting can produce corneal constructs that balance biological function with mechanical integrity. This hybrid approach enhances the potential for developing functional, multi-layered corneal tissue models suitable for transplantation and regenerative medicine applications.

Biodegradability

Optical transparency is crucial for corneal bioinks to ensure unhindered light transmission, vital for vision. Materials like collagen, gelatin, and silk fibroin are transparent and suitable for corneal applications. Bioinks should degrade at a rate matching tissue formation, ensuring gradual replacement by the cells' extracellular matrix. Fabricating organs and biomaterials using 3D printing and bioinks is essential due to their similarity to natural extracellular matrices, promoting cell proliferation. For instance, synthetic hydrogels offer excellent biocompatibility but require a thorough assessment of their degradation rates before application (Donderwinkel et al., 2017).

Gelatin

Gelatin, a biodegradable polypeptide derived from collagen, supports tissues like blood vessels, cartilage, corneas, tendons, ligaments, and dentin. Despite collagen's importance, its biomedical use is limited by low antigenicity from specific polypeptide patterns in its structure. Gelatin dissolves in water, absorbs significant water content, and degrades faster at higher temperatures. It is amphoteric with varying isoelectric values depending on extraction methods (J. Li et al., 2020).

Collagen

Collagen may be found throughout mammalian tissues in many structural and hierarchical arrangements. The most prevalent kind of collagen is type I, primarily found in the cornea, tendons, ligaments, skin, and bone tissue. Type IV collagen is found in basement membranes, whereas type II collagen is typically found in cartilage. Polypeptide chains with different amino acids arranged in glycine X-Y tripeptides make up collagen, where X and Y are usually proline and hydroxyproline.

Mechanical Properties

The cornea needs bioinks with high mechanical strength to endure intraocular pressure while maintaining its form. To achieve optimum operation and integration, the bioink's elastic modulus should be like that of the native cornea. Crosslinking chemicals and procedures, such as UV crosslinking for methacrylated gelatin, can improve the mechanical properties of the bioink. A 3D fiber hydrogel construct was created by Chen et al. using a pneumatic extrusion approach to create GelMA hydrogel scaffolds reinforced with poly(ε-caprolactone)-poly(ethylene glycol) (Kong et al., 2020). Researchers tested fiber spacings from 50 to 500 μm to mimic the natural shape of the corneal stroma. They discovered that changing the fiber spacing influenced attributes like swelling, light transmittance, and mechanical strength. This work demonstrated that fiber design within the hydrogel structure is critical for facilitating the regeneration of injured corneal stroma in both laboratory and live environments.

In addition, Bektas et al. created a 3D bioprinted corneal stroma using GelMA hydrogels (Kilic Bektas & Hasirci, 2020). They used the extrusion approach to produce stromal keratocyte-loaded 3D hydrogels. After three weeks of declining testing, the findings showed improved cell viability (98%), with just an 8% weight loss recorded. After three weeks, the bioprinted hydrogels not only retained acceptable mechanical characteristics but also demonstrated exceptional transparency (more than 80%) in both cell-loaded and cell-free hydrogels.

Rheological Properties

For successful bioprinting, bioinks must exhibit appropriate rheological properties. They should be shear-thinning to allow smooth extrusion through the printer nozzle and quickly solidify to maintain the printed structure. Collagen-based materials can be used for bioprinting, although there are certain limitations. For example, they remain liquid at low temperatures but transform into a fibrous structure at elevated temperatures or neutral pH (H. Li et al., 2018). Numerous strategies have been created to get over these limitations and enhance the printing qualities of collagen-based bioinks. Collagen can be printed onto a sacrificial support gel or combined with synthetic polymers to improve its rheological characteristics (Gelinsky, 2017).

For instance, calcium alginate hydrogels are commonly used in bioprinting. However, their limited rheological properties make it difficult to print well-defined 3D constructs. To overcome this, one strategy is to use slightly precross-linked alginate bioink (Chung et al., 2013). Another approach is to blend alginate with other (bio)polymers, such as methylcellulose (MC) (Schütz et al., 2017). Since MC is not affected by calcium cross-linking, it temporarily increases the viscosity of the bioink during extrusion without changing the final hydrogel's stiffness. This blend also provides shear-thinning properties.

CURRENT BIOPRINTING TECHNIQUES FOR CORNEAL TISSUE ENGINEERING

Bioprinting techniques for corneal tissue engineering aim to create three-dimensional, functional, and biocompatible corneal constructs capable of replacing damaged or diseased corneal tissue. The choice of bioprinting technique is critical, as it affects the resolution, cell viability, and structural integrity of the printed corneal tissues. The most popular bioprinting techniques for corneal tissue engineering include inkjet and extrusion-based techniques, which have pros and disadvantages, detailed below.

Inkjet Bioprinting

Inkjet-based bioprinting utilizes an inkjet printer to deposit bioinks onto a substrate, making it particularly useful for printing corneal epithelial layers essential for artificial corneal substitutes. This technique offers high resolution and precision, which is advantageous for creating complex structures necessary for corneal tissue engineering (Jia et al., 2023). Inkjet bioprinting employs thermal or piezoelectric actuators to produce and deposit tiny bioink droplets onto a substrate. These droplets are precisely controlled and ejected through a nozzle, creating intricate and high-resolution patterns. Mechanical piezoelectric inkjet printers use a charge to compress a piezoelectric crystal. This contraction triggers a movement of the plate, which applies mechanical stress to the nozzle, resulting in droplet extrusion.

Extrusion-Based Bioprinting

Extrusion-based bioprinting involves using a bioprinter that extrudes bioinks through a nozzle to create layers of the desired structure. This method is commonly used for printing corneal stromal equivalents, which is essential for developing artificial corneal substitutes. The bioinks utilized in this method usually consist of hydrogels or composite materials, which offer the mechanical strength and transparency required for the printed structures (Jia et al.,2023).

Laser-Assisted Bioprinting

Droplets of bioink are driven into a substrate by the creation of microbubbles in a bioink layer by laser-assisted bioprinting (LAB). This method offers fine control over the placement and generation of droplets (Zhang et al., 2019). This method uses an extremely strong laser pointed across transparent glass onto an energy-absorbing layer of titanium, gold, or another metal to prevent nozzle clogging (Delaporte & Alloncle, 2016; Duocastella et al., 2007).

Once a laser pulses, it transmits energy to the bioink via the energy-absorbing layer, allowing for exact release. Modifications to Laser-Induced Forward Transfer (LIFT) have been made to protect bioinks from photons and hazardous particles while retaining viability. While Matrix-Assisted Pulsed Laser Evaporation (MAPLE) technology reduces exposure to harmful particles by transmitting kinetic energy through a biopolymer matrix, thicker energy-absorbing layers (100 nm) shield the biomaterial from photo exposure. Laser printers can print cell-dense bioinks at high resolution, despite their high cost and challenging scaling (Delaporte & Alloncle, 2016).

Comparison and Suitability for Corneal Tissue

Every bioprinting method has unique strengths that can be adapted to the specific demands of corneal tissue engineering. For instance, extrusion-based bioprinting is optimal for creating durable stromal layers, whereas inkjet bioprinting is highly effective in producing detailed epithelial and endothelial layers. Laser-assisted bioprinting is distinguished by its precision, making it ideal for accurately replicating intricate structures and preserving cell viability. Different bioprinting techniques offer unique advantages and are chosen based on the specific requirements of the tissue or organ being developed. Below is an overview of three prominent bioprinting methods, highlighting their strengths and limitations:

- Inkjet Bioprinting: Optimal for high-resolution and rapid printing of low viscosity bioinks, though cell viability can be a concern.
- Extrusion-Based Bioprinting: Best for versatile, scalable constructs with good cell viability but limited resolution.
- Laser-Assisted Bioprinting: Provides the highest precision and cell viability, suitable for detailed structures but is complex and costly.

CHALLENGES AND FUTURE PERSPECTIVES

The field of 3D bioprinting has witnessed remarkable advancements over the past decade, driven by innovations in bioink formulations, printing technologies, and tissue engineering approaches. However, despite significant progress, several challenges hinder the development of fully functional organs and vascularized tissues, which are critical for long-term tissue viability and integration. One of the foremost obstacles is the difficulty in replicating the complex hierarchical organization of native tissues, particularly the precise arrangement of multiple cell types within three-dimensional structures (Groll et al., 2016). Although studies have demonstrated the successful bioprinting of small-scale tissues such as skin, cartilage, and corneal models, scaling up to larger, more complex organs remains a considerable challenge.

A major focus of ongoing research is the integration of vascular networks into 3D-printed constructs to facilitate nutrient and oxygen diffusion, which are essential for maintaining cell viability beyond superficial layers. Current advancements have employed co-axial extrusion, sacrificial bioinks, and endothelial progenitor cells (EPCs) to fabricate perfusable vascular channels, with promising results in preclinical models (Wang et al., 2024). Nevertheless, ensuring stable vascularization on a large scale continues to be a bottleneck, as scaffold-free approaches often lead to structural instability and insufficient mechanical strength (Nguyen & Pentoney, 2017).

Despite these challenges, the field has seen notable successes, particularly in the development of organ models and tissue constructs for drug testing and regenerative medicine. Bioprinted liver and kidney tissue models have demonstrated

partial functional capabilities, serving as valuable tools for pharmaceutical research and personalized medicine (Matai et al., 2020). However, translating these advancements into clinically viable, transplantable organs requires further innovation in biofabrication techniques, along with comprehensive studies addressing long-term functionality and host integration.

The future of 3D bioprinting hinges on interdisciplinary collaboration, integrating expertise from materials science, cell biology, and engineering to refine existing technologies and overcome current limitations. By addressing the challenges of vascularization, mechanical strength, and scalability, 3D bioprinting is poised to revolutionize regenerative medicine, offering hope for the eventual fabrication of fully functional, patient-specific organs.

CONCLUSION

Corneal disease is a leading cause of global blindness, with a severe shortage of donated corneas compared to demand (approximately 1:70). Tissue engineering and regenerative medicine offer promising solutions by restoring damaged tissues. The cornea's unique transparency and complex structure pose challenges for conventional scaffold fabrication. 3D bioprinting addresses these challenges by enabling the creation of intricate scaffolds with customizable geometries. Bioinks used in bioprinting must be biocompatible, printable, and capable of controlled degradation and swelling. Hydrogels, recognized for their versatility over the past 50 years, are ideal bioinks for bioprinting corneal scaffolds. They enable the printing of complex geometries and facilitate cell and growth factor incorporation, advancing corneal tissue engineering capabilities.

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