Review

Recent Approaches of Collagen Biomatrix Modification as a Corneal Biomatrix and its Cellular Interaction.

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Abstract: Since the past few decades, numerous modifications and innovations have been done to design the optimal corneal biomatrix for corneal epithelial cells (CECs) or limbal epithelial stem cells (LESCs) carriers. However, researchers have yet to discover the ideal optimization strategies in the development of corneal biomatrix design and its effects on cultured CECs or LESCs. This review further discusses and summarizes recent optimization strategies to develop an ideal collagen biomatrix and its interaction with CECs and LESCs. Using PRISMA guidelines, the articles published from June 2012 to June 2022 were systematically searched using Web of Science (WoS), Scopus, PubMed, Wiley, and EBSCOhost databases. The literature search identified 444 potential relevant published articles, with 29 relevant articles selected based on the inclusion and exclusion criteria after the screening and appraising processes. The current paper highlights the physicochemical and biocompatibility (in vitro and in vivo) characterization methods, which were inconsistent throughout the different studies. Despite the variability in the methodology approach, the reviewer postulated that the modification of the collagen biomatrix improves its mechanical and biocompatibility properties toward CECs and LESCs. All findings were discussed in this review; thus, it provides a general view of up-to-date trends in this field.

Keywords: collagen biomatrix; optimization; modification; corneal epithelial cells; limbal epithelial stem cells; biocompatibility

1. Introduction

The cornea is a transparent window to the eyes that maintains the refractive properties of light transmission to the retina [1-3]. The cornea is a multilayered component and is enclosed by a non-keratinized stratified epithelium layer, continuously providing a smooth ocular surface [1-4], as shown in Figure 1. The smoothness and integrity of corneal epithelium are essential for transparency, providing adequate light refraction, and homeostasis [1,5]. These important roles depend on a balanced corneal epithelial cells (CECs) turnover, as the new CECs originated from the limbal epithelial stem cells (LESCs), which were located at the periphery of the cornea and replaced the older CECs [1,2,6]. LESCs maintain the self-renewal of CECs by continuous and slow epithelization processes which are involved in the proliferation and differentiation of LESCs into CECs, followed by centripetal migration of CECs towards the central region of the cornea [1,2,6].

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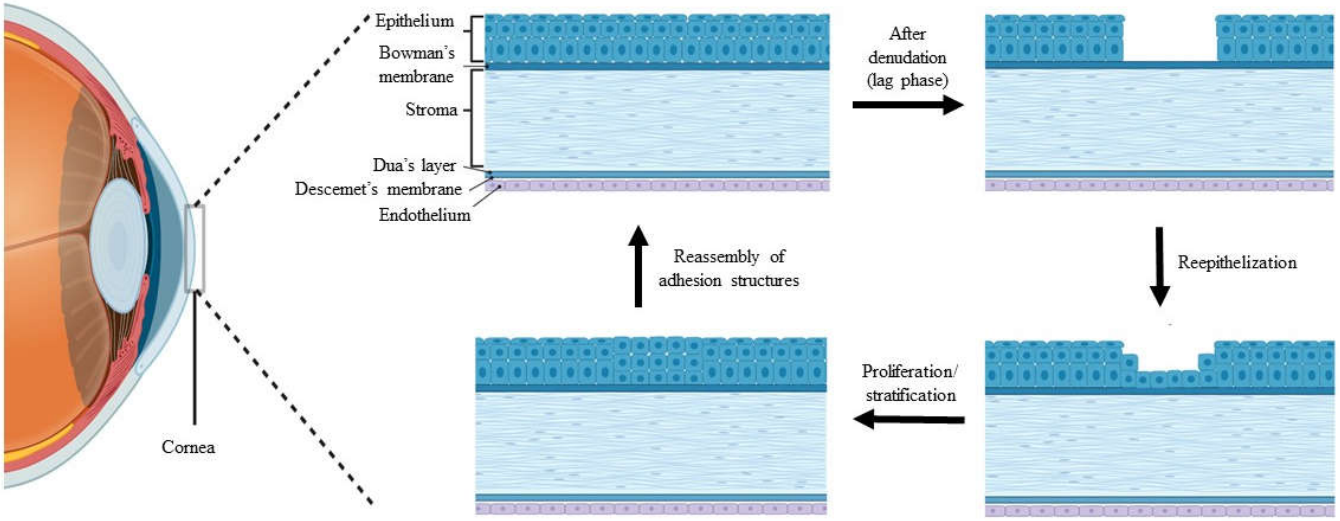

Figure 1. Stages of the wound healing process after corneal epithelial injury, created by BioRender.

The cornea is also responsible for the frontal barrier that allows the diffusion of oxygen and essential nutrient from the tear film, against invading pathogens, debris, chemical agents, and trauma [7-10]. Thus, the corneal epithelium is vulnerable to external injury, which compromises its first line defense against corneal damage and can be overcome by a rapid healing process through re-epithelialization activity [7,9-14]. The stages of the wound healing process following corneal epithelial injury were illustrated in Figure 1.

However, various potential problems such as delay in cell migration, epithelial hypertrophy and recurrent corneal erosion are prone to occur during the epithelialization process, which eventually leads to the scarring of the stroma, reducing the vision quality and corneal damage [11,15-17]. It may be due to the dysfunction, destruction, or deficiency of LESCs, which is also known as limbal stem cell deficiency (LSCD). Recent studies also reported that LSCD can induce unstable production of corneal epithelium, followed by corneal ulceration, conjunctival invasion into the cornea and neovascularization on the corneal surface that leads to inflammation and chronic pain, thus, ultimately to vision loss [18,19].

The only recognized treatment strategy for corneal blindness or vision loss is through corneal transplant but is limited due to the corneal shortage [20,21]. Another alternative solution was by tissue engineering approach which replaces the damaged cornea with a biomaterial-based biomatrix combined with cells to replicate the corneal tissue [22-25]. Many corneal biomatrices have been developed to replace all or only a part of the cornea depending on the patient's requirement [26]. The development of cornea biodegradable biomatrix (specific for the epithelial layer damage treatment) was focusing on regenerating the damaged epithelial layer either from transplanted CECs or differentiated from transplanted LESCs. Thus, the LESCs biomatrix is important in supporting the expansion, stratification and maintaining LESCs functions [27].

Biological biomatrices such as the human amniotic membrane (HAM) [28,29], fibrin [30-32] and feeder layers such as 3T3 fibroblast [33] are gold standard treatments and widely used as cell carriers as they promote cell expansion. However, as natural carriers, their potential drawbacks such as the tendency to carry infection, not being optically transparent and inadequate structural compaction and rigidity as a corneal biomatrix were reported [34,35]. The high economical cost of these natural biomatrix needs to be overcome by discovering new biomaterials for CECs and LESCs [36].

Many studies were focusing on biomaterial for CECs and LESCs including collagen, silk fibroin [37], gelatin [38], chitosan [39], alginate [40], hyaluronic acid [41] and also decellularized cornea [27]. Collagen is one of the well-known biomaterials for corneal biomatrix which mimics native corneal structure [4,42-44]. Collagen makes up about 70%

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of the dry-wet of the cornea and plays an important role in supporting CECs, LESCs and corneal fibroblast cell growth [4,6,9,10,23]. Three forms of collagen biomatrix are normally used in tissue engineering research, such as collagen sponge [45-48], collagen hydrogel [49-51] and collagen film [52-54].

The important aspect that needs to be considered during the collagen biomatrix development is the collagen biomatrix interaction with CECs and LESCs which is influenced by the physicochemical properties of the biomatrix [26,55]. The source of biomaterial, mechanical strength, biodegradation rate, optical characteristics and biocompatibility properties need to be tailored during the development of corneal biomatrix [55-57]. Considering the aforementioned characterization of the biomatrix, modification or optimization process is crucial for achieving optimal biomatrix designs [26]. Modifications of collagen biomatrix such as crosslinking, physical modification, incorporation of other biomolecules or cells into collagen biomatrix or incorporation of the collagen into another biomatrix, give different effects in terms of interaction between the biomatrix and cells. Since the main outcome of the produced corneal biomatrix is a prolonged effect after implantation, thus it is important to ensure its ability to regenerate into corneal native tissue.

The mechanical strength of the biomatrix is an important aspect that needs to be optimized as it must resist the high tension during implant, and also in vivo dynamic environments such as physiologic intraocular pressure and constant eyelid motion [58], which is closely related to the biocompatibility towards LESCs and CECs [59-61]. Modification through the cross-linking process will produce a mechanically strong cross-linked biomatrix [62], which results in multidimensional polymeric chain extension of the biomatrix. Unfortunately, the crosslinker could reduce biomatrix's transparency and cause cell toxicity that will overshadow their cross-linking potential [51].

Recently, several studies have reported the improvement of cell–biomatrix interactions via surface alterations of the biomatrix. The biomatrix surface with moderate hydrophilicity, irregular structure and the cationic charge was likely to be attached and grown by the LESCs and CECs [63,64]. Some studies have exploited the biocompatibility properties of collagen by incorporating or coating the collagen on another biomatrix to increase the biomatrix biocompatibility [40,65]. All modified biomatrix with their desirable properties for corneal engineering are shown in Figure 2.

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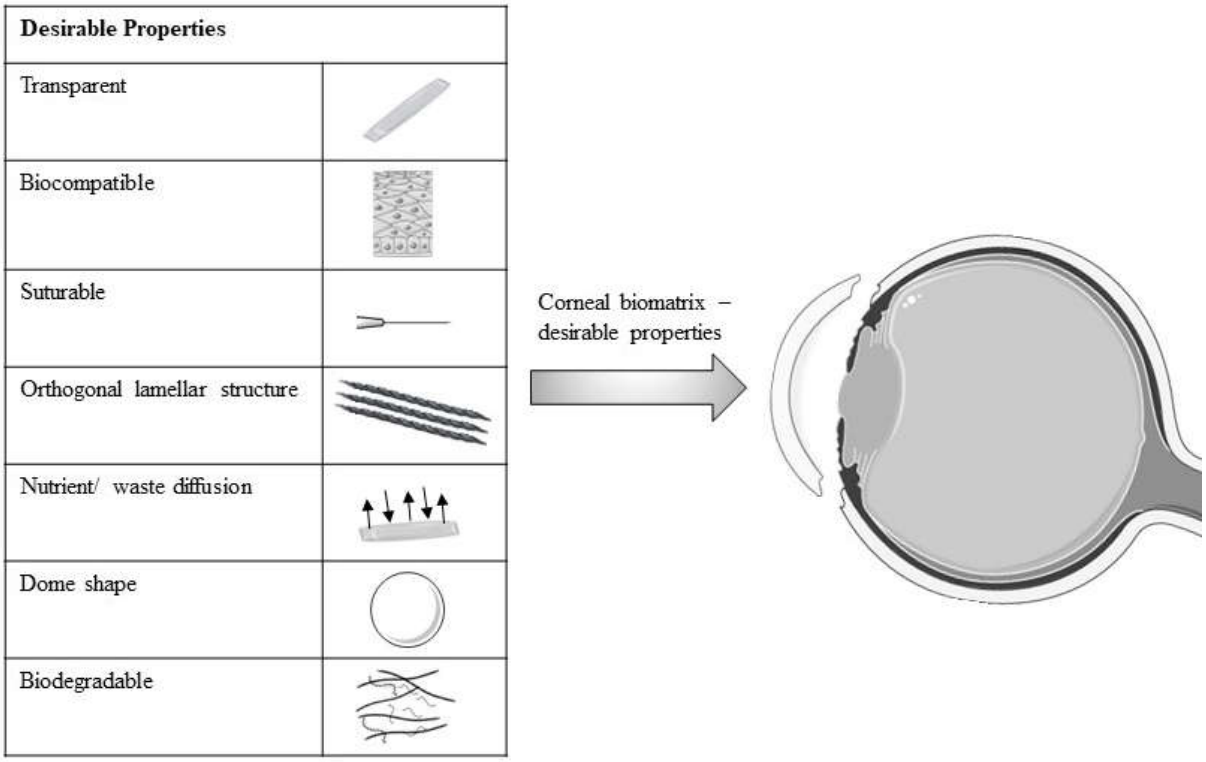

Figure 2. Schematic diagram of desirable properties of modified biomatrix for corneal engineering. Figure 2 was partly generated using Servier Medical Art, provided by Servier, licensed under a Creative Commons Attribution 3.0 unported license.

A literature search was conducted to identify the recent modifications that have been performed on collagen biomatrix within recent 10 years. This systematic review aims to discover the modification strategies to optimize collagen biomatrix for CECs and LESCs carriers in the treatment of LSCD. This review will also provide insight to further explore a better and safe modification to the collagen biomatrix for CECs and LESCs in future studies.

2. Materials and Methods

2.1. Search Strategy

This systematic review was performed to identify the relevant studies of recent modifications on collagen biomatrix and the efficacy of these modifications on the physicochemical and biocompatibility of the biomatrix toward CECs or LESCs, both in vitro and in vivo. Briefly, it was constructed based on PRISMA guidelines to ensure the quality and transparency of this review [66]. Five separate databases including Scopus (Elsevier, Amsterdam, NH, The Netherlands), Web of Science (WoS) (Clarivate Analytics, Philadelphia, PA, USA), PubMed (National Center for Biotechnology Information, NCBI, Bethesda, MD, USA), Wiley (John Wiley and Sons, Inc, New Jersey, USA) and EBSCOhost (EBSCO Information Services, Massachusetts, USA) were systematically searched to discover the studies related to the biomaterial or bioengineering, especially in collagen biomatrix as a biomatrix of CECs and LESCs in corneal therapy.

These databases have screened all related published journal articles. This article search was guided by the focus question formulated using the PICO strategy whereby Population (P) was in vitro and in vivo study on collagen biomatrix for CECs and LESCs transplantation; Intervention (I) was different modification strategies on the collagen biomatrix; Comparison (C) with other biomaterials was not applicable; and Outcome (O) was physicochemical and cellular characteristics of the biomaterials studied towards CECs or LESCs (in vitro and in vivo).

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The combination of three sets of keywords (corneal epithelial cells OR CECs OR corneal epithelium OR limbal epithelium OR limbal epithelial cells OR limbal epithelial stem cells OR LESCs) AND (limbal deficiency OR limbal stem cell deficiency OR LSCD OR corneal limbal stem cell deficiency OR corneal epithelial injury) AND (collagen OR collagen biomatrix OR collagen bio scaffold OR collagen scaffold) were used during the searching process of the relevant articles published.

2.2. Criteria of Selection

Only English articles were included due to limited resources for translation. Studies that provide free full-text articles published within 10 years, with a limit ranging from 2012 to 2022 were considered. Titles and abstracts that have fulfilled the topic requirements were systematically screened. The articles related to humans were included as the relevant basis for the scope of this review. All research articles related to collagen as a component or a part of the biomatrix for CECs and LESCs were also included. All secondary literature and any original article that involved clinical studies were removed. Any studies that focused on the other fields except for physicochemical properties and biocompatibility (in vitro and in vivo) were also omitted.

2.3. Management of Data Extraction Table

Articles were screened and underwent three phases to be selected as part of this systematic review. The first phase involved article title screening that meets the requirement of the topic of interest. The title that did not match the inclusion criteria was removed. The next phase involved the elimination of unrelated articles based on inclusion criteria followed by the removal of all identical articles. The last steps involved omitting the articles that did not meet the inclusion criteria after full-text reading by two independent reviewers. Two reviewers were independently assessing the specified inclusion and exclusion criteria of selected published articles to guarantee neutrality in the selection of the final articles.

This was accompanied by a discussion among the reviewers to get a consensus on the discrepancies that emerged during the assessment of the articles. Extracted information as outlined in the data extraction table for in vitro study are as follows (1) Author and year published (2) Type of biomatrix (3) Modification techniques (4) Type of cells used (5) Test and result (physicochemical properties) (6) Test and result (in vitro biocompatibility) and (7) Conclusion. The data extraction table for in vivo study was outlined as follows (1) Author and year published (2) Type of biomatrix (3) Modification techniques (4) Animal model/injury (5) Test and result (in vivo) and (6) Conclusion. This review is not suitable to be published in PROSPERO as it is included in vitro studies. For quality assessment, this review was carried out systematically, in which employing the critical appraisal instrument [67]. Each item in the appraisal instrument for each selected study was also discussed by independent reviewers.

3. Results

3.1 Searching Result

The combination of three sets of keywords during the searching process successfully identified 444 articles as potentially relevant. A total of 409 articles that have not fulfilled the inclusion criteria and identical were removed during the title and abstract screening process. From the remaining 35 articles, the reviewers omitted six more articles that did not meet the requirement of the inclusion criteria. After the selection process, 29 articles were included in the data extraction table, of which 10 articles were acquired from WOS, seven articles from Scopus, 10 articles from PubMed, one article from Wiley and one article from EBSCOhost, respectively. The article's screening and selection process were summarized in Figure 3.

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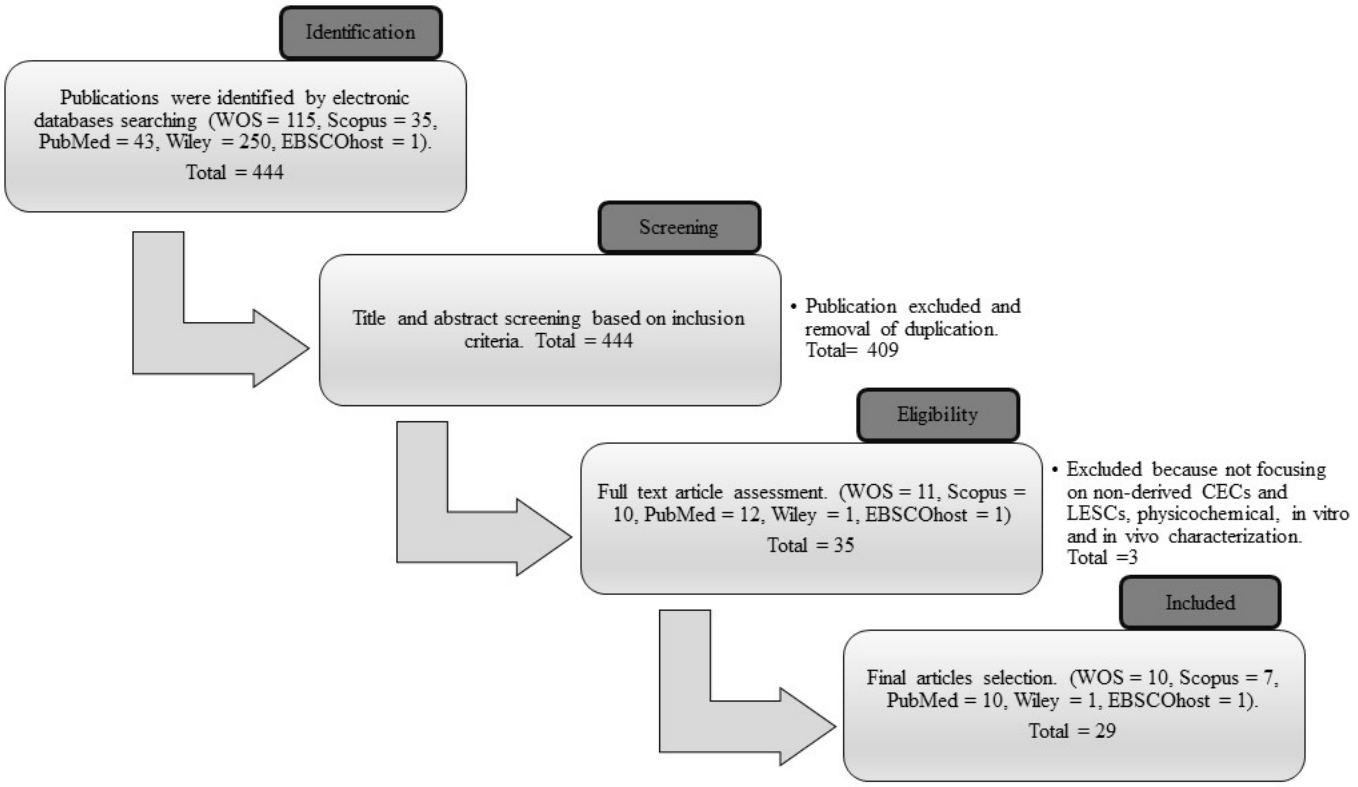

Figure 3. The process flow of the selection of the final articles from Web of Science (WOS), Scopus, PubMed, Wiley, and EBSCOhost databases.

3.2. Study Characteristics

In this review, the search was successfully finalized the studies related to the modification of collagen biomatrix and its effect on CECs or LESCs. To summarize the selected articles from 2012 to 2022, eight studies aimed to develop a new formulation for collagen biomaterials [68-75], whereas 12 studies aimed in improving or characterizing current collagen biomaterials, including the improvement of fabrication methods to produce better collagen biomatrix [27,35,51,58-61,76-80].

Four studies investigated the interaction of corneal cells on collagen biomaterials with other biological molecules and cells [81-84]. Meanwhile, four studies exploited the biological benefit function of collagen by combination of different biomatrix to improve its function as a cell carrier [40,85-87]. From these articles, several types of cells were used such as CECs, LESCs alone or co-culture of both cells and the combination of one of these cells with stromal cells (corneal stromal stem cell or limbal fibroblast). Most of these cells are primarily acquired from the cornea of the human cadaveric donor, rabbit, porcine, mini pig (Gottingen), bovine and mouse. It was either freshly obtained from the corneal rim, immortalized, primary or cell line.

All these studies reported various modifications on the collagen biomatrix either by extraction of collagen from new sources, physical modification, crosslinkers, or incorporation with other cells and biomolecules. Some researchers also incorporate collagen into other biomatrix. The new source of collagen biomatrix that has been obtained is through the decellularization of the bovine eyeballs, porcine conjunctiva and fish scale, production of the synthetic collagen peptide or modification of collagen's methacrylate group. The physical modification that has been done was compression by using the different compressors, embedding the decellularized corneal lenticule (dCL) with compressed collagen, surface patterning or vitrification process.

Since last 10 years, some researchers used the following crosslinker: polyethene glycol (PEG), N-hydroxysuccinimide (NHS), 1–Ethyl–3-(3-dimethylaminopropyl) carbodiimide (EDC), 1–Ethyl–3-(3-dimethylaminopropyl) carbodiimide-N-

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hydroxysuccinimide (EDC-NHS), 4-(4,6-dimethoxy-1,3,5-triazin-2-yl)-4-methyl-morpholinium chloride (DMTMM), methacyloyloxyethyl phosphorylcholine (MPC) or hybrid crosslinker. Few researchers used other components such as fibronectin (FN), laminin, stromal cells, ascorbic acid, or stem cell factor (SCF)/ C- kit into the collagen biomatrix. Some studies also incorporated the collagen into another biomatrix such as incorporation of collagen into poly(lactide-co-glycolide) (PLGA) biomatrix, dopamine hydrazone biomatrix-crosslinked hyaluronic acid (HA-DOPA), poly-L/DL-lactic acid (PLA) films or silk film.

Ten studies were conducted both in vitro and in vivo, two articles only involved in vivo study, whereas the rest of the articles only involved in vitro study. Ultimately, the selected articles have shown optimization of collagen biomatrix and its effect on the physicochemical and biocompatibility of biomatrix towards the CECs and LESCs which affect its cellular biocompatibility differently. All articles were summarized in Table 1 for in vitro study and Table 2 for in vivo study.

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Table 1. Description of selected in vitro studies on the modified collagen biomatrix.

Authors	Type of Biomatrix	Modification Techniques	Type of Cell	Test and Result (Physicochemical Properties)	Test and Result (In Vitro Biocompatibility)	Conclusion
Krishnan et al., 2012 [68]	FSC-PE	Decalcification and demineralization of the biomatrix, followed by coated with PE.	Limbal tissue	• Tensile strength: 5 times higher than HAM. • Tearing strength: double the value of HAM (3.8 N). • Optical clarity: 73%. • Collagenase assay: 13.25% content of hydroxyproline (Hyp) and 96.06% of collagen content. • Microbial resistance: two-fold resistance compared to HAM. ($1.9 \pm 3.7 \times 10^{-3}$ cfu/mL). • Fourier-transform infrared spectroscopy (FTIR): amide 1, 2, 3. • Scanning electron microscope (SEM): fibrous and porous structure.	• Microscopy: monolayer covering the entire FSC-PE surface on days 15. • Growth kinetic: FSC-PE (425 mm^2) higher compared to HAM (300mm^2) covering on day 10. • Reverse transcription polymerase chain reaction (RT PCR): High keratin K3/12, but low p63 & ABCG2.	FSC-PE has good mechanical properties and supports the differentiation of LESCs and the proliferation of differentiated CECs.
Zhao et al., 2014 [70]	aCM	Xenogeneic decellularization of the conjunctiva with 0.1% sodium dodecyl-sulphate (SDS).	iCECs and primary rabbit corneal epithelial cells (rCECs).	• Optical transmittance: transparent (87.86 ± 3.9%). • Transmission electron microscopy (TEM): collagen fibril tightly arranged and regular with no cellular debris in the aCM. • Fourier-domain-optical coherence tomography (OCT): thicker compared to dCM (52.66 ± 4.8 mm versus 35.46 ± 3.7 mm). • Stretch stress test: high tensile strength (7.96 ± 0.6 gf versus 5.86 ± 0.5 gf) and tensile elastic modulus compared to the dAM. (23.66 ± 3.4 MPa and 14.36 ± 2.1 MPa). • Biodegradation: Start degraded after 20 min, completely 40-60 min).	• Tetrazolium salt (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay: high cell viability. • Trypan blue alizarin red staining: CECs grew confluent. • Immunohistochemistry (IHC): K3/12. • Haematoxylin and Eosin stain (H&E): formation of 2-3 CECs layer. • TEM: tight junction between the cells.	aCM possesses favourable physical properties and supports multi-layered CECs growth.

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Sánchez-Porras et al., 2021 [75]	Porcine limbus	Decellularization (4 methods) and recellularization.	SIRC and hADSCs	• Transparency: Highly transparent. • Picrosirius red and alcian blue staining: high intensity staining of SIRC compared to the hADSCs.	• IHC: p63, pan-cytokeratin, crystalline Z (post days 7 – SIRC and days 14 to 21 - hADSCs), laminin and collagen IV (post 14 - 21 days of both).	0.1 % SDS is the best way to decellularized limbal. This biomatrix able to regenerate the stratified epithelium.
Naresh et al., 2021 [88]	Decellularized human corneal tissue remnants (DHC)	Decellularization (1% sodium deoxycholate, DNAse I, and 4% dextran), followed by recellularization.	limbal epithelial progenitor cells (LEPC), limbal mesenchymal stromal cells (LMSC), and limbal melanocytes (LM)	• Anterior segment-OCT: The mean thickness of DHC with 4% dextran (679.2 ± 59.7 μm) and 6% dextran (649.4 ± 76.9 μm) was best comparable to without dextran (711.2 ± 86.6 μm). • H&E: Dextran reduces the corneal thickness but no differences in the remnant of cellular material. • SEM: - No epithelial cell detectable on all DHC, - no significant gross change of the collagen fibres on all DHC, - 4% dextran DHC showed reduction of the collagen bundles distance. • Optical properties: Dextran improve the transparency. • ECM component: Presence of glycoprotein, glycosaminoglycan, agrin, heparan sulphate proteoglycan, collagen III, IV, XVIII, FN, junctional adhesion molecule C, tenascin C, vitronectin, laminin. • Mechanical properties: No major difference in the elastic moduli.	• H&E: - Monolayer of LEPC on DHC post 1 week and stratified epithelium layers observed post 3 weeks of cultivation. - Injected LMSCs spread to the posterior side of DHC post 1 week and migrated to the anterior part after 3 weeks. - DHC-stratified LEPC/LM produced after 3 weeks of cultivation. • IHC: - pan-cytokeratin (CK), Epithelial-cadherin, Melan-A+, Ki-67+ and CK3(epithelial layer), - CK15 and p63 (basal layer), - Vimentin+ (stromal layer close to pan CK+).	DHC (with 4% dextran) complete the removal of cellular component, maintain the tissue architecture, ECM composition and biocompatible with LEPCs and LMs.
Zhou et	acellular	Decellulari-	rCECs	• Light transmittance: High	• MTT: High proliferation rate of	APCS-gel is suitable

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al., 2021 [73]	porcine corneal stroma hydrogels (APCS-gel)	zation	and rabbit corneal stromal cells (rCSCs)	• Nutrition rate: fast • Proteomic: 106 proteins, collagen I, IV, V, fibroblast growth factor (FGF), bone morphogenetic protein (BMP). • SEM: High porosity • Permeability: Highly permeable	rCECs and rCSCs • Live and dead assay: Highly viable • Immunofluorescence staining: - 3-4 layers of rCECs formed on APCS-gel (post 7 days), - The presence of K12-, p63+, ABCG2+, Ki67+ detected. • Corneal wound healing assays: Rapid re-epithelization 72 ± 3% (24 h), 90 ± 3% (30 h)	for CECs reconstruction by maintaining stemness and enhanced proliferation of the ocular surface.
Baratta et al., 2021 [72]	CMP	Damaged collagen type 1 coated petri dish treated with CMP.	N/A	• Differential interference contrast optic: CMP promotes collagen alignment in the parallel orientation of previously highly disoriented collagen strand coated plate damaged by collagenase.	N/A	CMP re-aligns the damaged collagen by enzymatic digestion.
Jones et al., 2012 [59]	RTCI - gels	Compression by nylon mesh (50 µm mesh size, 134 g) for 5 min at room temperature	hLESCs	• SEM: Compressed hydrogel improves surface topography and creates a similar surface to the bovine cornea. • Storage modulus: Compressed hydrogel has high values (1500 Pa) compared to uncompressed hydrogel (30 Pa).	• IHC and western blotting: Compressed hydrogel has high CK3 (94%), and ZO1 but low CK14 compared to uncompressed hydrogels. • MTT: Compressed hydrogel > uncompressed hydrogel at week 2.	The compression improved the mechanical strength, surface topography and capacity of the RTC-gels to support the attachment, differentiation of LESCs and viability of differentiated CECs.
Gouveia et al., 2019 [60]	RAFT TE	Treated with: collagenase I (RAFT TE-CI), Phosphate-buffered saline (PBS) (RAFT TE – PBS) or none	hLESCs	• Mechanical strength: limbus-like compliance (15 kPa), stiffer (65 kPa).	• Migration assay: RAFT TE-C1 (20 ± 2 µm/h^{-1}) migrated slower compared to RAFT TE–NT (26 ± 2 µm/h^{-1}). • Live and dead assay: Viability RAFT TE-CI > RAFT TE-NT • Phase contrast microscope: RAFT TE-PBS & RAFT TE-NT maintained a single monolayer with round, flatter, stretched morphology and de-	RAFT TE-CI supports LESCs compared to RAFT TE-PBS and RAFT TE-NT. RAFT TE-PBS and RAFT TE-NT (stiffer hydrogel supports the differentiation via mechanotransduction factor YAP and BMP4.

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		(RAFT TE-NT). Laminin surface coating.			tach from the basal sheet compared to RAFT TE-CI • IHC: RAFT TE-CI high levels of Np63, ABCG2, CK15, Ki67 and β-Catenin and lower expression of CK3, BMP4 and YAP compared to RAFT TE-PBS and RAFT TE-NT on days 15.
Massie et al., 2015 [61]	RAFT TE	Different concentration and volume of collagen used.	hLESCs	• The 'optimal' RAFT TE is 0.6 ml of 3 mg/ml col with transparent, thin (OCT: 52.5 ± 8.9 um) but handleable (break force: 0.167 ± 0.055 N). • Degradation rates: uniform and comparable to HAM.	• Optimal RAFT TE (0.6 ml of 3 mg/ml): • Phase contrast microscope: 8.0 ± 3.0 days to achieve confluence comparable rates to HAM (10.5 ± 0.5 days). • Morphology: Small, tightly packed, scant cytoplasm with cobblestone shape. • IHC: high p63a. Superficial layer: high K3/K12	Optimal RAFT TE (0.6 ml of 3 mg/ml collagen) has suitable physical properties and supports hLESCs growth.
Kureshi et al., 2014 [78]	RAFT TE	Incorporated with hLF and DMEM. 1mm wide strip defect was created on the epithelial surface on the construct (using algerbrush II corneal rust ring removal) and continue with analy sis.	LESCs	• Light microscope: complete re-epithelization varying 7-2 days. • H&E: multi-layered cells • IHC: high p63a in the wound edge (continue fell to 6.1 ± 2.8% after 50% wound closure but increase to 33.4 ± 11.8% after 100% wound closure).	• Light microscope: - achieve confluence by days 13, - basal layer-small, round, cobblestone, high nuclear to cytoplasm ratio. • MTT: High proliferative capacity • H&E: - multilayer cells (days 19). - Small basal cells with round-shaped, 'cobblestone' morphology (high nucleus-to-cytoplasm ratio) adjacent to the collagen stroma with flattened squamous cells lying on the apical surface. • IHC: high p63α (65 ± 10%) at days 19.	RAFT TE incorporated with hLF support the cultivation LESCs but is poorly differentiated and promotes wound closure.

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Hong et al., 2018 [27]	COLLEN	dCL embedded by compressed collagen	hCECs, rabbit LESCs	• Suture retention test: 0.56 ± 0.12 N • Biodegradation rate: improved biodegradation up to 16 h (in early-stage, rapidly degraded within 4 h, then the rate become slow until 16 h to degrade completely).	• MTT: 2.4 times higher than dCL alone • H&E: Well-spread hCECs attached and formation of non-keratinizing multilayered epithelium, the stratified squamous of LESCs attached on the COLLEN. • IHC: high CK3, 4, 5, 12, 15 and p63α	COLLEN has suturable mechanical properties, resistant to degradation, and supports LESCs and CECs growth.
Jangamreddy et al., 2018 [69]	CLP-PEG-EDC-NHS & RHCIII-MPC (control)	Crosslinked to MPC & EDC-NHS. Conjugated to PEG	ihCECs	• Light transmission: > 90%. • TEM: comprised very fine fibril. • Biodegradation rate: resist collagenase even after 30 months of storage. • Flexure test: RHCIII-MPC has higher tensile strength, but CLP-PEG is more elastic and 4 times the percentage of elongation. • Water content: > 90% water. • FTIR: Amides A, B, I, II.	• Live and dead assay: minimal cell death (post 48 h culture on hydrogel). • PrestoBlue cell viability reagent: no significant difference to the RHCIII-MPC.	CLP-PEG-EDC-NHS are functionally equivalent to control, RHCIII-MPC biomatrix and biocompatible to the corneal cells.
Fernandes-Cunha et al., 2020 [51]	Bovine collagen type 1 hydrogels cross-linked to PEG - NHS (BCI-gels-PEG-NHS)	Crosslinked to NHS, Conjugated to PEG (4 or 8 arms & 4%, 8% or 16% concentration of PEG	iCECs and corneal stromal stem cells (CSSCs)	• Storage modulus: The increase of the PEG's arms and concentration, increase the storage modulus except for 16%. • Transparency: All hydrogel is transparent except 16% PEG (8 arms). • Degradation rate (collagenase): non-crosslinked hydrogel degrades 50% after 8 h. Degradation does not depend on the PEG's arms and concentration. After 4 h (0% biomatrix degrade), 8 h (20% degrade), 12 h (30-40% degrade). • EGF released: It does not depend on the PEG's arms and concentration.	• MTT: - iCECs adhesion does not depend on PEG arm numbers. - The increase of PEG's concentration increases the iCECs adhesion. - iCECs proliferation on 4% and 8% PEG higher compared to non-crosslinked hydrogel. - The proliferation of iCECs is higher on 8 arms compared to 4 arms but, these are not observed in 16%. • Live/dead assay: all hydrogel has high cell viability. 100% cell viable post day 2.	Mechanical properties of BC 1-gels-PEG-NHS depend on PEG's arms and concentration. BC 1-gels-PEG-NHS support the iCECs and CSSCs proliferation, adherence and morphology compared to the non-crosslink hydrogel.

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				After 7 days, 95% EGF is still encapsulated in BCI-gels-PEG-NHS.	• Cell morphology (F-actin): the presence of lamellipodia and only a few confluent areas on the non-crosslink hydrogel. • IHC: ZO-1 of iCECs highly expressed on both arms at 4% and 8% of PEG's concentration.	
Haagdorens et al., 2019 [77]	Unmodified RHCI, RHCI FN-pattern, CLP-12-EDC/NHS, CLP-12-DMTMM, CLP-12-FN-pattern, CLP-12-3D grooved, CLP-18-DMTMM.	Different crosslinker: EDC, DMTMM. Surface modification: FN surface pattern, 3D grooved surface topography.	iCECs primary hLESCs	• Water content: All hydrogel (88%-93%). • Light transmittance: CLP (>91%), RHCI (84.8±1.45) higher than HAM. • refractive index: All hydrogel (1.34-1.35) higher than HAM (1.33) • Permeability: All hydrogels are comparable to the HAM.	• Presto blue assay: Comparable on all hydrogels. • Live and dead assay: all hydrogel has minimal cell death. • Live cell imaging: support attachment (post 3 h seeding) and proliferation of the cells. FN-pattern / 3D grooves on CLP influence cell proliferation. (Attach FN /grooves first before spreading to the rest). • Confluence (post 72 h): CLP-12-EDC-NHS (91.0%±1.3)> CLP-12-3D (90.0%±2.7) > CLP-12-FN pattern (85.0%±3.3) > DMTMM (≥80%)> RHCI (71.4%±4.1) > RHCI-F-μCP (66.27%±8.3) > CLP-12-EDC (65.1%±18.3). • TEM: iCECs monolayer with apical microvilli but no expression of gap junction. • SEM: Post 4 days, cobblestone morphology, but iCECs on RHCI and RHCI-FN hydrogel displayed heterogeneous morphology (singular elongated cells in between squamous iCECs). The cell on CLP-12-3D was observed mainly in the grooves and not on the ridges.	RHCI and CLP-12 DMTMM, irrespective of surface modification, support the cultivation of primary hLESCs and iCECs. The regenerated epithelium maintained similar characteristics to HAM-based cultures.

 doi:10.20944/preprints202302.0371.v1

						• Phase contrast microscope: Post days 14, >15mm diameter of cell outgrowth on RHCI, RHCI-FN and =15mm on CLP-DMTMM • IHC: RHCI-DMTMM & CLP has low KRT3 & DSG3 and high ΔNp63, KRT14, INTB4 & E-cad. KRT3 is high on CLP compared to RHCI and HAM.	
Xeroudaki et al., 2020 [58]	BPC-EDC-NHS	Crosslinked with EDC-NHS Compression by compress mould method	Primary hCECs	• Optical transmission: >90% • Mechanical strength: Improved ultimate stress, stiffness, and toughness. • SEM: smooth surface with a fine structure. Collagen fibre is arranged parallel, unidirectional and has a porous structure (nanoscale). Presence of microfracture on the biomatrix induced by suture material during implant of the BPC by suture.		• MTT: High proliferation rate comparable to positive control at day 14. • Live and dead: ≈ 87.33±2.65% relative to positive controls.	BPC-EDC-NHS is transparent, has regularly arranged collagen, optimal mechanical properties and is biocompatible with CECs in vitro.
Chen et al., 2017 [81]	Collagen type 1 coated 6 well plate	A2-P	TKE2	N/A		• Clone formation assays: High clone formation ability without varying cell sizes. • IF: High p63, ABCG2, TCF4, SOX2, OCT4, Ki67, PCN, p63, SOX2 • Akt inhibition study: activates Akt Phosphorylation, • Western blot: Presence of collagen I & IV, laminin, and FN • Antioxidative study: possessed antioxidative properties MTT: High proliferation rate	A2-P and collagen 1 enhanced the stemness and proliferation of TKE2 which depends on its regulation of ECM components including collagen I and IV.
Miyamoto et al., 2012	Collagen type 1V coated	Exposure to anti-SCF antibody,	Mouse CECs	N/A		• MTT: Anti-SCF antibodies inhibited the attachment of hCECs on type IV collagen. SCF/c-kit enhanced CECs	SCF and c-kit play a role in the cornea wound healing by al-

 doi:10.20944/preprints202302.0371.v1

[89]	dished.	genistein, and Arg-Gly-Asp peptide	iCECs.		adherence to collagen IV coated dished.	tering CECs attachment.
Lake et al., 2015 [79]	Culture plates coated with 2 - 200 lg/cm² collagen I, III, IV and VI.	Transfect a5 promoter/ chloramphenicol acetyltransferase (CAT) plasmids into CECs cultured on collagen.	hCECs rabbit CECs.	N/A	• Electrophoretic mobility shift assays: - High a5 promoter activity in sub confluent cultured rabbit CECs compared to confluent rabbit CECs. - All collagen altered Sp1/Sp3, NFI, and AP-1. - Collagen I and IV repressed the a5 basal promoter segment. Collagen I repressed a1 integrin transcription of hCECs. • Microarray: collagen I upregulate 3252 genes and col IV deregulated 349 genes. • PCR: No obvious alteration at the protein level. • Morphology, IHC: - Small sizes and highly proliferative hCECs grown on collagen IV but round morphology hCECs have grown on collagen I (detached from culture plates). - Sub confluent rabbit CECs are moderate increase grown on collagen IV but not on collagen I. - FN promoted the adhesive and migration of CECs.	FN promoted the adhesive and migratory properties of CECs which were then altered by collagen which suppressed a5 gene expression, especially during confluence rabbit CECs.
Chakraborty et al., 2013	A variety of substrates	-	Primary hLESCs	N/A	• MTT assay: collagen IV improved the LESCs viability and proliferation compared to the plastic Petri plate.	Collagen IV support the viability and proliferation of LESCs

 doi:10.20944/preprints202302.0371.v1

[76]	including collagen IV coated the dishes.				• Thymidine incorporation: High level in LESCs cultured on collagen IV. • Matrix metalloproteinases (MMP)-9 assay: the presence of 92-, 82- and 72kDa. • Zynogram and western blot: Presence of MMP-9 but no detectable amount of MMP-2.	supported by the MMP-9 and MMP-2 (a key regulator of LESCs migration and proliferation).
Qin et al., 2021 [80]	ColMA	Modifying collagen with methacrylate group, followed by photo cross-linking – photopolymerized in situ.	hCECs	• Gelation point: ColMA5 (3.27 ± 0.03 s) ColMA10 (1.33 ± 0.03 s) • Burst pressure test: ColMA5 (63.50 ± 10.40 mmHg) ColMA10 (48.25 ± 9.61 mmHg) • Light transmission: 89-95% • FTIR: amide I, II, III	• Cell migration: 100% (40 h)	ColMA is a transparent biomatrix, with high-pressure overload capacity and is compatible with hCECs.
Wilson et al., 2014 [82]	RTCI-gels-FN-coated-AHDCS.	FN-coating encapsulated the AHDCS, treated with transforming growth factor beta-1 (TGF-β1) media followed by wortmannin.	AHDCS, CECs (3 different co-culture on the biomatrix: explant, transwell, and conditioned media co-cultured)	• Construct contraction (OCT): - All cellular biomatrix in TGF-β1 media show 15% thickness reduction for the first 24h, followed by 30% for a biomatrix that remained in that medium while remaining constant for the biomatrix that change to the CnT20 culture. - Acellular biomatrix thickness remains constant for 14 days. • Modulus measurement: - Increase with time for biomatrix cultured in serum-containing fibroblast media (post 2 days). - Transwell and acellular biomatrix remained constant for 14	• Live/ dead assay: High cell viability in all culture environments (days 7 and 14) • Light microscope: CECs display cobblestone morphology, with tight cell-cell junction. TGF-β1 reduced CECs viability, outgrowth, and proliferation. • IHC: Presence of CK3 in cultured environment.	Mutual interactions between CECs and CSSCs. A collagen hydrogel environment can retain the plasticity of CSSCs, and the mechanical properties of the cornea are defined by epithelial-stromal interactions.

 doi:10.20944/preprints202302.0371.v1

				days. - CnT20 monoculture and conditioned media biomatrix's modulus post 9 days.		
Kureshi et al., 2015 [83]	RAFT TE	-	Human CSSCs, hLESCs	N/A	• TEM: - monolayer (cobblestone) hLESCs on the RAFT TE surface. - Superficial hLESCs appeared stratified with microvilli on the apical surface. • IHC: - The presence of p63α, ABCB5, CK8, CK15, CD73 and CD90 formed on the surface of RAFT TE. - CK3 on the superficial hLESCs. - CSSCs remained close to hLESCs, pushed to the edges of RAFT TE.	Cultivation of CSSCs support hLESCs on RAFT TE.
Massie et al., 2015 [84]	RAFT TE-dFib RAFT TE-hLF	Incorporated with hLF or dFib.	hLESCs,	N/A	• MMP activity: MMP-2 and -9 activity increase in dFib RAFT TE • Sircol assay kit: de novo collagen synthesis increases in dFib RAFT TE. • IHC: a-SMA high in dFib RAFT TE compared to hLF in RAFT TEs.	hLF remained quiescent while dFib maintained activated, pro-scarring phenotype properties in RAFT TE.
De La Mata et al., 2019 [85]	PLA-collagen IV film	Functionalization of PLA film (70:30)	hCECs hLESCs	• Optical transmittance: transparent. • Mechanical strength: handleable, suturable. Contact angle: low contact angle (53.0 ± 11.0°). • IF, protein assay kit: 8.3 ± 1.3 μg/cm^2 of collagen IV were grafted to the surface of the PLA-collagen IV field (73.5% grafting yield).	• Fluorometric Alamar Blue assay: - hCECs viability 90% (post 8 days), - hCECs density on tissue culture plastic is higher than PLA-collagen IV (post 8 days). - hLESCs adhered within 2 h and confluence at 9.4 ± 1.0 days on	PLA-collagen IV has suitable physical properties to support the attachment, viability, and proliferation of CECs and LESCs. It also maintains undifferentiated LESCs.

 doi:10.20944/preprints202302.0371.v1

					PLA-collagen IV. • Bright-field microscope: Monolayer of homogenous polygonal cell formed. • IHC, RT PCR on cultivation hLESCs on PLA-collagen IV: Highly expressed K15, P63α, ABCG2 compared to K3, K12 in LESCs cultured on PLA-collagen IV.	
Wright et al., 2014 [40]	Oxidized alginate hydrogel -collagen 1V (OA-gels-CIV)	Incorporated by collagen IV	Primary bovine LESCs and hCECs	• SEM: Internal pore diameter size: 0.2–0.8 mm. • Rheology: OA-gels-CIV less stiff compared to non-oxidized hydrogel. • Lowry protein assay: The concentration of collagen IV lost from OA-gels-CIV was greater after 48h than 24 h, but no difference in protein loss.	• MTT: Oxidation (5%) and incorporation of collagen IV further increase CECs viability. • Trypan blue exclusion: CECs released from OA-gels-CIV can grow in colonies. • IHC: High CK3, CK14	OA-gels- CIV enhanced CECs viability but does not influence LESCs viability and differentiation.
Kayiran Celebier et al., 2020 [86]	PLGA-collagen I	Incorporated by collagen I	Primary rabbit CECs	• SEM: non-uniform pore distribution and pore wall thickness but architecture still maintained. • FTIR: Amine group. • Water uptake study: high water uptake capacity • Biodegradation rate: incorporation of the collagen but did not affect the degradation rate. • Tensile strength: PLGA (75:25)-collagen I >PLGA (50:50)- collagen I > PLGA (50:50) >PLGA (50:50)-NS> PLGA (50:50)-collagen I-NS > PLGA (75:25)-collagen I-NS.	• MTT: High CECs adhesion rate and proliferation rate (79% after 10 days). • SEM: CECs densely packed	PLGA-collagen I promote CECs adhesion, viability and proliferation without causing toxic effects for at least 10 days.
Yuncin	Silk film	Nanotopog-	Primary	N/A	• Phase-contrast microscopy: CECs	Collagen 1 coating and

 doi:10.20944/preprints202302.0371.v1

et al., 2021 [87]	coated collagen 1	raphy: flat, 2000, 1000, 80nm parallel ridge. Coating with ECM (including collagen I)	mouse CECs, primary rabbit CECs		elongated and aligned parallel to the direction of the pattern. CECs adherence, 800nm ridge > other topography. Collagen 1 coating increases cell number. • Focal adhesion localization: coated with collagen 1 and 800nm ridge increase focal adhesion area. • Scratch assay: Recovery rate (1000nm>800nm>2000nm> flat> glass, uncoated > coated collagen). • Ingenuity Pathway Analysis: topography regulates filopodia formation of the cell via actin nucleation ARP-WASP complex pathway (Cdc42).	800 nm ridge enhanced CECs growth, better cell spreading and wound recovery.

2

Table 2. Description of selected in vivo studies on the modified collagen biomatrix. 3

Authors	Type of Biomatrix	Modification Techniques	Animal Model/ Injury	Test and Result (in vivo)	Conclusion
Zhao et al., 2014 [70]	aCM	Xenogeneic decellularization of the conjunctiva with 0.1% sodium dodecyl-sulphate (SDS).	Mechanical injury by deep limbal lamellar keratectomy and chemical trauma on the CECs with n-heptanol.	• Biodegradation rate: aCM began to degrade on days 21-28. • Slit lamp: corneal opacity was restored completely on days 30. • H&E: restoration of corneal epithelium began on days 7 and was completed on days 30. • Corneal impression cytology: More donor cells were detected in the peripheral cornea. The number of donor cells on the recipient cornea at days 30 was higher on aCM compared with dAM (control).	aCM support multi-layered epithelial structure and is effective in the reconstruction of the ocular surface for the rabbit with the LSCD model compared to dAM
Zhou et al., 2021 [73]	APCS-gel	Decellularization	Removal of 2 mm central corneal epithelium in mice.	• Fast wound healing is 72 ± 3% after 24 h and 90 ± 3% after 30 h. • Gelation time longer compared to control. • MTT: Differentiation: (45 ± 13 %) after 18 h, highly	APCS-gel is suitable for CECs reconstruction by maintaining stemness and enhanced prolifera-

 doi:10.20944/preprints202302.0371.v1

				viable, proliferation. • Wound healing assay: fast	tion of the ocular surface.
Park et al., 2019 [71]	3D-BDCS	Encapsulated human turbinate-derived mesenchymal stem cells (TMSCs) and followed by cross-linked in vivo.	Mechanical injury by the lamellar dissection by using a crescent knife.	• OCT: Presence of low-intensity thin layer. • H&E: the broken epithelium layer may be due to histologic processing, and the presence of a few inflammatory cells. Changes in corneal thickness show the biocompatibility of the 3D-BDCS towards endothelial cell.	The changes in corneal thickness and the distributions of inflammatory cells and histology confirmed the biocompatibility of the 3D-BDCS
Baratta et al., 2021 [72]	CMP	Short synthetic collagen peptide	Removal of epithelium and epithelial basement membranes of the mouse (360° lamellar keratectomy) by using an Algerbrush with a 0.5 mm burr.	• Fluorescein Sodium Ophthalmic USP strip and stereoscopic zoom microscope: Wound closure within 24 h period. Wound closure is 15–20% slower for a higher concentration of 250 nM of CMP compared to 25 nM of CMP. • H&E: - The basal epithelium adheres to the anterior stroma surface. - Re-epithelization with minimal variability in the regenerating layer. - 250nM CMP increases the number of CECs than naive. - At the proliferative edge of the epithelium, the basal layer in vehicle-treated eyes appeared thinner and less organized than that in the CMP-treated ones.	CMP re-aligns the damaged collagen. CMP enhanced the closure of the wound process and promoted the re-epithelization process with forming of organized epithelium layers.
Qin et al., 2021 [80]	ColMA	Modifying collagen with methacrylate group, followed by photo-crosslinking-photopolymerized in situ.	Rabbit and pig corneal defect (partial thickness corneal defect).	• Fluorescein isothiocyanate-dextran, SEM: ColMA nanogranules left and attached to the collagen fibrils after the removal of ColMA hydrogel. • Slit lamp: post day 13, transparent, decrease of the epithelial defect. • H&E: 4-5 epithelium layer formed with few white blood cells and fibroblast (post days 31).	ColMA has a high-pressure overload capacity, a barrier against bacterial penetration, and dehydration. Nanogranules from dislodging ColMA adhere to stro-

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				• Masson's trichrome stain: collagen deposition in the anterior stroma. • TEM: Compacted alignment of collagen fibrils. • Immunofluorescence (IF): Less expression of α-SMA myofibroblasts.	mal tissue promote re-epithelization, reduces myofibroblast activation and decrease scar formation.
Hong et al., 2018 [27]	COLLEN-based limbal graft	dCL embedded by compressed collagen	Rabbit model of LSCD (induced by alkali burn), COLLEN was sutured onto the incised bed with 10-0 thilon nylon suture.	• Mechanical strength: Sufficient for in vivo, • Slit lamp: no neovascularization, no oedema and inflammation. • H&E: Multi-layered CECs originated from the cultured LESCs formed on the corneal central region (post 2 weeks implanted). • Periodic acid-Schiff (PAS) staining: no conjunctivalization. • IHC: CK3/12 was slightly expressed in both the limbal and central cornea region. • High levels of CK15, p63α, ABCG2, and PCNA in the limbal region compared to the central cornea.	The COLLEN-based limbal graft was successfully transplanted and verified its clinical efficacy on the ocular surface reconstruction of LSCD in a rabbit model.
Chae et al., 2015 [35]	CV	Vitrification process	CV-fibrin glue group: stromal injury by lamellar keratectomy. CV-hLESCs group: LSCD induced by chemical injury.	In the CV- fibrin glue group • Fluorescence staining: native CECs reformed rapidly • Pathology examination: Presence of healthy CECs • H&E: Dense columnar CECs basement membrane-like found on the surface of the CV. The presence of 4-6 layers of CECs (post10 weeks). • IHC: The presence of K3/K12 in the stromal layer. CV-hLESCs group: • Pathological examination: Transparent, low inflammatory response and reduced neovascularization (5 weeks post-surgery). • H&E: epithelial cell, hemidesmosome, cell-cell junction and apical surface covered with microvilli can be seen. • The ultrastructure: CV has organized meshwork collagen fibril.	CV support CECs and prevents epithelial hypertrophy, shows no complication after implantation, and can serve as an hLESCs carrier.
Jangamreddy	CLP-	Crosslinked to	Mechanical surgery of	• In vivo confocal microscopy: biomatrix stably grafted	CLP-PEG-EDC-NHS are

 doi:10.20944/preprints202302.0371.v1

et al., 2018 [69]	PEG-EDC NHS & RHC III-MPC (control)	MPC & EDC-NHS, conjugated to PEG	the mini pig's cornea	(6 months post-operation), with no excessive redness, swelling or inflammation. • Neo cornea regenerated and stably integrated, optical transparent without any sustained immune suppression (12 months post-operation). Regeneration rate similar to control. • H&E, TEM: Presence of epithelial hyperplasia, arranged proteoglycan. • Ultrastructural analysis: Vast quantities of extracellular vesicles. • IHC: High K3, collagen I, III and V at cornea stroma. CD9, Rab-7 in the epithelial layer. High CD9 below the basal epithelium of the limbus.	functionally equivalent to RHCIII-MPC (control) and have pro-regenerative effects by stimulating the in-growing endogenous host cells to produce ECM via secretory extracellular vesicle.
Fernandes-Cunha et al., 2020 [51]	BC1-gels-PEG-NHS	Crosslinked to NHS, conjugated to PEG (4 or 8 arms & 4%, 8% or 16% concentration of PEG).	Mechanical injury of the cornea of adult white rabbit by lamellar keratectomy	• Clinical observation: BC 1-gels-PEG- NHS bound to the stromal bed and low degradation rate, the defect area re-epithelized and formation of multi-layered CECs, no hyperplasia (post 1 week implanted). • IHC: the presence of Z0-1 and CK3 markers.	BC 1-gels-PEG- NHS is safely integrated and supports the multilayers of CECs.
Xeroudaki et al., 2020 [58]	BPC-EDC-NHS	Crosslinked with EDC-NHS Compression by compress mould method	Subcutaneous and rabbit's cornea (epithelial and stroma layer damaged)	Subcutaneous implantation. • H&E: wound completely healed 1-3 weeks post-implantation (minimal cellular infiltration at the interface with host tissue), gradual biodegradation of the BPC-EDC-NHS promoted the new collagen synthesis. • IHC: absence of CD45+, α-SMA, partial expression of β-III tubulin and collagen III deposition present at the implant-to-host interface region. Implantation into the rabbit corneal. • Clinical assessment: Presence of host stromal cell, stratified epithelium layers and nerve regeneration while maintaining corneal shape and thickness (6-month postoperative) period.	BPC-EDC-NHS has suitable mechanical properties, is safely integrated and is biocompatible with native corneal cells in vivo.
Chen et al., 2017 [81]	A2-P eye drop	A stable form of ascorbic acid.	Mechanical injury (epithelium layer)	• Slit lamp: A2-P accelerate the closure of the corneal epithelium wound after 48 h.	A2-P promoted corneal wound healing and sup-

 doi:10.20944/preprints202302.0371.v1

	induced by using algerbrush II corneal rust remover.	• IF: High NP63, Ki67. • Western blot analysis: Presence of collagen I, IV, laminin and FN, high level of Akt phosphorylation, PCNA	ported viability and the proliferation of LESCs. A2-P also promoted endogenous ECM production of LESCs.

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4. Discussion

4.1 New Bioresource and Its Efficacy on CECs and LESCs.

It is vital to tailor the collagen source during the development of the biocompatible biomatrix due to the presence of various amino acids in the collagen depending on the species and tissues sources [1,6,9,10]. This affects the final characteristic, physical properties and biocompatibility of the biomatrix [90,91]. Decellularization is one of the current approaches in producing a new collagen source for the biomatrix.

A previous study by Zhao and colleagues decellularized conjunctiva to produce an acellular conjunctiva matrix (aCM) as LESCs carrier [70]. The conjunctiva has a high degree of similarity to the cornea as both are derived from the epidermal ectoderm. Compared to the denuded amniotic membrane (dAM), the aCM biomatrix has better physical characteristics and is biocompatible with CECs. In vivo, the aCM could reconstruct the ocular surface in LSCD rabbits without neovascularization, inflammation or oedema [70].

A study by Park and colleagues also decellularized corneal stromal tissue from bovine eyeballs and produced three dimensional (3D) bioprinting decellularized collagen sheet (3D-BDCS), which could re-epithelialize the damaged epithelial layer within a few days [71]. Moreover, the decellularization of the porcine limbus and re-cellularized Statens Seruminstitut Rabbit Cornea (SIRC) limbal epithelial cell line and human adipose-derived mesenchymal stem cells (hADSCs) could produce a biomatrix with high content of collagen IV, and are able to regenerate stratified epithelium [75]. This is due to collagen IV affecting CECs transcriptional factor, which plays a significant role in CECs adhesion and migration properties [92,93].

Fish scale collagen was isolated from fish scale of the fresh fish (*L. calcarifer*) of sea catch, was one of the other new collagen bio sources that is rich in collagen I, followed by coated with polyethene (FSC-PE) possesses favorable physical strength, transparent and biocompatible with corneal cells [68,94]. FSC-PE allows proper epithelialization due to collagen I up-regulation of the specific gene, which regulates cell viability, attachment, and differentiation. The porous nature of FSC-PE also supported the viability and differentiation of LESCs and enhanced CECs migration and proliferation [35,68,94,95].

Collagen mimetic peptides (CMP) or collagen-like peptide (CLP) is another alternative of collagen source for the development of biomatrix [72]. CMP promotes realignment of damaged collagen, which accelerates wound closure in vivo. This is due to CMP is a short synthetic collagen peptide that able intercalate into damaged endogenous collagen I in vivo [96,97]. CMP is also recognized to enhance CECs density and re-epithelialization process with a better organization of epithelial layers [69,72,77].

Moreover, Qin and friends (2021) [80] produced Collagen Methacrylate (ColMA) by modifying collagen with a methacrylate group, followed by photo crosslinking. ColMA is a transparent biomatrix, with high-pressure overload capacity and is compatible with hCECs. Nanogranules from dislodging ColMA adhere to stromal tissue promoting re-epithelization, reducing myofibroblast activation and decreasing scar formation.

Within these recent 10 years, all collagen new bioresources including FSC-PE, aCM and 3D-BDCS potential to be developed as CECs or LESCs carriers. The presence of alternative extracellular matrix (ECM) to the macro-molecule collagen including CMP and ColMA is an attractive biomaterial and suitable to be developed as a biocompatible biomatrix for CECs or LESCs.

4.2. Physical Modification of Biomatrix

From the past 10 years, some researchers have performed physical modifications to improve the mechanical stability of the collagen biomatrix. The collagen biomatrix is also modified through compression technique by Jones and colleagues (2012) [59] and Xeroudaki and colleagues (2020) [58] modified. They found that compression of collagen hydrogel bioengineered porcine collagen (BPC) crosslinked with EDC-NHS reduced the water content, thus permitting control of the collagen concentration, stiffness, mechani-

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cal strength and surface topography, which contributes to its biocompatibility with corneal cells [49,59-61]. The compressed BPC supports the proliferation and maintains the normal morphology of hCECs (in vitro and in vivo) [58,59].

A study by Gouveia and colleagues [60] have reported that plastic compression of the collagen hydrogel which is Real Architecture for 3-Dimensional Tissue Equivalent (RAFT TE) also likely supported the differentiation of the LESCs via mechanotransduction-dependent pathways, Yes-associated protein (YAP) support the viability of the differentiated CECs. However, RAFT TE promotes the migration of LESCs which maintained a single monolayer, but few stratification cells became round and detached from the basal sheet. These cause RAFT TE to have a low viable differentiated CECs number and flat stretched CECs morphology compared to uncompressed softer collagen hydrogel [60].

Therefore, LESCs maintenance is highly dependent on the softer biomechanical limbus niche region properties which are opposed to the relatively stiff corneal central [98-101]. This is due to the LESCs niche requiring a specialized microenvironment to maintain the undifferentiated LESCs by slowing their migration rate and preserving their proliferative and stratification capabilities [60,79]. But these are opposed by studies by Massie et al., (2015) [61] and Kureshi et al., (2014) [78]. They showed the compressed RAFT TE supported the attachment, viability and proliferation of undifferentiated LESCs in vitro [78], [59].

Hong and colleagues (2018) [27] have conducted another physical modification of collagen bio-composite which embedded the decellularized corneal lenticule (dCL) with compressed collagen as a LESCs carrier known as COLLEN. COLLEN take the biological advantages from the compressed collagen and the mechanical properties of the dCL. COLLEN support the LESCs and hCECs attachment, expansion, morphology, and functions which were similar to the compressed collagen. In vivo, COLLEN-based limbal graft was stably grafted with native limbal region tissues without neovascularization, oedema, conjunctivalization and inflammation. COLLEN also support multilayered differentiated CECs after 2 weeks post-implantation while maintaining the putative stem cell markers on the limbal region compared to central cornea [27].

Another study by Haagdorens and colleagues [77] has reported that the surface modification which is FN pattern on CLP hydrogels influenced the pattern of cell proliferation. Yuncin and friends (2021) [102] stated that parallel ridge on silk film-coated collagen I enhanced CECs growth, and spreading and promotes wound recovery. Surface patterning and surface topography including the curvature of the biomatrix also affects the adhesion, proliferation, and gene expression of CECs [103].

The vitrification process is another physical modification approach to develop a rigid glassy material collagen vitrigel (CV) biomatrix. It is a thin membrane composed of high-density organized meshwork type I collagen fibrils with superior mechanical properties, non-degradable, stable water content, and optically transparent and supporting corneal cells [35,104]. In vivo, CV promotes the regeneration of healthy CECs and LESCs with low inflammatory response and reduced neovascularization (5 weeks post-surgery) [35].

In conclusion, the compression on the collagen biomatrix was able to increase the physical strength and affected the behavior of cultivated CECs or LESCs. Most of the researchers reported that compressed collagen biomatrix supported the viability of CECs, but it is not suitable for LESCs growth. This is due to the LESCs required soft biomechanical region properties of the collagen biomatrix to preserve proliferative and stratification capabilities of the undifferentiated LESCs. But these were opposed to the result obtained by [83]. Thus, further study is needed to explore the mechanism behind these corneal cells' behavior. In addition, the surface modification of collagen hydrogel also affects the growth and behavior of cultivated CECs and LESCs. CV and COLLEN are other creative approaches in developing optimal designs of CECs or LESCs biomatrix.

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4.3. Crosslinking Effect on the Biocompatibility of the construct towards CECs/LESCs.

Crosslinking is another approach in improving the mechanical stability of the collagen biomatrix. Different cross-linkers have different effects on the mechanical properties, in terms of biocompatibility of the biomatrix towards corneal cells, especially CECs and LESCs. Figure 4 showed the overview of the chemical cross-linker used in recent 10 years based on selected articles. Amide-based crosslinker such as EDC-NHS is commonly used with collagen biomatrix as it mimics the lysine-based crosslinker which is naturally present in collagen [105]. EDC-NHS affect the GxOGER sequences of collagen molecules [58], not remained as a part of the protein structure post-crosslinking [106,107] and improved the porous and interconnected structure of the collagen biomatrix [43], promoting cell attachment, proliferation and viability of the attached cells.

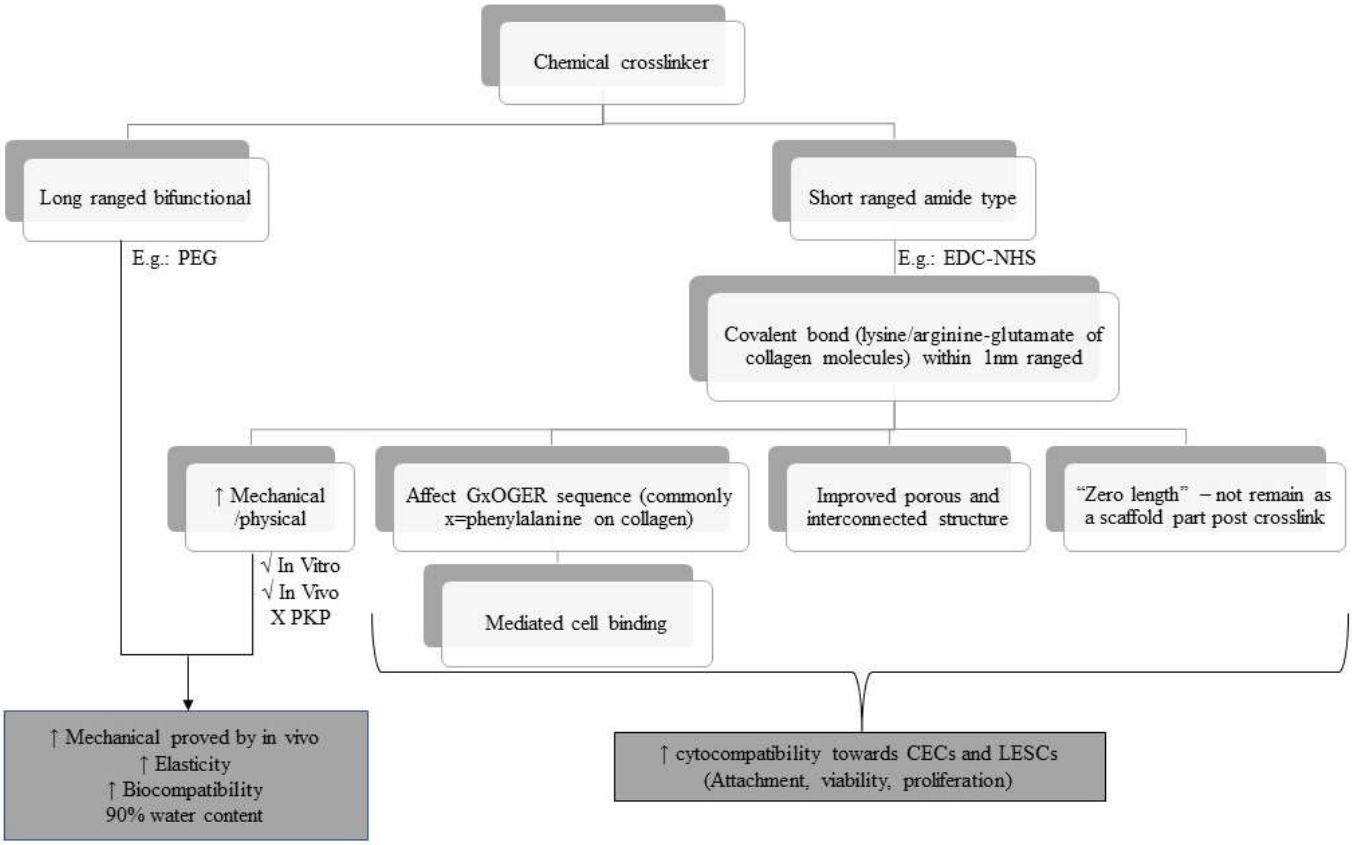

Figure 4. Overview of the chemical cross-linker used for recent 10 years based on selected articles.

EDC-NHS enhances the physical properties of the collagen biomatrix which has sufficient mechanical strength during subcutaneous implantation and implant in the rabbit cornea but was not strong enough for penetrating keratoplasty (PKP) [58,107-109]. This is due to the functional group located on the adjacent collagen microfibril being too far to be bridged by EDC-NHS as EDC-NHS only can link within 1.0 nm from each other [109]. These drawbacks could be overcome by hybridization of EDC-NHS (amide-type crosslinker) with a bifunctional cross-linker such as PEG, which provides synergistic effects on the physical and biological properties of collagen biomatrix [110,111].

Study by Jangamreddy and colleagues [69], Rafat and colleagues [112] have managed to conjugate the CLP-EDC-NHS to PEG (4 arms or 8 arms) to further improve the mechanical strength and promote a stable biomatrix for regeneration. This hybrid crosslinked hydrogel (CLP-PEG-EDC-NHS) enhanced the mechanical strength and elasticity by 100% and 20% respectively, compared to the non-hybrid biomatrix. The hydrogel comprised over 90% water, compared to the cornea that has 78% water, which contributes to its' biocompatibility. It also did not cause any cytotoxicity effects toward CECs as

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there was minimal CECs death at 48 hours (h) post cell culture and was able to regenerate the neo cornea with healthy regenerated functional CECs in vivo [69]. Although CLP-PEG-EDC-NHS did not have high tensile strength as control recombinant human collagen type III (RHCIII) conjugated to MPC (RHCIII-MPC) implants, but it was more elastic compared to the RHCIII-MPC, which allowed them to withstand the grafting procedure [69,113].

Regarding Fernandes-Cunha and colleagues [51], the concentration and arm number of PEG affect the transparency, directly proportional to the storage modulus and degradation profile of PEG crosslinked collagen biomatrix. The highest storage moduli achieved was at 8% (8-arm of PEG) but decreased at 16% PEG content. It may be due to the saturation effect, where beyond a certain concentration of PEG, no further crosslinking was achieved, and any additional PEG reduced the crosslinking density and transparency via inadequate macromolecular mixing leading to matrix heterogeneity.

In addition, this biomatrix has low cytotoxicity effects as almost 100% of human immortalized corneal epithelial cells (iCECs) confluent after 2 days post cultivated on all biomatrix. PEG arms concentration and arm number were directly proportional to the iCECs adhesion and proliferation except in 16% of PEG respectively. The high PEG concentration causes the reduction of biomatrix porosity which restrains the mobility of the polymer network and thus, reduced the proliferation of corneal cells [112]. The presence of iCECs improved the transmittance in the 4 and 6 arms by 16% PEG. PEG also affect the alignment of the collagen fiber which affects the iCECs behaviors [113].

Haagdorens and colleagues [77] also used an EDC-NHS crosslinker for the biomatrix of human corneal epithelial cells (hCECs) and human limbal epithelial stem cells (hLESCs). They used seven different collagen-derived hydrogels (recombinant human collagen type I (RHCI) and CLP hydrogel) with EDC-NHS or DMTMM cross-linker as a carrier of immortalized human corneal epithelial cells (ihCECs) and primary hLESCs. All these collagen hydrogels met the physical criteria of a good biomatrix for CECs and LESCs carriers. RHCI and CLP hydrogel irrespective of the crosslinker type except for CLP-12-EDC is biocompatible towards ihCECs as there was minimal cell death that supported the metabolic activity, attachment, and proliferation of ihCECs and primary hLESCs. However, these biomatrix did not promote LESCs differentiation except for CLP [77].

Overall, the crosslinker use on collagen biomatrix as a carrier of CECs or LESCs within these recent 10 years at its optimal crosslinker concentration was able to improve the mechanical stability of the biomatrix and biocompatible with CECs or LESCs. The use of high concentration and arm numbers of the crosslinker leads to the saturation effect which could cause a decrease in the transparency and porosity of the biomatrix and reduce the proliferation of the corneal cells. More novel crosslinkers and techniques need to be explored, either alone or in combination between chemical, enzymatic or physical crosslinker methods, which may give synergistic effects as a CECs or LESCs carrier.

4.4. Interaction of CECs/LESCs with Other Cells and Molecules in a Collagen Biomatrix

Other components such as FN, laminin, stromal cells, ascorbic acid, and SCF/C-kit have been incorporated into the biomatrix as they are able to promote re-epithelization [6,10,13,79]. Wilson and colleagues (2014) [82] investigated the effects of FN on the cultured CECs by seeding the adult porcine CECs on the FN-coating rat tail collagen type 1 hydrogel (RTCI-gels) encapsulated adult human derived corneal stromal (AHDCS) (RTCI-gels-FN-coated–AHDCS). RTCI-gels-FN-coated–AHDCS supported the CECs viability and maintained the normal cobblestone morphology with tight cell-cell junction. This is due to FN-binding integrin a5b1 promoting the CECs adhesion and migrating to cover the uncovered surface FN [13,114-117]. It mimics the physiological cornea as FN is a temporary ECM that presents abundantly during early corneal wound healing and is

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progressively replaced by collagen and laminin from the basal membrane as wound healing progressed [10,13,77,118-120].

In contrast, CECs appeared to be much smaller, less flattened and lacking the tight cell-cell junction when cultured on the biomatrix treated with wortmannin (epithelial stroma interaction inhibitor) [82]. These indicated the mutual interaction of CECs with stromal cells encapsulated in the hydrogel that was needed to support the CECs growth and enhanced epithelium multilayered organization [82,83,121].

This is also supported by Kureshi et al., 2010 [122], Massie et al., 2015 [84] and Zhang et al., 2015 [123]. Their studies showed that stromal cells/limbal fibroblast cells successfully enhanced the LESCs growth on the collagen biomatrix, which mimics in vivo corneal arrangement. It also was due to the LESCs differentiation depended on the mediated expression of the Wnt/B catenin of bone morphogenetic protein (proliferative marker) secreted by the stromal cell [60]. Massie and colleagues [84] have reported that human limbal fibroblast (hLF) (quiescent) was quite safe to be transplanted with hLESCs on the collagen biomatrix. hLF was less activated in terms of stroma and basement membrane remodeling and did not progress toward a scarring-like phenotype compared to diseased fibroblasts (dFib).

Another molecule that is important for corneal wound healing process is ascorbic acid. According to Chen and colleagues [81], the presence of L-ascorbic acid 2-phosphate (A2-P), the stable form of derivative ascorbic acid increased the stemness of mouse corneal epithelial stem/progenitor cells (TKE-2) by regulating the ECM components (collagen). A2-P also enhanced the stemness and proliferation of TKE2. SCF/C-Kit are another biomolecule that is present in normal mouse cornea and plays an important role in promoting corneal wound healing. According to Miyamoto and colleagues [89], SCF/C-kit enhanced cell attachment to FN, laminin and collagen type IV during corneal wound healing via the induction of the avidity and affinity of integrin members in vitro.

In conclusion, corneal stromal/keratocyte cells promoted CECs or LESCs growth on the collagen biomatrix. The presence of other components including FN, ascorbic acid, and SCF/C-kit enhanced the biocompatibility of collagen biomatrix towards CECs or LESCs.

4.5. Collagen as a Subtitute for Biomatrix.

A study by Chakraborty and colleagues [76] reported that collagen IV coated surface improved LESCs attachment, growth, and proliferation when compared to the untreated plastic surface. Many researchers have ventured into the benefit of biocompatibility properties of collagen type IV as the main component of the biomatrix. This includes incorporating or coating the collagen IV into another biomatrix to develop the optimal corneal biomatrix design.

De la Mata and colleagues [85] studied the biological properties of collagen IV by incorporating the collagen into a poly-L/DL- lactic acid (PLA) biomatrix. Functionalizing PLA films with collagen IV (70:30) improved LESCs attachment, selection, and enrichment and maintained the undifferentiated LESCs phenotype with homogenous polygonal morphology [85]. This finding was supported by Wright, De Bank, Luetchford, Acosta and Connon [40] who incorporated collagen IV into an oxidized alginate biomatrix. Incorporating collagen IV further enhanced the CECs viability and provide niche environment that supports the re-epithelization process and may serve as viable wound healing bandages for the damaged cornea.

Moreover, a study by Kayiran Celebier and colleagues [86] exploited the biological benefits of collagen I by incorporating collagen I into poly(lactide-co-glycolide) (PLGA) polymers loaded with naproxen sodium (NS). This incorporation of collagen I did not affect the degradation period or the mechanical strength, but improved hydrophilicity and enhanced the water uptake capacity compared to the plain biomatrix [64,124]. As a result, the PLGA biomatrix coated with collagen I improved CECs attachment, prolifera-

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tion, and viability [86,125], whereas incorporation of collagen I coating the silk film also enhanced biomatrix biocompatibility toward CECs [87].

In conclusion, exploitation of the biological benefit of collagen IV or collagen I by incorporating it into another biomatrix can improve the biomatrix biocompatibility toward CECs or LESCs, which have a great potential to be further used in in vivo study and clinically.

5. Conclusions

In conclusion, various modification strategies have been done to optimize the collagen biomatrix for CECs and LESCs carriers in the treatment of corneal defects. After summarizing and comparing the modification strategies and their effects on the biomatrix for each study included in this review. Despite the variability in the methodology approach, the reviewer postulated that the modification of the collagen biomatrix overall improves its mechanical and biocompatibility properties toward CECs and LESCs. The presence of the new bioresource of collagen within these 10 years potential to be developed as a biocompatible biomatrix. Physical modification and crosslinking methods improved the physical strength of the collagen biomatrix and affects the behavior of cultivated CECs and LESCs. The incorporation of the corneal stromal/keratocyte, FN, ascorbic acid, laminin, and SCF/C-kit into the collagen biomatrix enhanced its biocompatibility towards CECs and LESCs. Some studies also exploited the biological benefit of collagen by incorporating it into another biomatrix greatly improving the biocompatibility of the biomatrix towards CECs and LESCs. Our findings field provide insight into the field of current modifications on collagen biomatrix for the corneal defect. We believe the results highlight the need for modification of collagen biomatrix and these ultimately support the translation of collagen biomatrix-based therapies to the clinical setting.

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Abbreviations

3D	Three-dimensional
3D-BDCS	3D bioprinting decellularized collagen sheet
A2-P	L-ascorbic acid 2-phosphate
aCM	Acellular conjunctiva matrix
AHDCS	Adult human derived corneal stromal
APCS-gel	Acellular porcine corneal stroma hydrogels
BC1-gels-PEG-NHS	Bovine collagen type 1 hydrogel crosslinked to PEG-NHS
BMP	Bone morphogenetic protein
BPC	Bioengineered porcine collagen
CAT	Chloramphenicol acetyltransferase
CECs	Corneal epithelial cells
CK	Cytokeratin
CLP	Collagen-like peptide
CLP-12 EDC	Collagen-like peptide type 12 crosslinked with 1-Ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride
CLP-PEG-EDC-NHS	Collagen-like peptide crosslinked with 1–Ethyl–3-(3-dimethylaminopropyl) carbodiimide-N-hydroxy succinimide and conjugated to the polyethene glycol
CMP	Collagen mimetic peptides
COLLEN	Decellularized corneal lenticule embedded compressed collagen
ColMA	Collagen Methacrylate
CSSCs	Corneal stromal stem cells
CV	Collagen vitrigel
dAM	Denuded amniotic membrane
dCL	Decellularized corneal lenticule
dFib	Diseased fibroblasts
DHC	Decellularized human corneal tissue remnants
DMTMM	4-(4,6-dimethoxy-1,3,5-triazin-2-yl)-4-methyl-morpholinium chloride
ECM	Extracellular matrix
EDC	1-Ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride
EDC-NHS	1–Ethyl–3-(3-dimethylaminopropyl) carbodiimide-N-hydroxysuccinimide
FGF	Fibroblast growth factor
FN	Fibronectin
FSC-PE	Fish scale collagen coated with polyethene
FTIR	Fourier-transform infrared spectroscopy
H&E	Haematoxylin and eosin stain
HA-DOPA	Dopamine hydrazone scaffold- crosslinked hyaluronic acid

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hADSCs	Human adipose-derived mesenchymal stem cells
HAM	Human amniotic membrane
hCECs	Human corneal epithelial cells
hLESCs	Human limbal epithelial stem cells
hLF	Human limbal fibroblast
Hyp	Hydroxyproline
IF	Immunofluorescence
IHC	Immunohistochemistry
ihCECs	Immortalized human corneal epithelial cells
K	Keratin
LEPC	Limbal epithelial progenitor cells
LESCs	Limbal epithelial stem cells
LM	Limbal melanocytes
LMSC	Limbal mesenchymal stromal cells
LSCD	Limbal stem cell deficiency
MMP	Matrix metalloproteinases
MPC	Methacyloyloxyethyl phosphorylcholine
MTT	Tetrazolium salt (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
NHS	N-hydroxysuccinimide
NS	Naproxen sodium
OA-gels-CIV	Oxidized alginate hydrogel incorporated with collagen IV
OCT	Optical coherence tomography
PE	Polyethene
PEG	polyethene glycol
PKP	Penetrating keratoplasty
PLA	Poly-L/DL- lactic acid
PLGA	Poly lactic-co-glycolic acid
RAFT TE	Real Architecture for 3-Dimensional Tissue Equivalent
RAFT TE-CI	Real Architecture for 3-Dimensional Tissue Equivalent treated with collagenase I
RAFT TE-NT	Real Architecture for 3-Dimensional Tissue Equivalent treated without treatment.
RAFT TE-PBS	Real Architecture for 3-Dimensional Tissue Equivalent treated with phosphate-buffered saline
RAFT-TE	Real Architecture for 3-Dimensional Tissue Equivalent
rCECs	Rabbit corneal epithelial cells
RHC	Recombinant human collagen
RHCI	Recombinant human collagen type I
RHCIII	Recombinant human collagen type III

RTCI-gels	Rat tail collagen type I hydrogel
RT-PCR	Reverse transcription polymerase chain reaction
SCF	Stem cell factor
SDS	Sodium dodecyl-sulphate
SEM	Scanning electron microscope
SIRC	Statens Seruminstitut Rabbit Cornea
TEM	Transmission electron microscopy
TGF-β1	Transforming growth factor beta 1
TKE2	Mouse corneal epithelial stem/progenitor cells
TMSCs	Human turbinate-derived mesenchymal stem cells
WOS	Web of Science
YAP	Yes-associated protein

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