Exploring the potential of the recombinant human collagens for biomedical and clinical: a short review

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Abstract

Natural animal collagen and its recombinant collagen are favorable replacements in human tissue engineering due to their remarkable biomedical property. However, this exploitation is largely restricted due to the potential of immunogenicity and virus contamination. Exploring new ways to produce human collagen is a fundamental key to its biomedical and clinical application. All human fibrillar collagen molecules have three polypeptide chains constructed from a repeating Gly-Xaa-Yaa triplet, where Xaa and Yaa represent one random amino acid. Using cDNA techniques to modify several repeat sequences of the cDNA fragment, a novel human collagen named recombinant human-like collagen (HLC), with low immunogenicity and little risk from hidden virus can be engineered and notably tailored to specific applications. HLC was initially used as a coating to modify the tissue engineering scaffold, and then used as the scaffold after cross link agents were added to increase its mechanical strength. Due to its good biocompatibility, low immunogenicity, stabilised property and the ability of mass production, HLC has been widely considered to use in skin injury treatments, vascular scaffolds engineering, cartilage, bone defect repair, skincare, haemostatic sponge, and drug delivery including coating with medical nanoparticles. In this review, we symmetrically reviewed the development, recent advances in design and application of HLC, and other recombinant human collagen-based biomedicine potentials. For comparison and providing basic background information about the techniques, we start with recombinant human collagens. In the end, future improvements in using HLC are also discussed.

Keywords: Recombinant human collagen, human-like collagen (HLC), tissue engineering, drug delivery, medical nanoparticle

1. Introduction

Collagen, a fibrillar protein, is found in abundance in the body and plays an important connective role in skin, joints, and bones [1]. Natural human collagens have been explored in biomedical and clinical applications, particularly in tissue repair, regeneration, and replacement but they are difficult to obtain [2]. The fact that collagen sponge converted from natural collagen solution after lyophilisation lacks mechanical strength and with unpredictable biodegradability [3] further hinders its applications in hard tissue. The replacement of natural human collagens with extracted animal collagens appears to be an avoidable natural solution but using animal collagen has the potential risk of immunogenicity and virus contamination, and its mechanical strength and biodegradability still remains an issue [4].

There are twenty-nine different collagens classified in eight families based on their structure, chain bonding, and locations in the human body [5]. They all have a simple repetitive $(Gly\text{-}Xaa\text{-}Yaa)_n$ sequence motif, where X and Y are often proline and hydroxyproline, that can be used as a building block for self-assembly into complex hierarchical structures such as fibrils, fibers, and bundles [5]. Using standard recombinant methods, targeted human collagen cDNA fragment is selected to use as a backbone gene [6]. This gene is then cloned into a selected expression vector and transformed into expression cells. After purification, recombinant human collagen is obtained [7-10]. This recombinant human collagen is very similar to the natural human collage and its purification process can potentially minimize or even eliminate viral contamination. As a result, the recombinant human collagen normally has a good biocompatibility such as low immunogenicity and little risk from the hidden virus [11]. Numerous commercially available collagens (e.g. FibroGen, CollPant, etc) have been developed over the years. Due to their improved properties, recombinant human collagen have been successfully used in biomedical engineering [12, 13], especially in

skin repair, tendinopathy, cornea and some cardiac repair therapy [14-18].

However, studies also find that recombinant human collagen lacks stability and often degrades quickly during the purification process [7-10]. These properties of recombinant human collagen make it difficult to be obtained and used. At the meantime, the low yield and high production costs limit its applications further. These are probable reasons why even though this standard recombinant method has been around for many years, it did not find, thus far, any widely used applications. On the contrary, utilizing native collagens from animal sources remains the main resource of applications. Therefore, a new approach is desperately needed.

In 2002, Fan *et al*. modified several repeat sequences of human collagen cDNA fragment, designed a novel collagen sequence and expressed it as a novel recombinant human collagen [19-21]. They named it as 'recombinant human-like collagen (HLC)' (China patent number: ZL01106757.8). Briefly, the human collagen sequence template is modified through changing the Xaa and Yaa in the repeat peptide $(Gly\text{-}Xaa\text{-}Yaa)_n$ sequence [22-24]. Foremost, the isoelectric point (pI) could be adjusted higher or lower through changing the amino acids of the $(Gly\text{-}Xaa\text{-}Yaa)_n$. This pI design therefore makes the HLC purification more achievable. Secondly, the hydrophilicity could also be adjusted though adding hydrophilic amino acid in the $(Gly\text{-}Xaa\text{-}Yaa)_n$. Thirdly, additional lysines [25] within the $(Gly\text{-}Xaa\text{-}Yaa)_n$ can be further used to promote the cells adhesion.

Notably, compared with the traditional recombinant human collagens, this novel HLC is more stable, degrades slowly, and therefore easy to purify [26-28]. Meanwhile, HLC scaffolds obtained through this method are suitable for cell homing and growth [11, 28]. Even strikingly, after many years of optimization on all producing process, the production of the HLCs is continuously increasing [20, 21, 29-32]. Currently, these

HLCs can be put into mass production with a high yield of 14 g/L and small costs which facilitate its applications in hemostatic sponge, skin injury treatments and skincare. The ability of mass production also open opportunities to explore its applications in many other aspects of biomedical and clinical areas.

This unique technique and great potential applications in biomedical and clinical sciences have attracted more and more research interests since our first publication in 2002 [19]. In this paper, we review the development of HLC, the "hobbled" mass production venture and fast-moving recent advances on its applications. In addition, the prospects for further development of HLC for therapeutic-related applications are also evaluated. For comparison and providing basic background information about the techniques, we start with recombinant human collagens.

2. Recombinant human collagens

Collagens play essential roles in cell attachment, migration, proliferation and differentiation [1]. They are classified as twenty-nine different types and grouped into eight families according to their structures, chain bonding, and locations in the human body [1]. Different types of recombinant human collagens can be designed accordingly to their locations in the body to meet their different functions. For example, recombinant human collagen type I has been used as membranes for guided tissue regeneration due to its wide distribution in dermis, tendon, ligament, bone, and cornea [33, 34]. Similarly, recombinant human collagen type III has been used as haemostats and tissue sealants due to its wide presence in tendon, cornea, reticular fibers, cartilage, vessel wall, nucleus pulpous, bone, vitreous body, and skin [35-37]. There are different human collagens continuously being developed through recombinant systems for purposed use [38].

2.1 Expression of the Recombinant Human Collagen

Typical expression systems used are mammalian cells [39], insect cells [40], transgenic tobacco [41, 42], transgenic mouse [43], and transgenic silkworms [44]. Microbial expression systems are also successfully employed to express various types of human collagens [45]. For example, human type I-III collagens have been successfully expressed by *yeast pichia pastoris* [8, 46-48], human type III collagen by *Escherichia coli* [49], hydroxylated human collagen III α1 chains by *P. pastoris* GS115 [50] and Escherichia coli [51], while recombinant human prolyl with human collagen a1 (III) chains has been co-expressed in two yeast systems [52]. Table 1 summarizes recombinant human collagens obtained using different expression systems (Table 1).

Table 1. Recombinant human collagens

Type	Host	Yield	Reference
Procollagen X	HEK293 cell	50 mg/L	Frischholz *et al.* [39]
Procollagen I	Insect cell	10~20 mg/L	Myllyharju *et al.* [40]
Collagen I	Transgenic tobacco	200 mg procollagen per kg of fresh leaves.	Stein *et al.* [41]
Procollagen	Transgenic mouse	50~200 mg/L	John *et al.* [43]
Procollagen III	Transgenic Silkworms	5 kg total collagen obtained from about 600 kg cocoon material	Tomita *et al.* [44]
Collagens I-III	*Yeast Pichia pastoris*	200~600 mg/L depending on the collagen type	Myllyharju *et al.* [8]
Collagen I α1	*Yeast Pichia pastoris* (SMD1168/pPIC9K)	1080 mg/L	Li *et al.* [46]
Collagen III α1	*Yeast Pichia pastoris* (GS115/pPIC9K)	1270 mg/L by using the *S. cerevisiae* alpha mating factor (α -MF)	Wang *et al.* [47]
		3360 mg/L (purity 94.6%)	Li *et al.* [48]
Collagen III	*E. coli.* BL21 (DE3)	900 mg/L	Rutschmann *et al.* [49]

Normally, the level of the protein expression depends on which type of collagen is chosen to express and which type of host cell is chosen to use. When using the microbial expression system to express the collagen, the condition of the fermentation can also

play a role. As shown in Table 1, recombinant human collagens express the lowest level [40] in insect cells (10 to 20 mg/L), slightly higher in mammalian cells [39], but still lower than that in the transgenic tobacco [41], transgenic mouse [43] and transgenic silkworm [44]. In general, all mentioned expression systems still need to be improved to achieve mass production. Meanwhile, production costs in these processes are considerable higher than those in the fermentation process used for other types of cells, such as yeast [8, 46-48] or *E. coli*. [49]. Nevertheless, the yield of the recombinant collagen expressed by *Yeast Pichia pastoris* and the *E. coli* is significantly higher than that by other expression systems. For example, the level of expression achieved in *Yeast Pichia pastoris* can range from 200 to 3360 mg/L [8, 46-48], and 900 mg/L in *E. coli* BL21 (DE3) [49]. Therefore, microbial fermentation seems to be a feasible method to achieve a relative mass production.

2.2 Application of recombinant human collagens

Various recombinant human collagens have been obtained and applied in biomedical engineering since they were developed [7-10]. However, due to the limited mechanical strength, they were mainly used in repairing damages in neural, skin, cartilage, tendinopathy and cardiac therapy [14-18]. For example, Que *et al*. [14] introduced the full-length human collagen type III genes into CEN/ARS plasmids, the plasmids were then transformed into *S. cerevisiae strain* BYα2β2 to induce type III collagen expression. This recombinant collagen was then used as a scaffold for human neural stem/progenitor cells. Woodley *et al*. [15] obtained the recombinant human type VII collagen and used it to treat skin wounds such as dystrophic epidermolysis bullosa. James *et al*. [16] developed the recombinant human collagen type I and type III injectable hydrogels and used them for cardiac therapy. As such, several different types of recombinant human collagens were developed and used in repair and reconstruction of tissue [35]. For example, the recombinant collagen type III has been widely studied

in tissue engineering as collagen type III is mainly found in infant skin, intima, or intestine. The recombinant collagen type VII is increasingly being used for skin disease treatment due to collagen type VII being mainly found in healthy skin. Collagen type II has been extensively studied in cartilage defect repair [53-55]. The details of the applications of the recombinant human collagens in recent years were summarized in Table 2.

Table 2. Application of recombinant human collagens

Recombinant collagen type	Application	Properties and Effects	Ref.	Year
collagen type I and type III	For cardiac therapy	As scaffolds to support endogenous cells and to promote the regeneration of ischemic tissue	[16]	2017
collagen type I	Treatment of lateral epicondylar tendinopathy	The efficacy of this new treatment is better than others currently available injection treatments in chronic lateral epicondylitis	[17]	2019
collagen type II	Cartilage tissue engineering	Chondrocytes retained their round shape, proliferated, and produced an extracellular matrix typical of articular cartilage when grown in the recombinant human type II collagen gels.	[53]	2008
collagen type II	Cartilage in nude mouse model	Recombinant human type II collagen gel as a scaffold for chondrocytes promoted a better maintenance of neotissue construct shape, compared with the cells without a scaffold. The soft gel material without any synthetic or animal-derived additives allows a safe and reproducible way of adding more structural competence to the implantation of chondrocytes.	[54]	2010
collagen type II	Providing 3D micro-environment for chondrogenesis of human bone marrow-derived mesenchymal stromal cells (BM-MSCs)	Recombinant human type II collagen hydrogel induces a transient, reversible catabolic disturbance in the chondrogenic differentiation of BM-MSCs, a process potentially beneficial in graft integration. This study further adds evidence on the role of 3D scaffolds as bioactive micro-environments and modulators of tissue reorganization, with important implications for cartilage tissue engineering.	[55]	2015

collagen type III	Corneal regeneration	This biosynthetic implant promoted endogenous regeneration of corneal tissue and nerves that were stable over four years, without any rejection episodes and in the absence of immunosuppression. In terms of aesthetic appearance, resolution of the initial blinding pathology and potential for restoration of vision in the long term.	[56]	2014
collagen type III	Corneal substitute	The structure of the biosynthetic hydrogels differs substantially from the human cornea, but both have a high transparency to visible light. The transparency of the hydrogel may be attributed primarily to its high-water content and narrow collagen filaments. Patients implanted with these hydrogels should exercise caution regarding UV exposure, particularly in the period prior to epithelial regrowth.	[57]	2015
collagen type III	As substitutes for the corneal extracellular matrix	Promote endogenous regeneration of corneal tissue	[18]	2015
collagen type III	As a scaffold for human neural stem/progenitor cells	These recombinant collagens can form hydrogel as a cell delivery scaffold and support differentiation of hNSPCs into neurons and astrocytes.	[14]	2018
collagen type VII	To treat skin wounds such as recessive dystrophic epidermolysis bullosa (RDEB)	The collagens can simultaneously migrate to the dermal epidermal junction (DEJ) throughout the RDEB patient's skin, reverse the 'subclinical', microscopic epidermal-dermal separation and prophylactically prevent frank skin blisters and erosions from forming.	[15]	2013
collagen type VII	To treat RDEB	Human mesenchymal stromal cells engineered to express collagen VII in skin graft chimeras to treat RDEB	[58]	2019

collagen type 7A1	Gene-corrected fibroblast therapy for RDEB using a self-inactivating COL7A1 retroviral vector	The efficacy and safety of gene-corrected fibroblast therapy using a self-inactivating vector that has now been good manufacturing grade-certified and pave the way for clinical translation to treat non-healing wounds in RDEB patients.	[59]	2016
collagen type 7A1	Ex vivo COL7A1 correction for RDEB using CRISPR/Cas9 and homology-directed repair	Precise genome editing in primary RDEB cells is a relevant strategy to genetically correct COL7A1 mutations	[60]	2018

2.3 Challenges for the application of recombinant human collagens

All applications of recombinant human collagens are mainly based on the type of collagen used and rely on the properties of related tissue structure on which collagens are applied to. Although an improved efficacy and effect is achieved, the systematical use of recombinant human collagens still face lots of challenges in both producing recombinant human colleagues itself such as a low-yield and high production cost due to recombinant human collagens lacking of stability and quick degrading during the purification process, and in introducing new methods and techniques to produce new structure of collagens with enhanced properties. Due to these reasons, their applications are still limited, especially in clinical environments. Most applications of the recombinant human collagens remain on animal studies.

However, there are still some excitements of some recombinant human collagens having been utilized in the clinics [17]. For example, recombinant human type I collagen has been applied for the treatment of lateral epicondylar tendinopathy (tennis elbow) and achieved a successful efficacy [17]. Another example is that FDA approved collagen type I nerve conduits have been implemented for defects smaller than 3 cm in clinic conditions [61, 62]. However, the recombinant human type I collagen have not widely applied in the neuron outgrowth and enhance Schwann cell proliferation and extension due to the low expression level of recombinant human type I collagen in the host cells. There are also some controversial examples of its application. For example, in 2019 there was a report that human mesenchymal stromal cells engineered to express collagen VII can restore anchoring fibrils in the skin graft chimeras to treat patients with recessive dystrophic epidermolysis bullosa (RDEB). But actually it is unlikely that recombinant collagen VII could be delivered to the dermal-epidermal basement membrane, processed by C proteinase, and assembled into anchoring fibrils. Furthermore, RDEB patients frequently do not produce collagen VII. Thus, injecting

the recombinant version of this protein could lead to the production of antibodies and cause consequences similar to those seen in patients with epidermolysis bullosa (EB).

One of the fundamental properties of native collagens is their ability to assemble into specific structures, including fibrils, networks, and other. But there are reported examples of recombinant mutated collagens, e.g. collagen II, that lose their ability to form fibrillar structures [54]. The reasons for this are not fully understood. As most of the cell-collagen interactions depend on the binding of specific receptors with unique domains present within collagenous assemblies, the lack of ability to form supramolecular assemblies presents a potentially significant problem.

In addition, the recombinant collagen technology itself faces economic challenge to mass produce the recombinant collagen needed. The complicated production procedure and the cost of implementing it normally overtook the cost of products that utilize native collagens from animal sources.

Therefore, new approach is desperately needed to fundamentally to change, to improve and to extend the applications of recombinant human collagens. There are several factors which could be considered: (i) developing various recombinant collagens to meet the need of different tissue applications, (ii) using more expression systems to improve the expression level of recombinant collage, (iii) modifying the structure of recombinant collagen to obtain better properties for different purpose in applications, such as using linkers to link with other materials to improve its mechanical strength, (iv) combing with new genetic modification and biochemical methods to produce new structure of recombinant collage with enhanced properties.

3. Recombinant human-like collagen

Based on the human collagen repeat peptide sequence $(Gly\text{-}Xaa\text{-}Yaa)_n$ (where Xaa and Yaa represents one random amino acid), the collagen sequence template can be modified through changing the Xaa and Yaa of the $(Gly\text{-}Xaa\text{-}Yaa)_n$ sequence into any amino acid [22-24]. Using this modified collagen sequence as the backbone, novel recombinant human collagen can be designed to accommodate different types of proteins with different sequences and with different properties. As the backbone gene is not a natural human collagen gene code but is a novel sequence from modifying several repeat sequences of the human collagen cDNA, this novel modified collagen is therefore named as human-like collagen (HLC). There have been several types of HLCs produced in this way [11, 19-21, 26-32].

Different from the traditional recombinant human collagens, the cDNA of the human collagens can be modified within the HLCs, such as changing the Xaa and Yaa in the repeat peptide $(Gly\text{-}Xaa\text{-}Yaa)_n$ sequence to adjust the pI, the number of lysines, cell binding domain, etc. of the HLC. Therefore, the HLCs are more stable, degrade slowly and thus easier to purify [20, 26-32]. The yield of the HLC is also significantly higher than that of traditional recombinant human collagen. Additionally, more lysines added to the HLC sequence template promotes the cells adhesion [11, 26, 28], this consequently enhances the application of HLCs in the treatment of different injured tissues [11, 26, 28]. Normally the human-like collagen requires chemical cross-linking or mixing with other materials to improve mechanical integrity as do many other collagens. The unique properties of HLC contain more $-NH_2$ which make it much easier to cross-link and mix with other materials [28]. Therefore, this novel recombinant human collagen (HLC) can achieve improved stability, higher yield, slower biodegradation speed and better cell adhesion.

Due to its excellent biocompatibility, low immunogenicity, the stabilised property and mass production ability, the HLC has been successfully used in tissue engineering to repair organ tissue damage based on animal models and received growing extensive attention [63-65]. The following sections will focus on discussing how to express and purify the recombinant HLCs and how to facilitate them in biomedical and clinical applications.

3.1 Expression of recombinant human-like collagen

Designing and modifying several repeat sequences of the human collagen cDNA and choosing suitable expression system proves to be very challenging. Optimising a best fermentation condition is the practical endeavor for the success of obtaining HLC products. The standard procedure for producing the HLCs in Fan's lab was shown in Figure 1.

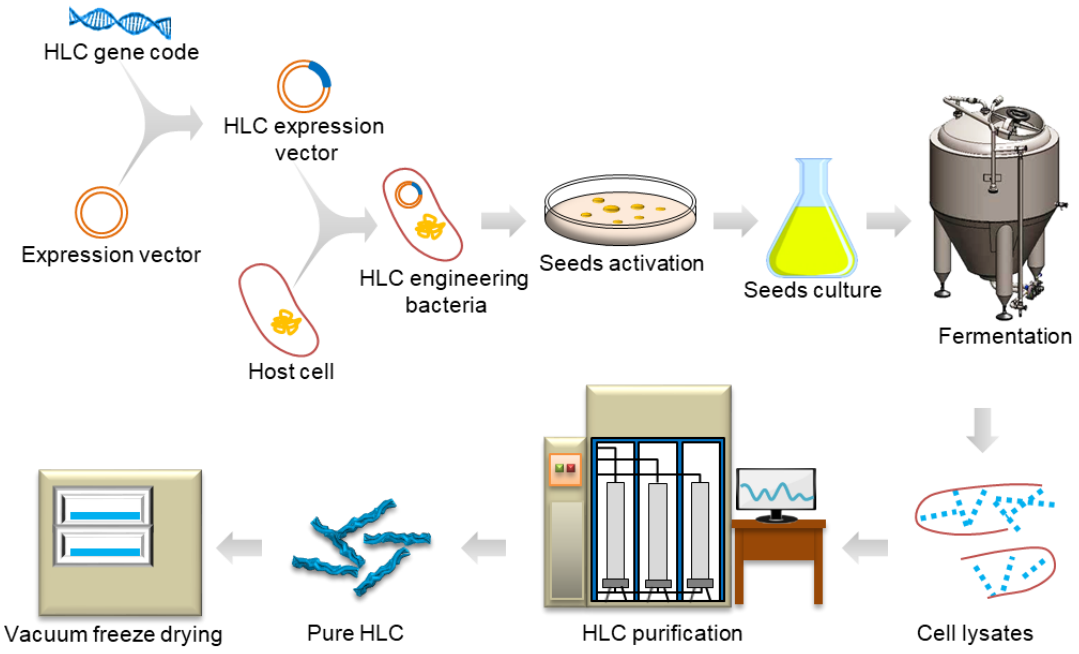

Figure 1. The standard procedure for producing the HLCs

Apart from three recent reports [66-69] of successful expression of HLCs, we are the only group which successfully express and produce the HLCs (Table 3).

Table 3. Recombinant human-like collagen

Host	Yield	MW.	Ref.
***E. coli.* origami (DE3)**	260 mg/L in a 10 L fermentor	35 kDa	Tang *et al*. [66]
***E. coli.* Rosetta (DE3)**	Shake flask culture	40 kDa	Yang *et al*. [67]
***E. coli.* BL21 (DE3)**	Shake flask culture	37 kDa	Peng *et al*. [68, 69]
***E. coli.* BL21 (DE3)**	14 g/L in a 500 L fermentor	93 kDa	Fan *et al*. [19, 30]

In Tang *et al*.'s study [66], the HLC gene, human prolyl 4-hydroxylase and D-arabinono-1, 4-lactone oxidase were co-expressed in *E. coli*. origami (DE3) to produce the hydroxylated HLC (approximate 35 kDa). The yield was up to 260 mg/L in a 10 L fermentor. In Yang *et al*.'s study [67], human-like type I collagen was expressed using Escherichia coli Rosetta (DE3) and had approximate 40 kDa molecular weight. In Peng *et al*.'s [61, 62] study, HLC was expressed using *E. coli*. BL21 (DE3) and had approximate 37 kDa molecular weight. While in Fan *et al*.' lab, the HLC (93 kDa) expressing recombinant *E. coli* BL21 (DE3) was constructed using a plasmid with kanamycin resistance and temperature induction genes [19, 30]. A stabilised high-yield HLCs production is routinely achieved. This level of expression, around 14g/L in 500 L fermentor is significantly higher than that of the above producer and the full-length human collagen (900 mg/L, Table 1).

Apart from the differences in host *E. coli*, the expression of HLC in Yang *et al*. [67] and Peng *et al*. [68, 69] was purely through shaking flask culture; and the yield of HLC in Tang *et al*. [66] was 260 mg/L using a 10 L fermentor. Notably, the yield of HLC in Fan *et al*. [19, 30] reached 14 g/L in a 500 L fermentor.

As it is expected that normally the technological requirement for recombinant collagens is high and there is a large amount of initial investment required to be able to start with. There are also lots of technical barriers need to be overcome before final mass production can be achieved, such as constructing the recombinant plasmid, obtaining engineered host cells to express mass target collagens, monitoring high-density fermentation and purification of the collagens etc. Initial setting up and producing reasonable amount of recombinant human collagens to use even on the animal experiments costed more than those of using animal-derived collagens directly. This is probably reason for the delayed broadcasting of this new technology. However, the advantages of the recombinant human collagens in better biocompatibility and lower immunogenicity is much better than those of native collagens from animal sources and attracted scientists to continuously develop this technology. Considering all the benefits and its cost-efficient in the production process, human-like collagen if successful certainly has great advantage than its animal extracted counterparts.

The reasons for the success of the high level of the HLC product in Fan *et al*.'lab are due to the following key considerations: (i) Choosing the right expression system, *E. coli* BL21 (DE3); (ii) Designing a suitable molecular weight of 93 kDa; (iii) Optimising a best fermentation condition. According to the metabolism characteristics of *E. coli*, a variety of regulatory strategies for HLC production were performed in our laboratory. For example, the high-density fermentation of recombinant *E. coli*. had been studied, including the effects of different oxygen control methods, nitrogen supplementation methods and different induction conditions on bacterial growth and HLC expression. Meanwhile, the kinetic and metabolic pathway fluxes of the bacterial growth and HLC expression were analysed in Fan *et al*.'s previous studies. Presently, Fan *et al*. have promoted the level of HLC expression to around 14g/L in 500 L fermentor, and the HLC with further properties such as additional stabilization, slow degradation, easily purification [70].

As a consequence , sufficient supplement of HLC is available and make it possible for us to explore its various applications, including skin injury treatments [71-76], vascular scaffolds engineering [77-80], cartilage [81, 82] and bone defect repair [83-90], and haemostatic sponge [28, 91, 92] etc.

3.2 Potential application of the human-like collagen in tissue engineering

In early studies, the HLC was not able to be used alone but as a coating to the scaffold due to its poor mechanical strength. For example, HLCs are usually used to coat or mix with the calcium phosphate ceramics, metals, or polymers to improve the compatibility and remain the stiffness of the substrate scaffold [79, 80, 82, 87, 88]. In order to increase the strength of the HLC to meet the requirement of the scaffold, compatible cross-link agents were used. For example, HLC was cross-linked by the dialdehyde starch (DAS) [71], oleuropein [89], 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC) [93, 94], EDC and adipic acid dihydrazide (ADH) [95], 1,4-butanediol diglycidyl ether (BDDE) [96-98], β-sodium glycerophosphate (β-GP) [99-101], and transglutaminase (TG) [11]. Cross-linked HLCs are in general mechanically strong and stable and therefore become the most popular scaffold option. Among them, the HLC cross-linked by TG is the most strong and stable, its mechanism and the chemical reaction is shown in Figure 2a. The wet HLC hydrogel obtained (Figure 2b) can achieve excellent flexiblity and water absorption capacity, and therefore can be made into various shapes to meet the different needs in the clinical transplantations.

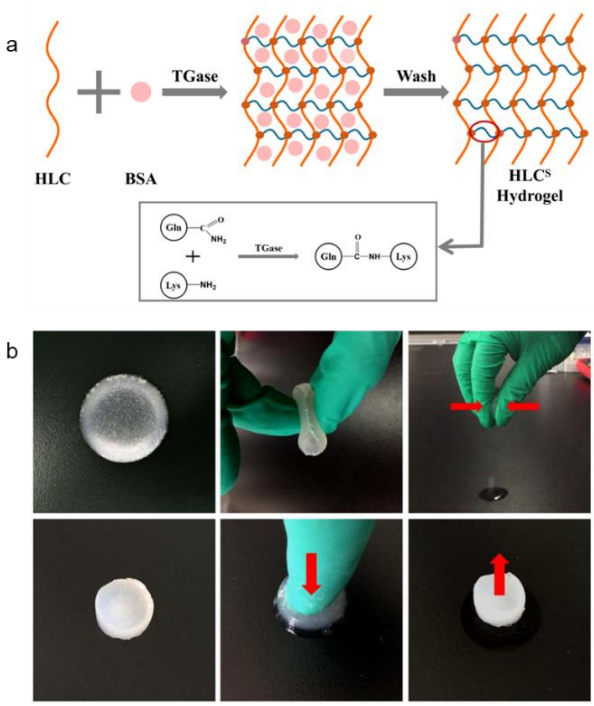

Figure 2. The cross-linked HLC hydrogel [11] (a) The mechanism and the chemical reaction of the HLC hydrogel cross-linked by TG. (b) The morphology of the HLC hydrogel.

In the following sections, we summarised the main applications of HLC in tissue engineering. Although most of the applications were studied on animal models, they presented the great potential for biomedical and clinical applications. The HLC used in all these applications is fundamentally the same. By choosing different cross-linkers and using different methods to link with HLC, the structure feature and performance of HLC are greatly improved with additional properties to fit for the intended applications. All the HLC sequence used in our studies can be required directly to us for research purpose.

3.2.1 Skin repair and skin regeneration

HLCs have been researched extensively on injured skin repair and skin regeneration due to their biocompatibility since it first produced. As early as 2014, Ma *et al*. mixed HLC with chitosan, cross-linked them by dialdehyde starch [71], and obtained a soft

biocompatible hydrogel. This soft hydrogel can effectively fill dermal voids with little or no inflammatory responses.

In 2016, Zhao *et al*. created an injectable HLC hydrogel cross-linked by microbial transglutaminase for skin regeneration [72]. Cross-linking microbial transglutaminase (MTGase, 40U/g of HLC), a nontoxic crosslinker with high specific activity and reaction rate under mild condition, improved the biocompatibility of the HLC hydrogel. In addition, compared with the commercial collagen (Collagen Implant I®, SUM) [72], this new injectable HLC hydrogel had a lower toxicity and better biocompatibility in animal experiments, and thus showed great potential as an injectable hydrogel applied in skin injury treatment.

In 2018, Zhu *et al*. [73] used the transglutaminase (TG) to cross-link the hyaluronic acid, carboxylated chitosan (CCS) and HLC. *In vivo* experiments on the rabbit skin defect model showed the hydrogels obtained in this way had powerful strength (Figure 3), could protect the wound from infection and fluid loss, and therefore effectively promote wound healing.

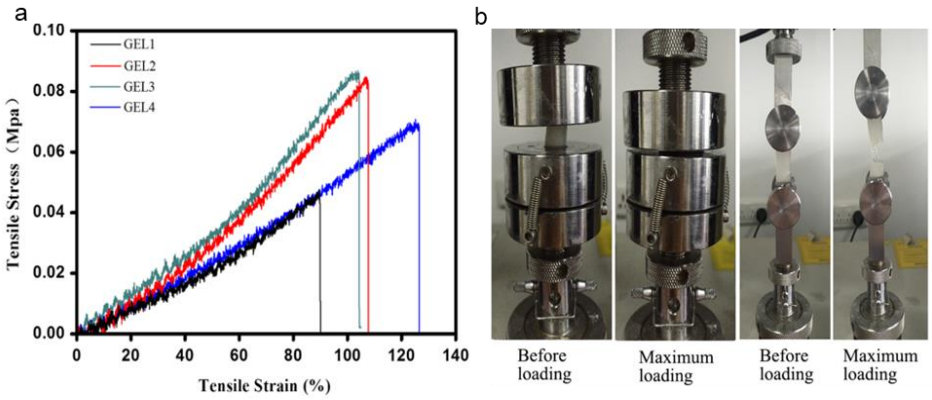

Figure 3. TG cross-linked HLC hydrogels were used in rabbit skin wound healing [73]. (a) Tensile stress-strain curves of the hydrogels; (b) Cyclic compressive mechanical tests.

In 2019, Guo *et al*. [74] developed a TG cross-linked HLC hydrogel loaded with basic

fibroblast growth factor (b-FGF) to repair the defect skin. *In vivo* findings showed that the b-FGF -containing HLC/TG hydrogel can dramatically promote the healing of defect skin and achieve scar less healing in a shorter time. Therefore, this hydrogel provided a safe, moist environment, and fully enhanced roles of b-FGF and HLC/TG hydrogel to promote wound healing. TG cross-linked HLC hydrogels have the advantage of mild reaction conditions which make it possible to be a growth factor and cell's carrier and to be implanted. Most importantly, TG cross-linked HLC hydrogel promotes wound healing without causing side effects. Thus, it provides significant potential for clinical use.

Recently, Pan *et al*. designed breathable haemostatic HLC-based hydrogel dressings and determined their effects on full-thickness defects [75, 76]. Initially, they fabricated a series of soft, flexible, porous non-stick hydrogel dressings through the repeated freeze-thawing of a mixed solution of poly(vinylalcohol) (PVA) and HLC with/without adding with carboxymethyl chitosan and then Tween80 was added as pore-forming agent for cutaneous wound healing (Figure 4a, b) [75]. The combined effects of various functions of the two hydrogels obviously promoted full-thickness skin wound healing when compared to the commercial dressing (Figure 4c). In 2019, they fabricated another novel HLC, PVA and sodium alginate (SA) composite hydrogel as wound dressings (Figure 4e-h) [76]. Their results showed that the hydrogel can accelerate wound contraction in full-thickness wounds of rabbits, promote wound healing and new skin formation, demonstrating its great potential as a wound dressing for skin wound healing (Figure 4d).

In summary, HLCs have great biocompatibility and when aided with proper cross-linkers it can be successfully used on injured skin repair and skin regeneration. In fact, Fan *et al.'s* lab [71-76] has been collaborating with hospitals to start clinical trials of using HLC dressing for skin repair and skin regeneration. There are also some cosmetic

products of HLC to be put on the market.

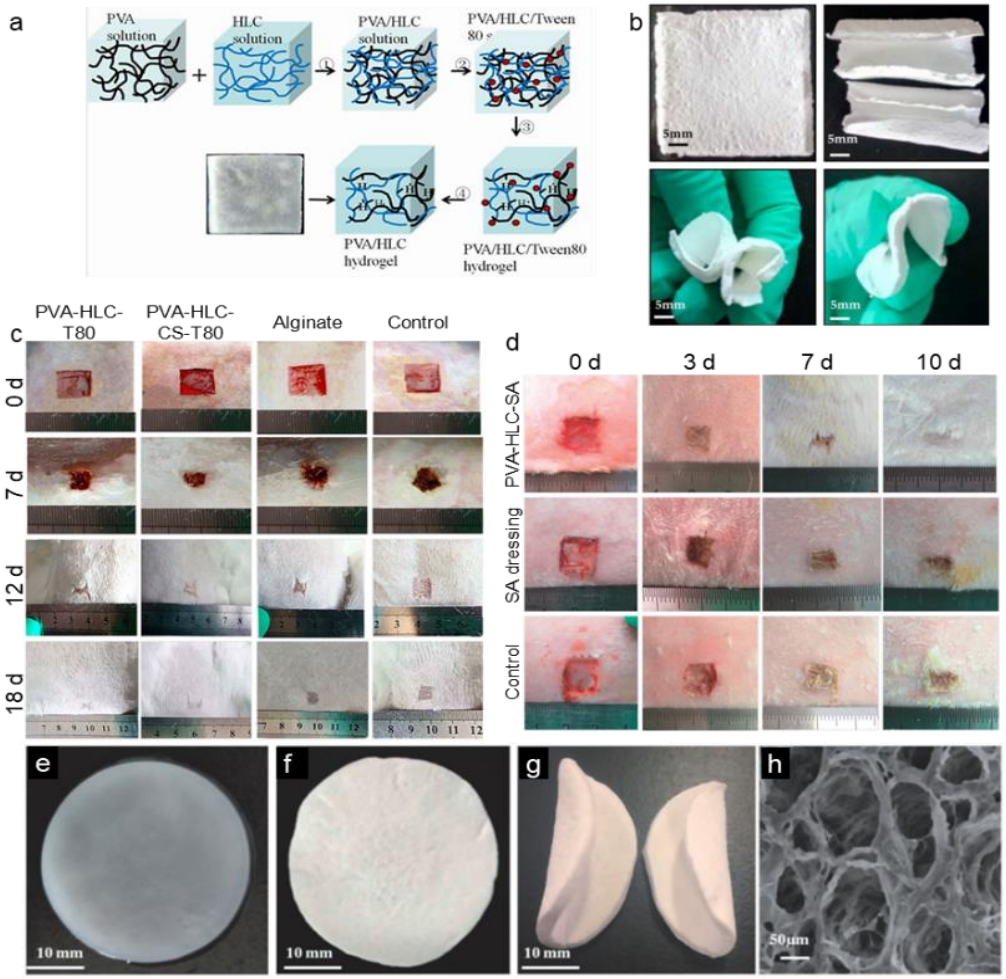

Figure 4. HLC-based hydrogel and its effects on full-thickness skin defects [75, 76]. (a) The formation process of PVA-HLC-T80 ① Solution blending process ② Addition of Tween80 ③ Repeated freeze-thawing process ④ The process of removing Tween80; (b) The hydrogels with Tween80 kept soft after lyophilization; (c) The wound after different time intervals; Morphology of the PVA-HLC-SA hydrogel (e) The hydrogel (f) Lyophilized hydrogel (g) Kept soft after lyophilized (h) SEM images; (d) The wound after different time intervals. PVA: Poly (vinyl alcohol). HLC: Human-like collagen. CS: Chitosan. T80: Tween80. SA: Sodium alginate.

3.2.2 Vascular scaffolds engineering

Series studies since 2009 show that the HLC is an ideal "raw" material on which vascular scaffolds can be fabricated through cross-linking with appropriate materials and by different cross-linking methods [77-78]. This therefore provides great potential for HLC to be used in vascular scaffolds engineering.

As early as 2009, the blood vessel tubular grafts were fabricated by cross-link HLC and hyaluronic acid (HA) with genipin and through the freeze-drying method [77]. This HLC-HA tubular graft had interconnected, well-distributed porous structure, and the porosity reached 94.38%. It also achieved the desirable stress and burst press properties for application standard.

In 2014, Zhu *et al*. [79] investigated the strength variation of the HLC-HA scaffolds using different blending rate of the HLC and HA. They found that the ratio of 10 HLC to 1 HA was an optimal choice. The scaffolds obtained have advantageous properties such as the interconnected porous structure (Figure 5), high porosity (89.3%), better mechanical properties (stress of 321.7±15kPa and strain of 45.5±0.2%), less degradation rate (9%), and excellent biocompatibility. All of the above make it a broad prospective candidate for a blood vessel tubular graft.

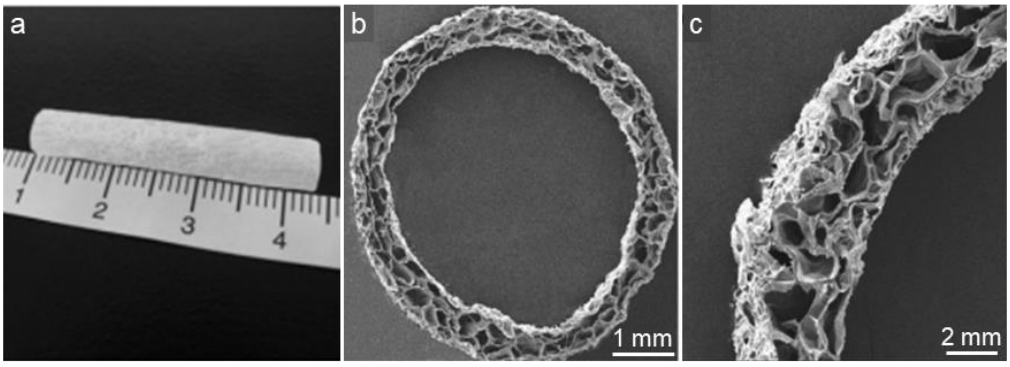

Figure 5. HLC-hyaluronic acid tubular vascular scaffolds [79] (a) Gross observation and (b,c) SEM imagines of the scaffolds.

In addition to using the freeze-drying method, Zhu *et al*. [80] attempted to construct the vascular tissue scaffold using co-electrospun of HLC/CS/PLA (human-like collagen/ chitosan/ poly lactic-acid). The use of PLA could improve the strength of the scaffold, and the CS and HLC could supply a more biomimetic structure and better hemocompatibility.

The natural blood vessel wall is flexible, and one of the main ECM in the vessel is collagen. The recombinant HLC and modified HLC scaffolds have the similar components and properties of the natural blood vessel wall. This makes it an ideal material for developing vascular regeneration.

3.2.3 Cartilage tissue engineering

Collagen is a main extracellular matrix protein in the cartilage, and the recombinant HLC is therefore appropriate for application in cartilage tissue engineering. However, the mechanical strength of the HLC cannot meet that of cartilage. Therefore hydroxyapatite (HA) is usually added into the HLC to form a complex scaffold in order to meet the requirement of the mechanical strength.

For example, in 2013, Jia *et al*. [81] used the HLC as basic materials, and mixed nano-hydroxyapatite (nHA) to fabricate a HLC/nHA composite scaffold with homogeneous and interconnected porous structure. This scaffold can withstand a higher compression stress (2.67 ± 0.37 MPa) than that of the Relive® Artificial Bone (RAB) scaffolds [81], and is beneficial to rabbit chondrocytes adhesion, glycosaminoglycan synthesis, and chondrocyte morphology maintaining (Figure 6a-d). Simultaneously, Fan *et al*. [82] also found that the HLC/nHA scaffolds had excellent cytocompatibility and could maintain chondrocyte spherical morphology.

All the above *in vitro* data warranted the HLC/nHA scaffold as a potential biomimetic platform for chondrocytes in cartilage tissue engineering. But *in vivo* verification is still needed as the HA cannot degrade *in vivo*, which may cause problems.

In 2017, Song *et al*. [11] prepared an HLC only hydrogel scaffold with unique porous structure and used it in rabbit articular cartilage defect repair. 12 weeks post implantation of the HLC scaffolds, the defect in the control group (none of implant) was not recovered (Figure 6e, g), but the defect in the HLC treated group returned to normal (Figure 6f, h). These results further support that the HLC hydrogel scaffold can encourage cartilage repair.

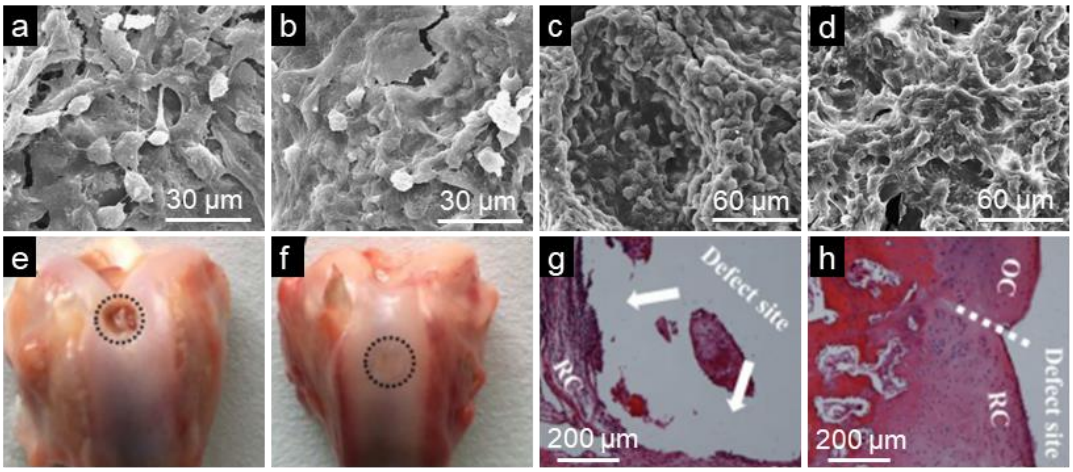

Figure 6. HLC as raw materials applied in cartilage tissue engineering. (a-d) were the *in vitro* experiment results of the HLC/nHA scaffold [81]. **(e-h) were the *in vivo* experiment results of the HLC scaffold** [11]. Chondrocytes on (a) HLC/nHA scaffold, (b) RAB scaffold for 7 days and (c) HLC/nHA scaffold, (d) RAB scaffold for 21 days by SEM. (e, f) were macroscopic images of the cartilage joint 12 weeks after surgery: (e) The control group; (f) The HLC group. (g, h) were histological analysis of the cartilage defect area 12 weeks after surgery: The control group (g) and the HLC group (h) stained with H&E.

Improving the mechanical strength of HLCs is the key for it to be used in cartilage tissue engineering. Choosing suitable materials to meet the requirement of stiffness for the cartilage tissue engineering scaffold is continuous efforts to pursue. This principle also applies to other applications of HLC as well.

3.2.4 Bone defect repair

HLC can be used to repair not only skin, vascular and cartilage tissue damage, but also bone tissue damage. When HLC is combined with natural materials, synthetic materials or nanomaterials, its mechanical strength can be greatly enhanced, and its biodegradation can also be controlled so that they can be used in the bone defect repair.

There are many publications based on HLC added with tough component and used in bone repairing. Calcium phosphate ceramics, metals, or synthetic polymers, such as HA, Ti, NiTi alloy and PLA are usually used to composite scaffolds for bone defect repair [83-90]. For example, NiTi shape memory alloy was used as the basic scaffold. The HLC was then used to modify the surface of the NiTi so as to better biofunctionalise the alloy [87]. The HLC coating benefits the application of NiTi alloy in the orthopaedic field. Li *et al*. [88] further investigated the effect of osteogenesis of the titanium scaffold with HA and HLC coating. Compared with the HA surface layer, the HA/HLC surface layer could promote the survival of the osteoblasts. Notably, the varied concentrations and the methods of HLC addition could all significantly improve the survival rate of the osteoblasts. Therefore, HLC molecules could homogeneously distribute on the HA-coated titanium scaffold and provide a favourable position for the osteoblasts.

Although metal materials such as Ti and NiTi alloy can supply the “hard scaffold” for the bone repair, they will not degrade and will exist in the host for a lifetime once implanted. In order to avoid the foreign body implantation, Fan *et al*. tried to fabricate

a porous composite scaffold with HLC and nHA through cross-linking by oleuropein for bone tissue engineering [89]. When adding the oleuropein solution (2% w/v) to cross-link the HLC and nHA, the compressive strength and Young's modulus of the scaffold reached maximums of 2.97 ± 0.19 MPa and 43.03 ± 6.17 MPa, respectively. Due to the success of the HLC/nHA applied in bone tissue engineering [89], in 2017, Zhou *et al*. [83] fabricated the 3D porous scaffold based on HLC, nHA, biodegradable polylactic acid, and polydopamine (pDA)-assisted BMP-2-derived peptide (nHA/HLC/PLA-pDA-P24). This designed HLC complex scaffold has the stronger mechanical strength, controllable biodegradation rate, and could significantly enhance bone regeneration of the rat cranial defects. The results indicated that the novel nHA/RHLC/PLA-pDA-P24 scaffold was helpful for bone tissue regeneration.

In contrast to the above studies using traditional materials mixing with HLC to promote the mechanical strength and make biodegradation rate controllable, Chen *et al*. recently found that HLCs can specially combine with an osteoinductive agent (BMP-2) to induce bone regeneration rapidly [90]. Due to the special high binding affinity between HLC sponge and BMP-2, the HLC sponge can load with tiny BMP-2 of 1 μg enables complete repair of large areas of rat defective bone within 4 weeks. When the HLC-BMP implant is loaded with 5 μg BMP-2, it can introduce serious bone overgrowth which indicates that HLC seems to have an ability to "magnify" and "distinguish" the tiny changes of dose of BMP-2 used for maintaining osteoinduction and bone formation (Figure 7).

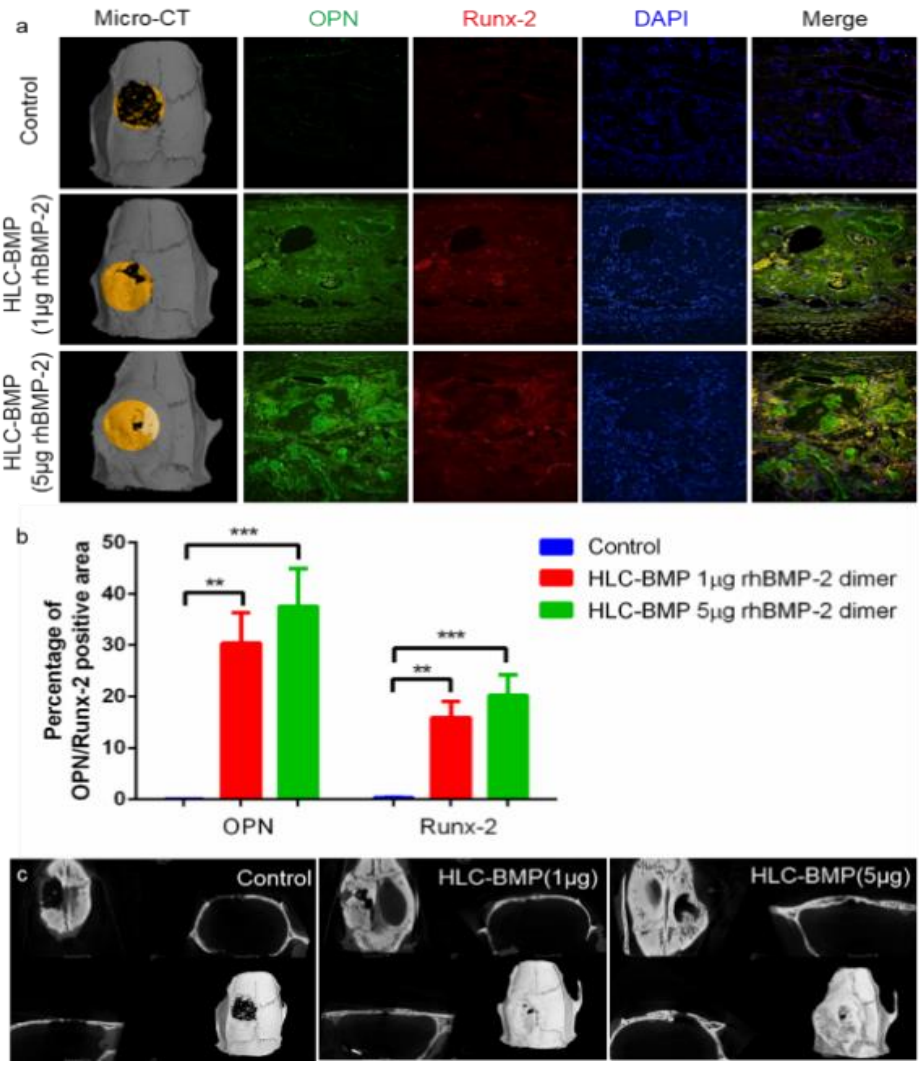

Figure 7. Micro-CT and fluorescent microscopy analysis of the rat cranial defect repair using different dose of BMP-2 at 8 weeks after implantation [90]. (a) Representative micro-CT images and immunofluorescent staining of implants (Control, HLC-BMP sponge loading with 1 μg and 5 μg rhBMP-2 dimer respectively) 8 weeks after implantation. Nuclei were stained blue with DAPI, OPN was stained green and Runx-2 was stained red. (b) Quantitative estimation of OPN and Runx-2 expression in each group (**$p < 0.01$, ***$p < 0.001$, n=18). (c) The raw data of Micro-CT of the rat cranial defect repair using 1 μg and 5μg dose of rhBMP-2 at 8 weeks after implantation. The detailed picture shows that the 1 μg HLC-BMP implant was safe, and that the percentage of repair was up to 88.13 ± 6.01 %. However, 5 μg HLC-BMP implant introduced serious bone overgrowth.

The above results showed that the HLCs could potentially not only be applied in bone tissue engineering but could also be developed into a customisable delivery system for other growth factors. Therefore, it will be extremely valuable for any defect repair and tissue regeneration in both research and clinical applications. We have summarised all these studies in below Table 4. Using different crosslink materials to produce a unique structure along with other growth factor make the HLC extremely valuable in tissue engineering. Continuously optimising the material design to produce more “powerful” HLC will certainly expand its applications in tissue engineering.

Table 4. Application of human-like collagen in Tissue engineering

Materials design	Application	Properties and Effects	Ref.	Year
Microbial transglutaminase - crosslinked HLC hydrogels	Skin tissue engineering	A lower toxicity and better biocompatibility compared with the commercial collagen (Collagen Implant I®, SUM)	[72]	2016
TG cross-linked HLC hydrogel loaded with b-FGF	Repair the defect skin	This hydrogel can dramatically promote the healing of defect skin and achieve scar less healing in a shorter time.	[74]	2019
HLC with hyaluronic acid cross-linked by genipin	Blood vessel tubular grafts	Interconnected and well-distributed porous structure; porosity of 94.38%; high stress of 1000.8 ± 7.9kPa and burst pressure of 1058.6 ± 8.2kPa; excellent biocompatibility and appropriate degradation rate.	[77]	2009
HLC/hyaluronic acid scaffolds	Intima layer for endothelialisation of a vascular scaffold	Interconnected porous network and porosity of 89.3%; high stress of 321.7±15kPa and strain of 45.5±0.2 %; only 9% degradation upon immersion in PBS for 45days at 37°C *in vitro*; excellent biocompatibility.	[79]	2014
HLC/nano-hydroxyapatite composite scaffolds	Cartilage tissue engineering	Homogeneous interconnected structure with withstand of a compression stress of 2.67 ± 0.37 MPa, which was higher than that of Relive® Artificial Bone Scaffolds. encouraged chondrocytes cell adhesion, glycosaminoglycan synthesis by the HLC/nHA scaffolds and maintained natural chondrocyte morphology maintaining	[81]	2013
HLC hydrogel cross-linked by transglutaminase	Cartilage tissue engineering	Effectively repairing rabbit articular cartilage damage with nearly 100% repairing of the defect in the HLC group and only a blurred boundary observed between the defect and the adjacent normal cartilage	[11]	2017
nHA/HLC/PLA-pDA-P24 scaffolds	Bone tissue regeneration	Significantly enhanced bone regeneration of the rat cranial defects by the nHA/HLC/PLA-pDA-P24 scaffolds	[83]	2017

HLC directed growth of hydroxyapatite nanocrystals	Bone repair	The artificial analog of bone fabricated by the collagen fibrils and the HA nanocrystals with a potential clinical application in bone repair	[84-86]	2005 - 2006
HLC modulated the growth of nHA on NiTi alloy	Orthopedic field	Beneficial application of NiTi alloy in the orthopedic field by the HLC and nHA coating with special structure	[87]	2009
HLC and HA -coated Titanium	Provide a favourable position for osteoblasts	Significantly promoted survival of the osteoblasts by the HA/HLC surface layer compared with the HA surface layer	[88]	2015
HLC and BMP-2 complex	A large area of defective bone repair	HLC sponge loaded with tiny BMP-2 of 1 μg, with profiles of a controlled release characterized by a short burst and a steady slow process. self-Osteogenesis *in situ* consequently facilitated with the ability of complete repair of large areas of defective bone	[90]	2019

3.2.5 Injectable human-like collagen used in tissue engineering

Another unique property of the HLC is its injectable. The HLC hydrogel in a syringe can be injected through a needle into any shape of the mold. Therefore, injectable HLC hydrogel can be used in the tissue filling and repairing. After adding crosslinkers, the shape and flexibility of the HLCs could also be changed by different conditions such as different pH and temperature for specific use.

For example, HLC and hyaluronic acid (HA) can be crosslinked with 1,4-butanediol diglycidyl ether (BDDE) to form an injectable HLC/HA hydrogel [96]. Another example of multifunctionalised injectable hydrogel was designed based on HLC and high molecular-weight pullulan [97]. Pullulan hydrogel can be formed through cross-linking by NaIO4, and then coupled with HLC by the reaction between the-NH2 end-group of HLC and the -CHO group present on the aldehyde pullulan to form the injectable HLC/pullulan hydrogel [102]. This injectable HLC/pullulan hydrogel was a safe, soft and suitable option for skin restoration, cartilage treatment, and lacrimal dryness therapy [97, 102].

Li *et al*. [99] designed a novel injectable pH/temperature sensitive chitosan-HLC/β-sodium glycerophosphate (CS-HLC /β -GP) hydrogel. Through controlling the pH and temperature, the shape and properties of the HLCs based hydrogel can be controlled at different conditions. For example, the HLC mixture was in solution when the environment temperature was lower than body temperature while it would become hydrogel at the body temperature [99].

Furthermore, in order to improve the sensitivity and the strength of the CS-HLC-β -GP hydrogel, Li *et al*. added the hyaluronic acid and prepared the chitosan-HLC-hyaluronic acid-β-sodium glycerophosphate (CS-HLC-HA-β-GP) hydrogel [100, 101] with a new

amide bond (-CONH) and -NRH2+. This new hydrogel can change the gelling time and swelling behaviors which are dependent on the intertwining, overlap and adsorption of the polymer chains at various temperatures and pH [100]. It also has a higher sensitivity than the CS-HLC-β -GP hydrogel and exhibits more adequate mechanical strength (1.5-2 MPa) and crosslinking densities (2.5-4.5×10^{-3} mol/cm^3). Therefore, this new hydrogel is more suitable in tissue defect filling.

Due to its sensitivity to pH and temperature changes, flexibility and plasticity, this hydrogel can also be easily used as an injectable filling biomaterial in plastic and reconstruction surgery, and drug delivery.

3.3 Human-like collagen-based hemostatic sponge

Using gradient freeze-drying method [103], HLCs can be processed to be sponges. Like the commercial collagen sponge, the HLC sponge can be used in the operation of the neurosurgery, orthopedics, gynecology, general surgery, operating room, and so on. The HLC sponge using as a filler can stop the bleeding rapidly, prevent wound adhesion, accelerate wound healing, and reduce the postoperative complications. The effects of the HLC haemostatic sponges have been intensively studied in the last decade [28, 91, 92], the representative studies were summarised as below.

As early as 2007, Mao *et al*. [91] investigated the haemostatic effect and histocompatibility of the chitosan-HLC on 1cm×1cm area of the wound surface of the liver of rabbits. In their study, bleeding time and histocompatibility were compared among Chitosan-HLC, HLC and gelatin sponges. Their results showed that the chitosan-HLC produced a shorter and visibly different bleeding time (78s ± 11s) from the gelatin sponge (115s ± 13s, $p < 0.05$). Histocompatibility of the chitosan-HLC was also noticeably better than the gelatin sponge.

In 2014, Meng *et al*. [92] used SD rats with liver haemorrhage model for comparative bleeding tests in order to investigate the haemostatic effect and histocompatibility of HLC. Blooding time (94 ± 13s) with HLC sponge was significantly shorter than that (121 ± 15s) with the purchased microporous starch (used as the control). This result showed that the HLC sponge had a positive effect on the haemostatic area.

In 2017, Jiang *et al*. [28] prepared a novel haemostatic sponge using HLC and glutamine transaminase (non-toxic cross-linker) via "two-step" freezing. The HLC sponge showed a uniform morphology, good biodegradability, and good biocompatibility in implantation tests and had a well haemostatic effect in the ear artery and liver injury models.

In addition, due to its high efficient biodegradability and easily digested though metabolism, the HLC sponge can facilitate seamless healing in the above injury models [28].

The above applications are typical example of the successful application of HLC. To achieve further various applications, modifying HLC with aided cross-linkers to obtain enhance properties will be the main efforts to focus on. With more developments in materials sciences, more new productions are to be expected.

3.4 Human-like collagen used in clinic for skincare

Diversely from being used in the dermis injury repair, HLC has been widely used in epidermis-care which contains promotion of skin elasticity, skin moisture, shrink pores, and so on [104]. In general, the HLCs can be directly daubed on the skin to protect the epidermis. Additionally, the HLCs can also be absorbed in non-woven fabrics and

formed the HLC dressing.

For example, Chen *et al*. [104] investigated the effect of infrared rays combined with HLC dressing to improve skin quality after photoaging with lattice CO_2 laser. In their study, pigment spots, wrinkles, texture, pores, moisture, elasticity and rhodopsin scores from the treatment with infrared ray combined with HLC dressing were significantly better than that with infrared ray alone. This suggested that HLC dressing can improve the quality of infrared ray treatment on the skin and thus provides a new method and a novelty agent for skincare.

The HLC dressings can also be used as a drug delivery system and applied in the treatment of skin diseases. For example, HLC dressings were used to slow-release a traditional Chinese medicine (Biantong Baihu decoction) to treat facial contact dermatitis [105]. The efficiency rate of Biantong Baihu decoction combined with the HLC dressings reached 87.88%, much higher than that of Biantong Baihu decoction alone. Therefore, HLC dressings can efficiently deliver Biantong Baihu decoction and has significant clinical efficacy on facial contact dermatitis [105].

Because the recombinant HLC is biocompatible and similar to natural collagen in the body, there is no allergic contact reaction for patients with sensitive skin. We have developed a skin care product, HLCs (COLLGENE®) and have been successfully used in clinic for cosmetic skin care for nearly 16 years.

3.5 Potential application of Human-like collagen in other diseases treatment

Because the HLC is an edible and biodegradable protein, it can therefore be used to encapsulate oral medicines [106, 107]. The biodegradable HLC is suitable for encapsulation of the intestinal probiotics, which could protect the probiotics from the

gastric environment and release the probiotics effectively in the intestinal tract.

For example, Su *et al*. [108] encapsulated the probiotic Bifidobacterium longum BIOMA 5920 using alginate-HLC (ALg-HLC) and evaluated the B. longum survival in simulated gastrointestinal conditions. Compared with ALg microspheres, ALg-HLC microspheres could better protect bifidobacteria from the simulated gastric juice (SGJ). When the encapsulated B. longum was exposed to simulated intestinal juice (SIJ) of pH 7.0, HLC was dissolved faster than that of ALg in SIJ, which lead to the treatment efficacy of ALg-HLC microspheres being higher than that of ALg microspheres alone in intestinal conditions.

Another example is microencapsulation of phosphorylated HLC-calcium chelates developed based on the phosphorylated HLC (PHLC) calcium complex [109], to control PHLC-Ca delivery and improve their bioavailability [110]. The ALG and CS were used as wall materials to protect and control the release of the PHLC-Ca. The PHLC-Ca was entrapped into the matrix of ALG through forming intermolecular hydrogen bonding or other interactions. Therefore, the bioavailability of the PHLC-Ca was improved by CS/ALG. This will stimulate new thoughts for future proteins calcium supplements.

Recently, Li *et al*. [111] fabricated HLC-based hydrogels using microbial transglutaminase (MTGase) and the 1-ethyl-3-methylimidazolium acetate ([EMIM][Ac]). They found that the development of this enzymatic hydrogel with controlled biodegradation rates can meet the needs of specific potential applications, such as tissue engineering and cancer therapy (Figure 8).

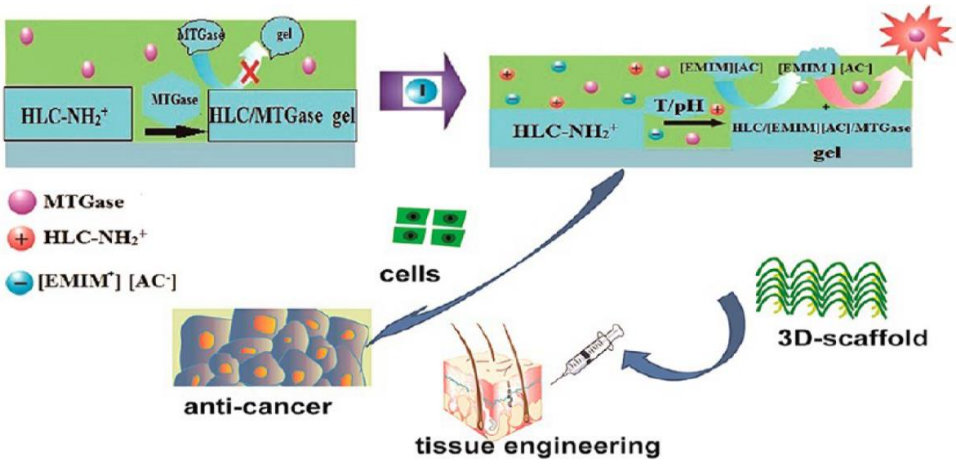

Figure 8. Synthetic mechanism and potential applications of HLC-based hydrogels by MTGase/[EMIM] [AC] [111].

Further application of HLC is to coat medical nanoparticles for the treatment of disease. After coating with the HLCs, the biocompatibility and bioavailability of nanoparticles can be improved which in turn greatly improve the efficiency of the medical nanoparticles [112-115]. For example, HLC-coated Fe_3O_4 nanoparticles were successfully prepared to test its effect on heat induction and cell toxicity [112, 113]. After coating of HLC, even with a reduced saturation, magnetisation temperature can increase at a faster rate under an alternating magnetic field. This might be due to the effective heat conduction and good colloid stability caused by the high surface charge of HLC. In addition, HLC-coated Fe_3O_4 nanoparticles can induce less cell toxicity at a higher concentration. Therefore, the effective heat induction can be achieved for example in cancer treatment with improved biocompatibility [112, 113].

Another example of this application is using the HLC to deliver iron effectively. Low iron intake and/or bioavailability cause worldwide iron deficiency. Fe_3O_4 are rich in iron but have poor bioavailability. Zhu *et al*. [114] used a thiolated HLC to protect the Fe_3O_4 and maintain the iron effectively. They found that the thiolated HLC-iron had less cytotoxicity and higher bioavailability than the bare iron. This ensures the thiolated

HLC-iron as a more effective iron supplement for clinic in the future [114, 115].

The above examples illustrated the great potential of using HLC as an effective deliver system in clinical applications. Mass production ability of HLC would make furthermore development possible and surely foresee more applications in the future.

4. Summary

Both the recombinant sources of human collagen and human-like collagen are reliable, predictable and chemically defined source of purified human collagens and they are animal-free. However, compared with the recombinant human collagens, the recombinant HLC is more stable, degrades slowly, and is easy to purify [20, 26-32]. The HLC has good biocompatibility, no immunogenicity, stabilized property and can be mass produced. It's exciting that some HLCs have already been utilized in the clinics. However, except applications in hemostatic sponge, skin injury treatments, and skin-care [104, 105], most of the HLCs applications still remain in the stage of animal testing, such as vascular scaffolds engineering [77-80], cartilage [81, 82] and bone defect repair [83-90], and other diseases treatment [106-111].

Using properly chosen cross-linkers, improved properties of HLC could be expected, and more biomedical and clinical applications of HLC should be able to be developed in the future (Figure 9).

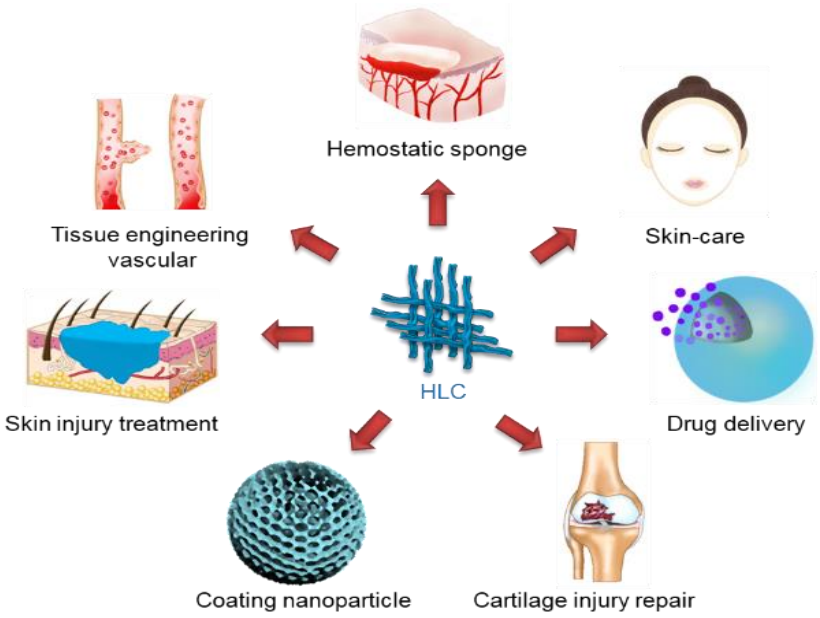

Figure 9. Potential applications of the HLCs in biomedicine. Due to its good biocompatibility, no immunogenicity, stabilised properties and mass production ability, HLC has been widely researched in skin injury treatments, vascular scaffolds engineering, cartilage and bone defect repair, haemostatic sponge, skincare, drug delivery, coating of medical nanoparticle, etc.

5. Future improvements

Compared to naturally derive animal collagen which show variation in quality, purity, predictability of performance and the risk of transmission of infectious, collagens defined recombinant human collagen eliminate disease risk. Additionally, recombinant technology allows collagen produced with significant quantities, new and non-native structures, including chimeric molecules and novel designed structures. Presently, many expression systems have allowed trials on concepts, but few systems have been proven to be successful as yet for commercial scale production of new biomaterials [116]. Full length recombinant human collagens have been successfully expressed in cell lines, yeast, and several plant systems, while collagen fragments have been

expressed in *E. coli*. In addition, bacterial collagen-like proteins (e.g. HLC) can be expressed in high yields in *E. coli* and easily manipulated to incorporate biologically active sequences from human collagens [117]. Although the yield of HLC in Fan *et al*. [19, 30] has reached 14 g/L in a 500 L fermentor and the HLC has many biomedical applications, there are still many aspects which can be improved so that the production of HLC can be easier, the quality of HLC can be more qualified, and the designing of HLC can be more flexible. With these improvements, the application of HLC will become more precise and efficient in each area of biomedical application. Here we have focused on some considerations displayed in our laboratory to improve the production of HLC as it is the key for the successful application. Without enough high quantity and easily made HLC there are limits to its potential applications.

5.1 Optimal use of collagen cross-linker

HLC need to cross-link with different cross-linkers such as the DAS [71], oleuropein [89], EDC [93, 94], EDC and ADH [95], BDDE [96-98], β-GP [99-101], and TG [11] to adjust the mechanical properties of the HLC scaffolds. Up to now, TG is one of the best cross-linkers for HLCs' crosslink. TG is an inherent enzyme in the human body, which is degradable, biocompatible. Most importantly, TG can work in the mild reaction condition (4℃ overnight) that can remain the activity of the protein. However, the mechanical strength of the HLC can't reach the stiffness of bone and cartilage simply through the cross-linker. Therefore, optimal use of cross linker with specific materials such as BMPs in different environment is the key to meet specific need in those applications [83, 90]. The fast reaction time during the cross-link is another future need to be improved. At the present, the time for HLC and the crosslinkers to form shape in normal human body environment is too long which greatly limits its applications. Therefore, it will be crucially important to develop novel cross-linkers and cross-link methods such as those can adapt the environment of human body to extend

the application of HLCs.

5.2 More choices of expression systems for HLC

Up to now, the HLCs are expressed by *E. coli* which belongs to prokaryotic cell. The prokaryotic cell may bring the prokaryotic cytoplasm when collecting the HLCs. Some of the products from the prokaryotic cell such as bacterial endotoxin is harmful for humans [118, 119]. In order to avoid this potential risk, a eukaryotic expression vector such as a yeast system needs to be considered to construct and express the HLCs. Besides, using yeast expression vectors to express HLCs in the supernatant of the fermented medium this could make HLCs purification easier than using *E. coli* vectors. Currently Fan *et al.*'s laboratory can express recombinant human prolyl with human collagen α1 (III) chains in Pichia pastoris GS115 [50, 52]. In the future, the safer HLCs will be expressed in more eukaryotic expression vectors and will be purified more easily.

5.3 Better purification and quality of HLC

The purity of the HLCs is still not very high in present purification methods, such as salting-out method, ultrafiltration, ion exchange chromatography and some traditional purification methods. In the future, new purification methods should be developed. Besides, the low purity of the HLCs leads to screen the active HLCs inefficiently. In order to obtain active HLCs more efficiently, the frontal affinity chromatography-mass spectrometry (FAC-MS) [120] needs to be used to study the interaction between the HLC and other cell markers and therefore to improve the effectiveness of screening the active HLCs.

5.4 Flexible designing of HLC

The last but not the least, the variability of the potential HLC could be further achieved. As the gene template of the HLC is adjustable, designing new sequences to get variable HLCs with new properties should be exploited. For example, the length of the sequence of the collagens can be made longer, the HLCs will then be more easily cross-linked and the HLCs hydrogels will be more flexible, elastic, and stronger. More lysines can be added in the HLC sequence to promote the cells adhesion. Active peptides (RGD, elastin-like polypeptide etc.) [121-123], growth factors (VEGF, EGF etc.) and other proteins (heparin, integrin etc.) [124] can be used to recombine with the HLCs and express the multifunction fusion proteins. Based on the variability of the HLCs, the HLCs' gene code can be designed to obtain recombinant HLCs with properties needed. For example, recombinant mini-collagen containing integrin binding site [125, 126], collagen-mimetic peptide sequences [127-130], etc. These purposed designed HLC could be used for optimising previous successful applications and further extending to other intended applications.

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