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REVIEW

Insights into the Emerging Collagen-based Bio-ink in 3D Bio-printing for Tissue Engineering: A Short Review

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ABSTRACT

3D-bioprinting is a new technology for creating precise computer-aided design and shape of any human organs, which has the potential to expedite wound coverage and closure. However, the development of complex tissues and organs in 3D printing is till at an infant stage, primarily due to several hurdles, such as optimization, biomechanical stability, and printing resolution. Collagen is natural polymer, which found abundantly in the extracellular matrix (ECM) and exhibit excellent biological properties. These collagen-based bio-inks can be tailored for different purposes, including wound healing, tissue engineering, organ transplantation and drug delivery systems. Until now, thermoplastic collagen/collagen bio-inks are limited to use in additive manufacturing (AM). The adaptation of thermoplastic collagen/ collagen bio-inks in AM techniques is therefore a great concern. The use of thermoplastic collagen and collagen-based bio-ink/powder in additive manufacturing can open up new applications in biomedical industries. In this context, this review summarizes the development of 3D bio-printing, its potential biomedical applications, and current challenges in the field.

KEYWORDS

Collagen; thermoplastic; 3D-bioprinting, bio-ink.

INTRODUCTION

To meet the extending demands, challenges, customization of product, low-cost market, and improved products futility, global corporate companies' goal to expedite their product designs, development and processing to grasp the market faster. To achieve this, the rapid prototype technology (RPT) was implemented for the first time for commercial purposes in additive manufacturing (AM) [**1**], also denoted as three-dimensional (3D) printing, which permits building up fragments by accumulation of materials such as metals, ceramics, polymers and origin of biological materials *via* layer-by-layer technique [**2**]. AM technology is potential and cost-effective, which provides various medical products by changing the supply chain of complex. Some of the additive manufacturing techniques such as Direct Metal Tooling, Fused Deposition Modelling, Powder Bed Inkjet, Selective Laser Sintering, Stereo-lithography and Inkjet printing [**3**]. Among them, 3D bio-printing is one of the emerging fields to fabricate different human organs/functional organs or tissue structures including bone tissues or implants. 3D bio-printing mimics biology through self-assembly and mini-tissue blocks. 3D bio-printing has brought about important advancement, empowering specific control over factors such as pore size distribution, complex structural designs, pore volume and the interconnectedness of pores within scaffolds/implants. This intricate tissue architecture can be meticulously connected using computer-aided design (CAD), leveraging complex geometric data attained from medical imaging techniques. Furthermore, bioprinting methods exhibit similarity to the fused deposition modelling (FDM) approach, as they involve depositing various materials, including aqueous solution, hydrogels, biopolymer dispersions or viable cells from a cartridge in the form of droplets into the building platform. Furthermore, it has the

potential to apply in tissue engineering using numerous biomaterials or bio-ink [**4**]. In this direction, researchers have focused mainly on collagen thermoplastics and collagen-based various bio-inks in additive manufacturing. Thermoplastic based collagen is derived from collagenous tissues, including bovine and porcine hide, often after the dehairing method and it forms an essential component of the extracellular matrix of connective tissues, such as bone and skins. The production process of thermoplastic collagen such as denaturation, milling and drying in order to get fine powder form, which is suitable for 3D-printing. An appropriate collagen blends for thermoplastic processing includes water as an important plasticizer, alongside additional optional additives like glycerol, dyes/inorganic salts. Through the process of extrusion and/or injection molding at a suitable temperature, thermoplastic collagen can be made into pellets, threads, sheets, films or any desired form. Thermoplastic collagen exhibits superior mechanical stability and rapid melt solidification, leading to exceptional dimensional stability. Accordingly, it holds significant promise for many biomedical related applications, including the growth of implants, tissue engineering and regenerative medicine [**5**]. Another interesting material is bio-ink, which is important in 3D bio-printing. Collagen and collagen-based materials have been employed as bio-inks owing to their excellent bioavailability and stability. Cameron *et. al.,* reported that the biopolymer gellan gum-based bio-ink was produced as microgel in standard cell culture media and used to print multicellular structures and functional tissues [**6**]. Sofia *et. al.,* developed silver-nanoparticles reinforced 3D-printed collagen for cost-effective antimicrobial scaffold. To the best of our knowledge, there are limited reports available on collagen thermoplastic and collagen-based inks for usage in additive manufacturing for biomedical applications [**7**]. Therefore, this review discusses the importance of collagen-based thermoplastics and various collagen-based bio-inks and their properties in additive manufacturing process for biomedical applications.

Collagen-based inks in 3D bio-printing for tissue engineering

Collagen-based inks have been used in both additive as well as non-additive manufacturing processes, which typically include castings, electro-spinning, wet-spinning and melt spinning techniques. Casting is performed by pouring a liquid material into a mold in order to get desired shapes before solidifying. A typical collagen-based biomaterials of highly porous 3D structures is obtained *via* freeze-drying process using a mold under vacuum. Collagen sponges are widely used in wound-healing and skin tissue-engineering as scaffolds for bone, skin, and soft tissues. Collagen-based inks in extrusion plays an important role during printing process. Particularly, collagen bio-ink blended with agarose gel in cell-laden printing increase on mesenchymal stem cells with banquet morphology, which results in osteogenic differentiation [**8**]. Successful 3D-printing approaches encompass a 3D printing step, which is mountable, reasonable, and adaptable to a wide range of bio-inks that are biodegradable, biocompatible and possess enhanced mechanical strength and biologically active mimicry. [**9**]. Type-I of collagen is the main protein that constitutes of soft tissues/base materials for tissue-engineering related applications. It also supports differentiation, cell adhesion, and other natural developments, which own the regenerative approach during wound healing. Very recently, the FDA approved the use of collagen membranes infused with autologous chondrocytes for the purpose of regenerating cartilage. Collagens are extensively used as sponges in tissue engineering applications. Type-I collagen is D-banded fibres under physiological condition, which are energetic for the natural bioactive epitopes that provide an anisotropic strength and stiffness. These properties can be significantly play role during 3D printing when the type-I collagen casted in specific geometrics for scaffold customization [**10,11**]. Kathryn *et. al*., reported that the thermo-responsive and photo-cross linkable collagen-based bioink for free-form production of scaffold for reformative medicine. They have used improved type-I collagen methacrylamide as a bio-inks for free-form construction of scaffold. The production of methacrylamide collagen using free-form manufacturing enables the bioprinting of macroscale structures and the customization of scaffolds with precise patterns, offering potential benefits in the field of skin tissue engineering [**12**].

The wound healing process traditionally entails excising a small piece of skin from a secondary surgical location, stretching it and re-applying the graft to the specific wound and/or burn. While this method often yields clinically realistic results, its effectiveness is constrained when dealing with extensive wounds due to limitations in donor sites. Allografts offer an alternative, but they necessitate immunosuppressive drugs to prevent skin graft rejection. Recent advancement in tissue engineering have led to the development of more intricate biological skin equivalents that are better suited for wound treatments.

Generally, Polymer scaffold patches like Apligraf, TransCyte and Dermagraft are pre-cultured with human fibroblasts before use. However, these graft patches are so expensive to produce and it suffers from the same immunological drawback.

Instead, cell-spraying and 3D bio-printing technologies have newly been commercial for wound treatments/tissue engineering applications. In this direction, Skardal *et. al.,* reported 3D bio-printed amniotic fluids from stem cells quicken the healing of skin wounds [**13**]. Bio-printing technique, shown in **Fig. 1(a)**, constantly yielded a fibrin and/or collagen-based gel, which provided 100% exposure over the wound area and effectively formed a close-fitting seal with the skins at their edges. Alternatively, the live cells are mixed with hydrogel and placed over the wounds *via* a nozzle-spray as shown in **Fig. 1(b)**.

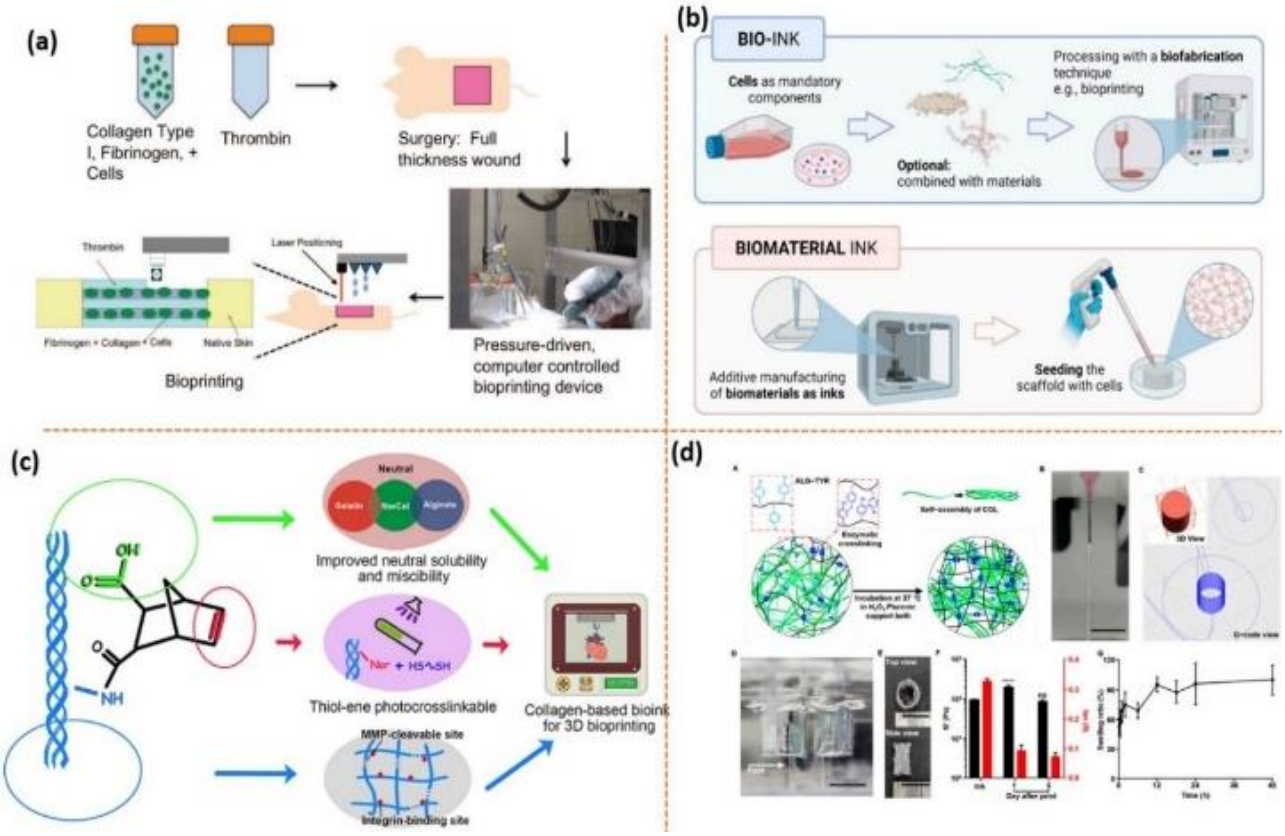

Fig. 1. Schematic diagram of fluid of amniotic from stem cells based bioprinting [13], (b) Bio-ink as cell laden and biomaterial inks used as cell free, cells were presented within the 3D bio-printed biomaterial based scaffold [14], (c) Structure of NorCol and design to expand printability and bio-activity [15] and (d) The 3D bio-printing of ALG-TYR/COL ink [16].

In the bioprinting field, it is essential to differentiate between bio-inks as cell-laden and biomaterials inks as cell-free, hence, biomaterials making bio-inks must serve as cell carriers for cell delivery during the preparation and bioprinting technique (see **Fig. 1(b)**). Kai *et.al.,* also reported that the collagen altered thiol functionalized norbornene photoclick based bio-ink for admirable bioprinting. Norbornene is chemically altered with neutrally soluble collagen (NorCol) *via* a reaction involving acidically soluble collagen and carbic anhydride within an aqueous environmental condition. NorCol can maintains collagens triple-helical structure and swifty forms a cell-laden hydrogel by using a cell friendly thiol-ene photo-click reactions. In the high temperature extrusion bio-printing technique, the bio-ink formulation consisted of 0.6 % w/v of NorCol, 3 % w/v of gelatin, 3 % v/v of glycerin, 0.025 % w/v of LAP and 4mM of HS-PEG-SH. A 0.5mL volume of this bio-ink was loaded onto syringe, allowed to equilibrate at 18 ^{o}C for 10 minutes, and then extruded at 18 ^{o}C, controlled by a temperature controller [**15**]. Sung *et. al*., also reported that the tyramine modified with alginate-based collagen hydrogel bio-inks for 3D bio-printing. 3D bio-printing of tubular concepts with tuneable gelation related kinetics *via* regulating the covalent bonding density and tyramine gelation time, which can be altered alginate hydrogels (ALG-TYR) through enzymatic-reaction *via* horseradish peroxide (HSP) and hydrogen peroxide. The bio-ink formulations are moderately cross-linked between phenol, which undergo further cross-linking after bio-printing, results continue filament in 26G needle extrusion *via* injectable hydrogel for 3D bio-printing, storage modulus and damping (tan δ) factor of the bioink before bio-printing with high resolution. (see **Fig. 1(d)**) [**16**]. Covalently crosslinked bioinks have high-shape reliability in dimensional printing (approximately 6 mm hight) and the cell adhesion locations of scaffold, which allow the cell proliferation. Also, various bio-inks used in different bio-printing techniques, cell type, growth factor, and cell types toward tissue engineering have been tabulated in **Table 1**.

Collagen thermoplastics in 3D-bioprinting

Among all the biomaterials, denatured thermoplastic collagens possess unique position. Thermoplastic based collagen can be in dry powder form, which prepared through thermoplastic techniques like extrusion or injection molding techniques. The production process of thermoplastic collagen can be converted into powder and the following steps can be adopted such as denaturation stages, drying followed by milling with appropriate plasticizer and other additives can be added and operated at

Table 1. Summary of various bio-inks used in different bio-printing techniques for tissue engineering [17].

Biomaterials/bio-inks	Cell types	Cell sources	Growth factors/ biomolecules	In-vivo method	Bio-printing technique
Collagen hydrogels	Keratinocyte fibroblast,	Human skins	Keratinocyte growth supplement	-	Solid free-form fabrication
Collagen hydrogel precursors	Melanocytes, fibroblast, keratinocyte	-	-	-	Extrusion
Collagen and fibrinogen	AFS	Amniotic based fluid	Thrombin	mice	Ink-jet
Collagen hydrogel/gelatin/ PCL	Fibroblast, keratinocyte	Human skin	-	-	Extrusion and ink-jet
Gelatin methacrylamide	keratinocyte	-	-	-	-
Gelatin-fibrinogen-alginate	Fibroblast, keratinocyte	Human skin	-	-	Extrusion
Collagen type-I and fibrinogen hydrogel	Fibroblast, keratinocyte	Human skin	Thrombins	Porcine wound model	Ink-jet
Gelatin-alginate	Mesenchymal stem cells	Rat	-	mouse	Extrusions
Gelatin-silk fibroin	Child foreskin fibroblast	Human	FGF-2	rat	Pneumatic bio printing

moderate temperature (90-100 °C). Currently, utilization of thermoplastic-collagen in additive manufacturing is very limited. The concurrent influence of thermal related energy and mechanical stress, which is characteristic of this technique, is considered a requirement for the melting of collagen thermoplastics. The use of collagen thermoplastic as a powder form in additive manufacturing will be a new paradigm to produce novel and cost-effective products. In this direction, for the first time, Meyer and co-workers used thermoplastic collagen in additive manufacturing process by using Bio scaffolder (GeSiM mbH). The molten collagen was dropped into a building platform, which forming different object shapes. Another recent study was reported on 3D printable thermoplastic collage (TC) for biomedical application by Marina and co-workers [**18**]. Further, they studied the TC scaffold and their mechanical properties such as increased tension/compression and decrease loss modulus (G''), dynamic viscosity (η), storage modulus (G') with compressive strength was between 3-10 MPa. On typical, the surface roughness of the sample was estimated to be around 2.15 ± 0.43 µm and different top view and side view of 3 D printed TC are shown in **Fig. 2**.

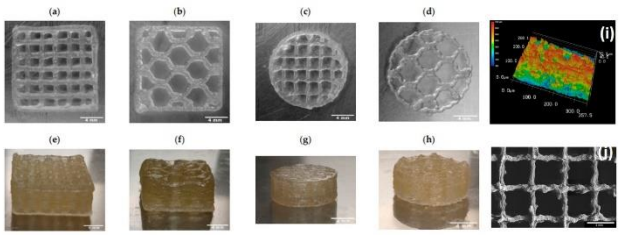

Fig. 2. Outline of the 3D bio-printed TC scaffold with 18 layers top view (a-d) and Tc scaffold side view (e-h), 3D laser scanning microscope with 200X magnification (i) and ESEM image of (j) 3D printed TC scaffold top view [18].

The TC scaffold could be used as a bone repairing material with biological properties due to their high porosity and higher pore connectivity, which are favourable for proliferation of cells and tissue in growth. Furthermore, thermoplastic collagen [**20**] is anticipated to be both bio-compatible and bio-degradable.

Lode *et. al.*, also [**19**] recently reported the additive manufacturing of collagen scaffold using 3D plotting of high viscous dispersions. Additive manufacturing enables the fabrication of patient-specific 3D based structures with defined geometry and interior pore architectures. However, creating pre-defined collagen scaffold through additive manufacturing poses challenges due to the lower viscosity of commonly used collagen solution, gels, and/or dispersions. The fabricated collagen scaffold displayed precise shapes, dimensional reliability and a hierarchical porosity with macro and interconnected micropores. Cultivating mesenchymal stromal cells (human) on these scaffolds showed excellent cytocompatibility for adipose and bone tissue engineering applications.

CONCLUSION & FUTURE PROSPECTIVE

One of the primary objectives of this review is to briefly highlight the developments in collagen-based ink and collagen thermoplastics, which are typically used in additive manufacturing. Mechanical characteristics of bio-ink demonstrate that research into the collagenous materials is necessary to use in the development of advanced 3D bio-printing techniques. Collagen biopolymer is not only bio-compatible but also has the advantages of encouraging improved cell-material relations after incorporated in polymeric solution. In spite of this exclusive advantages of collagen as bioink formulation, there is a requirement to further improve the dynamic interaction between natural

tissues and the environments. It is predictable that many research works will be started in the field of 3D bio-printing approaches. The growth of such smart bioinks/ thermoplastic collagen may afford a new avenue for intermediate changes within the cell laden, therefore enhancing efficacy in tissue engineering. Not only collagen-based bio-ink, collagen thermoplastic in the form of powder in additive manufacturing process is still very limited. Therefore, the significance of collagen ink as well as powder used in additive manufacturing process should be explored extensively.

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CONFLICTS OF INTEREST

All the authors approved that there is no conflict of interest of this manuscript.

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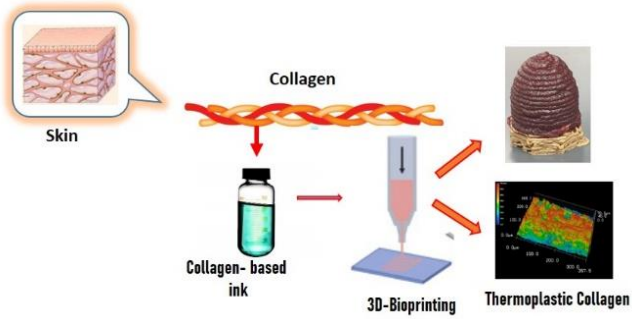
GRAPHICAL ABSTRACT



- Developments in collagen-based ink and collagen thermoplastics, which are typically used in 3D bio-printing.
- 3D bio-printing is one of the emerging fields to fabricate different tissue structures including bone tissues or implants.
- Cost effective Collagen scaffold in tissue engineering.