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3D printing tissue-engineered scaffolds for auricular reconstruction

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ABSTRACT

Congenital microtia is the most common cause of auricular defects, with a prevalence of approximately 5.18 per 10,000 individuals. Autologous rib cartilage grafting is the leading treatment modality at this stage of auricular reconstruction currently. However, harvesting rib cartilage may lead to donor site injuries, such as pneumothorax, postoperative pain, chest wall scarring, and deformity. Therefore, in the pursuit of better graft materials, biomaterial scaffolds with great histocompatibility, precise control of morphology, non-invasiveness properties are gradually becoming a new research hotspot in auricular reconstruction. This review collectively presents the exploit and application of 3D printing biomaterial scaffold in auricular reconstruction. Although the tissue-engineered ear still faces challenges before it can be widely applied to patients in clinical settings, and its long-term effects have yet to be evaluated, we aim to provide guidance for future research directions in 3D printing biomaterial scaffold for auricular reconstruction. This will ultimately benefit the translational and clinical application of cartilage tissue engineering and biomaterials in the treatment of auricular defects.

1. Introduction

Auricular defects with a prevalence of approximately 5.18 per 10,000 individuals are typically caused by congenital microtia, or acquired in the case of trauma, burns or following excision of skin cancers [1–3]. It is well established that the ear is divided into three parts: the outer ear, the middle ear and the inner ear. Each of them performs specific and unique function to ensure normal hearing. The outer ear is composed of the auricle and the external auditory canal. Auricle, consisting of skin, cartilage, and a small amount of fat is the initial component of the human hearing organ to facilitate the collection of sound and its subsequent transmission to the external auditory canal [4]. The external auditory canal is a tubular structure that extends from the auricle to the eardrum, which serves to convey sound from the external environment to the middle ear. Therefore, auricle plays a vital role not only in physiological processes but also in aesthetic considerations. The shape and appearance of the auricle are of significant importance to the patient's psychological and social adjustment. Furthermore, the outer ear serves social functions such as supporting eyeglasses, covering hearing aids or displaying jewelry [5,6]. Quality of life indicators as well as psychosocial state can be improved with auricular reconstruction or prostheses [7]. Due to the unique shape and delicate physiology of the auricle, auricular reconstruction is still a challenging treatment in clinic.

Currently, the most prevalent strategy for auricular defects in clinic

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is focused on the reconstruction of an ear scaffold platform using autologous rib cartilage or artificial scaffolds [8]. Draw back to 1959, Tanzer et al. [9] pioneered the use of autologous rib cartilage grafting to carve an ear scaffold, resulting in a well-shaped auricle. Autologous rib cartilage grafting thus became the leading treatment strategy for auricular reconstruction since then as its homogenous transplantation offers excellent biocompatibility and maximally eliminates the risk of immune rejection. However, the number of autologous rib cartilage is limited to think over the whole-body homeostasis. Besides that, the surgery requires a highly adept operator to collect the rib grafting in case of complications such as thoracic deformity, pulmonary atelectasis, and lung infection [10]. Therefore, artificial scaffold (*e.g.* Medpor) being made of porous high-density polyethylene is emerging as an alternative candidate and quickly became the more preferred choice in current clinical practice. Artificial scaffolds eliminates the pain and injury associated with obtaining autologous rib cartilage and significantly reduces the surgical difficulty in compare with autologous rib cartilage grafting. However, it is important to note that scaffold with such polyethylene-based matrix always exhibits high rigidity and may has issues such as poor flexibility, and associated problems like stent exposure, breakage, and infection after implantation [11,12]. As a result, artificial scaffolds made of other synthetic polymers have emerged as an alternative in recent decades, and many studies have begun to introduce chondrocytes in combination with suitable artificial scaffolds to construct new bio-scaffolds [13–16]. For instance, Prof. Jiang's team has successfully applied tissue-engineered ear cartilage based on polyglycolic acid/polylactic acid and chondrocytes to clinical applications [17]. The construction of scaffolds conventionally involves inoculating chondrocytes on bio-scaffolds for *in vitro* and *in vivo* subcutaneous cultures. However, conventional scaffolds are biodegradable and may collapse to varying degrees over time. Additionally, the acidic hydrolysis products of these polymeric materials may cause sterile inflammation in some cases [18,19]. Furthermore, conventional cell inoculation methods in scaffolds pose a significant challenge due to the difficulty in achieving uniform cell distribution and ensuring cell activity [20], which presents a major obstacle in auricular reconstruction. Besides that, in order to achieve uniform cell distribution, the personalized fabrication of such bio-scaffolds is time-consuming and the quality cannot be precisely controlled and adjusted.

As a result, the use of Computer-Aided Design/Manufacturing (CAD/CAM) techniques is becoming increasingly essential. In recent years, three-dimensional (3D) printing technology based on this CAD/CAM has been widely used in various medical fields [21–25]. The pursuit of better auricular reconstruction results and prognosis has led to a new research focus on improved graft materials. This technology which allows for the rapid production of patient-specific, anatomically realistic 3D bio-scaffolds has shown promise in the field of auricular reconstruction [26]. The aim of 3D-printing strategy for tissue-engineered auricular reconstruction is to creating human auricular cartilage with personalized morphology and fulfilled synchronous printing of seed cells with chondrogenic capabilities and the biomaterial scaffolds matrix. The resulting product is then implanted into the body to repair defects and replace autologous tissue [27–29]. The chondrocytes presented in the auricle are responsible for the synthesis and maintenance of the extracellular matrix (ECM). Chondrocytes play a crucial role in the maturation and repair of cartilage. The optimal characteristics of tissue-engineered auricles are defined by their biocompatibility, mechanical stability and biofunctionality. Currently, a team of researchers has used hybrid bioprinting for external auricle reconstruction [30], applying novel human auricular cartilage progenitor cells in bioprinting to construct an auricular structure, and the results show that this strategy not only well maintains the mechanical properties of the auricular structure, but also enables the chondrocyte progenitor cells to produce an abundant cartilage-like matrix throughout the auricular structure, which offers hope for regenerative medicine strategies for auricle reconstruction.

Hence, in this review, as indicated in Fig. 1, we systemically present the criteria construction for designing tissue-engineered auricles and provide a comprehensive overview of the recent advances and application areas of these biomaterial scaffold fabricated through 3D printing. Specifically, we firstly discussed the design of tissue-engineered auricles in terms of cell source and selection, scaffold material selection and design, and 3D printing technology. Then, we summarize the progresses of current research and provide an outlook on future research directions. Overall, this review is aim to provide guidance for future research directions in 3D printing biomaterial scaffold for auricular reconstruction. We hope this review will ultimately benefit the translational and clinical application of tissue-engineered auricles in the treatment of auricular defects.

2. Criteria construction of tissue-engineered auricles

2.1. Cell source and selection

Auricular chondrocytes are a type of elastic cartilage that contains an elastin network within extracellular matrix. This network helps maintain the auricular cartilage and prevents it from ossifying throughout life. Several studies have suggested that auricular-derived chondrocytes are the most suitable for auricular cartilage reconstruction due to their ability to spontaneously secrete cartilage-specific matrix [31–34].

From a clinical perspective, the best source of auricular chondrocytes is healthy auricular cavity cartilage or residual cartilage from deformed microtia [36–38]. However, the availability of autologous human auricular chondrocytes is limited. Based on current research, it is estimated that generating human auricular chondrocytes (HAuC) requires approximately 100–150 million chondro-forming cells, which can vary depending on the type and porosity of the scaffolding material used (Fig. 2) [39,40]. Furthermore, it has been suggested that auricular chondrocytes can be extracted from a patient's original pathological auricle through enzymatic digestion, indicating the possibility of treating microtia malformations through autologous auricular chondrocyte harvesting. For instance, He et al. [41] cultured dedifferentiated microtia chondrocytes in a three-dimensional chondrogenic culture system and generated re-differentiated microtia chondrocytes with the potential to regenerate mature cartilage. It's been confirmed that repetitive passaging of chondrocytes leads to dedifferentiation and loss of chondrogenic potential. However, there is limited data on the optimal number of passages for HAuC to maximize cell expansion while minimizing dedifferentiation. Bernstein et al. [42] isolated HAuC from discarded otoplasty specimens, expanded them, and encapsulated cells from generations 3, 4, and 5 into 8-mm-diameter discs made of type I collagen hydrogel with a cell density of 25 million cells/mL. Constructs were implanted subcutaneously into the backs of nude mice and were sampled and analyzed at 1 and 3 months after implantation. The study demonstrated that elastic cartilage formed by late-passaged HAuC (up to generation 5) was histologically, biochemically, and biomechanically similar to natural human elastic cartilage. This suggests that auricular chondrocytes could be expanded through *in vitro* passaging and used as seed cells for auricular cartilage tissue engineering. The findings indicate potential for the development of auricular cartilage engineering using HAuC.

Besides that, stem cells with potential to differentiate into various tissues or organs are another fascinating seed cells for auricular cartilage reconstruction [43,44]. Liu et al. [45] profoundly conducted a systematic evaluation of the application and progress of stem cells in ear cartilage regeneration, and indicated the potential of bone marrow mesenchymal stem cells (BMMSC), perichondrial stem/progenitor cells (PPC), and cartilage stem/progenitor cells (CSPC) to be used as seed cells in cartilage tissue engineering due to their abundant tissue sources, facile sampling, low immunogenicity, fast proliferation, and multidirectional differentiation potentials [46–48]. Hassan et al. [49] found that bone marrow-derived stem cells exhibited higher chondrogenic

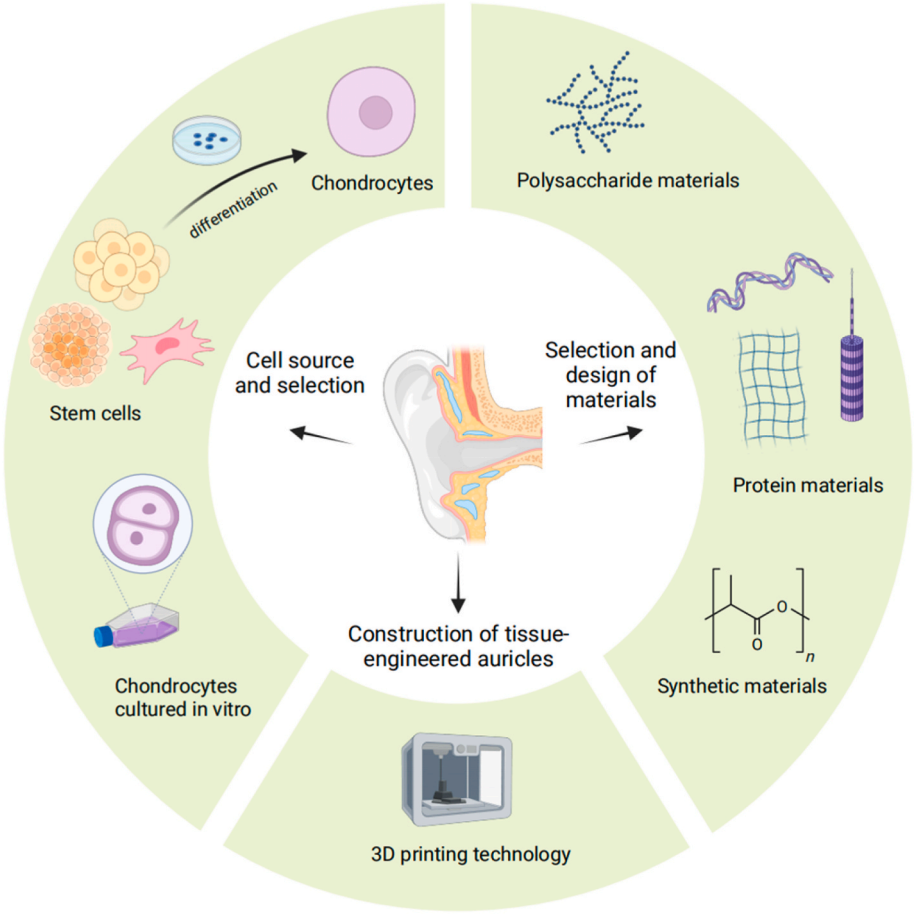

Fig. 1. Schematic illustration of criteria for tissue-engineered auricles construction.

potential compared to adipose-derived and mesenchymal stem cells. Among others, Zhang et al. [50] discovered that stem/progenitor cells from auricular cartilage exhibited superior cartilage formation compared to perichondrium *in vivo*. This was due to its good mechanical properties and showed more mature cartilage-like tissue formation with obvious cartilage lacuna in the histological examination. The Cartilage stem/progenitor cells group was consistent with the histologic analysis in terms of gene expression results, with higher expression of chondrogenic genes (ACAN, COL2A1, and COMP). These findings suggest that auricular cartilage stem/progenitor cells may be a promising candidate for cartilage tissue engineering. Current studies have reported three main methods for chondrogenic cell production: direct injection of stem cells into the cartilage microenvironment, co-culturing stem cells with ear chondrocytes, and inducing cells in cartilage-forming medium *in vitro*. *In vitro* observations have shown the chondrogenic capacity of these cells, and *in vivo* implantation has maintained their elasticity and morphology.

From a developmental biology perspective, stem cells of auricular cartilage tissue-specific origin are more advantageous for achieving auricular elastic cartilage tissue replacement. Dowthwaite et al. [51] discovered chondrogenic stem cells on the surface of articular cartilage in 2004, and these cells have since been found in auricular cartilage and cartilaginous membrane [52–54]. These cells have the ability to pass on repeatedly, expand in large numbers, differentiate into auricular chondrocytes, and form elastic cartilage. Therefore, they are a promising source of seed cells for auricular cartilage tissue engineering. However, their potential in regenerative medicine has not been fully investigated. Human auricular cartilage progenitor cells were only discovered in healthy cartilage and in cartilage remnants damaged by microtia malformations. Otto et al. [35] research found that auricular cartilage progenitor cells have the ability to supply the required cell numbers for tissue engineering of an auricular implant, while maintaining the chondrogenic phenotype and producing cartilage-like neotissue in a 3D hydrogel system. These cells can be easily obtained through a non-deforming biopsy of the normal ear or from the rudimentary microtia cartilage. As such, the availability of a potent progenitor subpopulation in the human auricular cartilage presents encouraging opportunities for the successful engineering of the human auricle and its translation toward the clinic. They could provide a valuable solution to the long-standing challenge of auricular cartilage tissue engineering. Dong et al. [55] efficiently generated shaped human elasticity in collagen within an external scaffold by co-culturing human MSCs with auricular chondrocytes in cartilage without volume loss. This marks a critical step towards clinical translation of auricular tissue engineering. However, the reconstruction of auricular stem cells is still in the early stages of animal experimentation, and transplantation in large animals is lacking. Further research is necessary.

2.2. *Selection of materials for tissue-engineered auricles*

The approach to total auricular reconstruction initiated with Tanzer using sculpted autologous rib cartilage (Fig. 3A), then the emergence and development of the alternative material MedPor (Fig. 3B), finally to the current popular biologic scaffold (Fig. 3C) [11]. Most scaffolds as the initial carriers of seed cells are used to temporarily replace the extracellular matrix and provide chondrocytes with a site for growth, proliferation, and secretion, which are absorbed and degraded as the newborn cartilage gradually develops and matures [56]. Generally, ideal scaffold for tissue-engineered auricles shall have the following properties [57]:(1) Good biocompatibility with negligible toxicity or

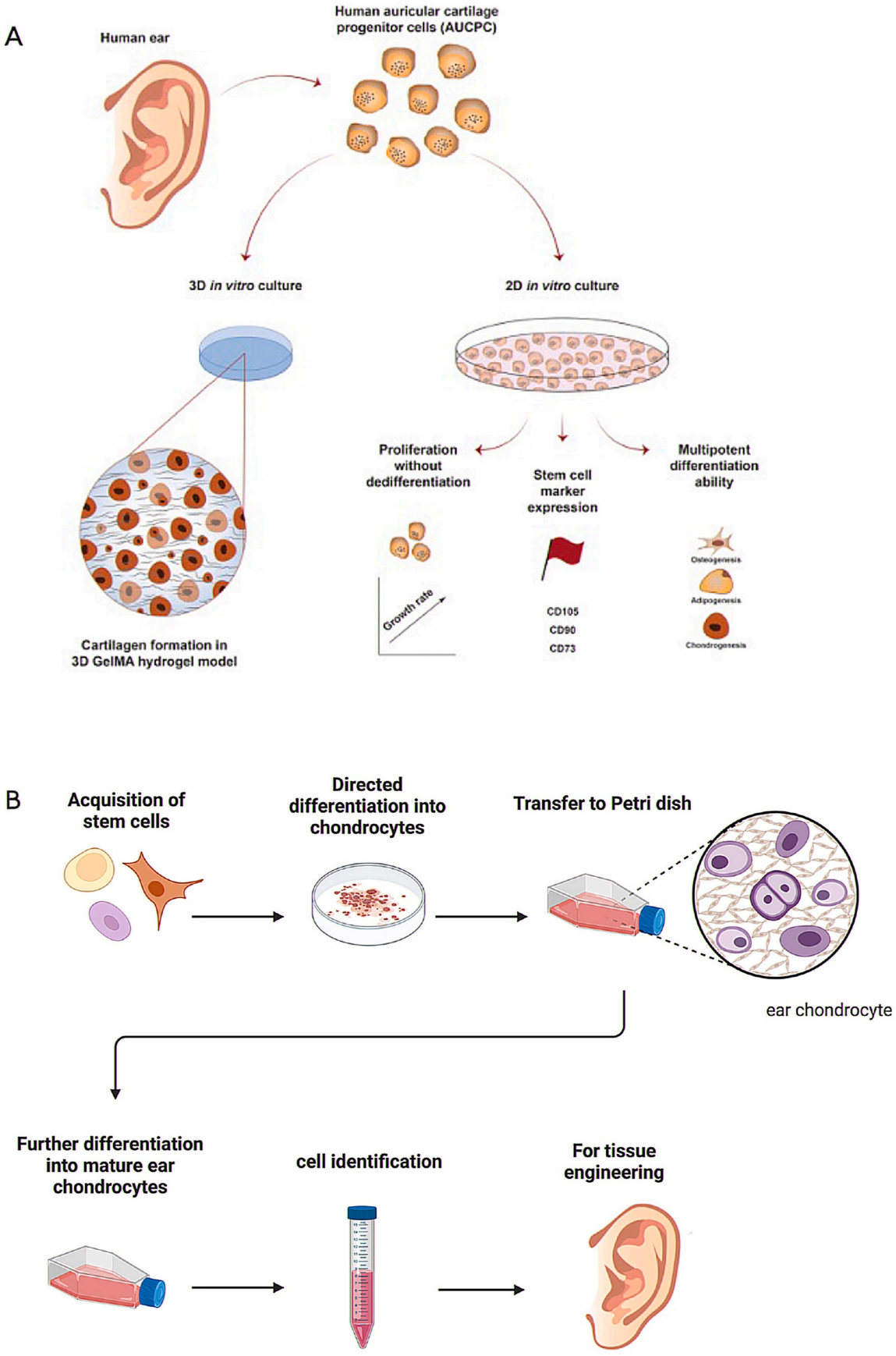

Fig. 2. (A) Human auricular cartilage progenitor cells culture *in vitro.* [35] Copyright 2022 iScience. (B) Directed differentiation of stem cells into ear chondrocytes.

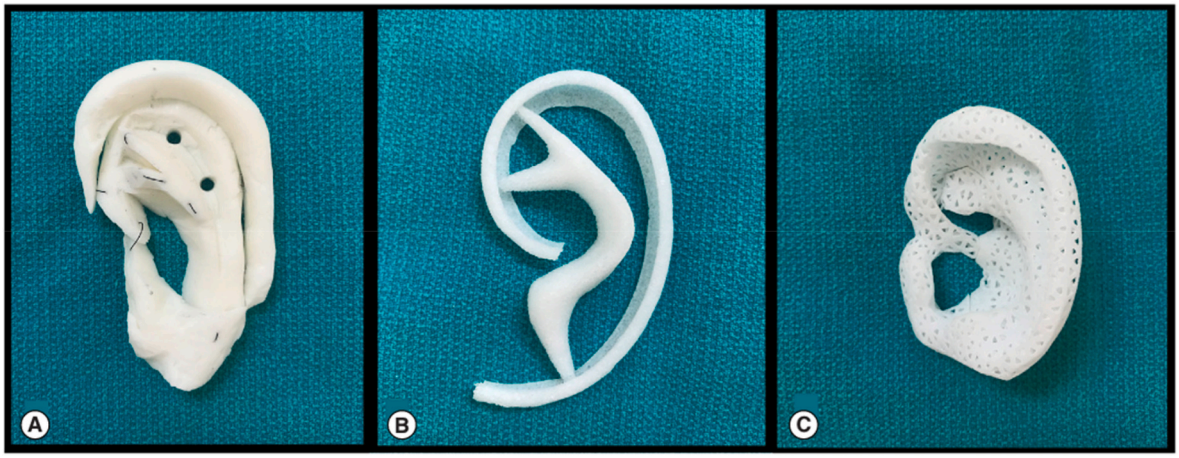

Fig. 3. Materials used for whole auricular reconstruction. Current techniques (A) autologous rib cartilage and (B) Medpor, and (C) porous bio-scaffolds [11]. Copyright 2018 J 3D Print Med.

immune rejection to embedded seed cells; (2) Adjustable biodegradability to orchestrate the whole regeneration process by seed cells; (3) Favorable surface activity for cell adhesion and expansion; (4) Three-dimensional porous structure, which is conducive to the cell migration and the nutrients transportation [58]; (5) Biomimetic mechanical strength and plasticity being similar to normal auricular cartilage, resistant to wear and support, and being able to maintain a certain cartilage morphology for a long time. Materials being used for biological scaffold would be discussed in the following in terms of its source (natural or synthetic).

Natural materials derived from natural plants or animals have the advantages such as great biocompatibility, biodegradability, wide range of sources and low cost, but limited mechanical strength and rapid degradation confined its application in tissue-engineered auricles [59,60]. Typically, protein (*e.g.* collagen, gelatin) and polysaccharide materials (*e.g.* alginate, chitosan, and bacterial nanofibrillar cellulose) are the most commonly used [61–64].

In the fields of protein-based materials, collagen, a protein found mainly in human connective tissues, is known for its good biocompatibility and biodegradability. Collagen-based scaffolds are widely used in tissue engineering owing to its promotion on cell adhesion, migration, and the formation of new tissues. Bichara et al. [65] discovered cartilage-like tissues after transplanting auricular chondrocytes loaded with collagen scaffolds following two weeks of *in vitro* culture. Moreover, Cohen et al. [66] implanted cell-scaffold complexes into nude mice for six months and the cartilage-like tissues were still visible, indicating that the collagen scaffolds have long-term stability. Likewise, gelatin, a collagen obtained from animal skin, bone, or cartilage, has good biocompatibility and biomimetic properties, providing an appropriate biochemical and biophysical environment. Gelatin scaffolds can be tailored to specific applications by adjusting their degradation rate. However, their mechanical properties are poor and they are prone to losing structural stability. Additionally, the degradation products of gelatin scaffolds may cause inflammatory reactions [67].

As for the polysaccharide-based materials, alginate is the most classic one for the construction of tissue-engineered auricles [68]. The scaffolds made from it are highly transparent and elastic, allowing for interaction with cells and promoting cell proliferation and differentiation. While collagen hydrogels have been successful in promoting new cartilage formation, their toughness and ductility do not match those of natural tissues. Slyker et al. [69] found that it could be functionalized with the N-terminus of collagen and could improve its toughness and ductility. Additionally, chitosan, a natural polysaccharide, has been found to have better mechanical properties and strong structural stability when used as a scaffold. It is commonly used in cartilage repair, bone repair, and skin regeneration. However, its mechanical strength and toughness are poor when used alone, so it is often combined with other materials. For instance, Sylvia et al. [70] developed a dialdehyde chitosan/hyaluronic acid scaffold, a porous structure with interconnected pores. The scaffold composition was found to have an effect on physicochemical properties, such as mechanical strength, heat resistance, porosity, and water content. And no significant difference was found between the cell viability proliferation of all scaffolds. Bacterial nanocellulose (BNC) is a polysaccharide produced by bacteria that readily binds water to form a hydrogel. The scaffolds made from BNC exhibit good mechanical properties and structural stability. However, the production and extraction costs of BNC are high, and there are limited studies on its applications [71]. Avila et al. [72] discovered that BNC with a 17% increase in cellulose content is a promising non-absorbable biomaterial for auricular cartilage tissue engineering. This is due to its similarity to auricular cartilage in terms of mechanical strength and host tissue response.

The decellularized scaffold is a scaffold made from natural auricular cartilage that has been decellularized, during which the elastic fibers was preserved. Therefore, its good biodegradability and biocompatibility together with reducing immune response is beneficial for promoting tissue regeneration and repair. Research has shown that the use of autologous or allogeneic bone marrow mesenchymal stem cell (BMSC)-derived decellularized nails, without the addition of exogenous growth factors, promotes the differentiation of BMSC to chondrocytes *in vitro* [73,74]. While natural materials have advantages such as low cytotoxicity and mild inflammatory response, they also have disadvantages such as lack of mechanical strength, rapid degradation rate *in vivo*, and difficulty in maintaining morphology [75–77]. This indicates that it is not sufficient to support and maintain the shape of the reconstructed auricle, and may be absorbed and degraded by the body over time, finally leading to failure of the outer ear reconstruction.

Currently, common synthetic materials including high-density polyethylene (Medpor), Poly-caprolactone (PCL), Poly-glycolic acid (PGA), Poly-lactic acid (PLA), *etc.* are widely used as biodegradable materials for tissue-engineered auricles [78,79]. However, some types of polymers are usually biologically inert [80], generally less biocompatible than scaffolds made up by natural materials. Besides, its degraded acidic products would also change the microenvironment of cell proliferation and differentiation [81–83]. For instance, Medpor has good mechanical properties and biocompatibility, such as high strength, rigidity and wear resistance, which provide good support and promote tissue repair and regeneration. However, Medpor stent degrades slowly and cannot be completely absorbed by the body. Its implantation requires surgical manipulation and is difficult to remove once implanted

[84]. PCL, PGA and PLA are synthetic polymers that are biodegradable. However, compared to natural materials, their biocompatibility is acceptable yet cases have reported harmful effect on their use in tissue engineering. They are known for their good mechanical properties and adjustable degradability, which makes them an indispensable option for biomimetic bioprinting. Polymeric materials can be effectively used to optimize the morphology and mechanical simulation of scaffolds. However, these grafts exhibit a non-homogeneous distribution of the extracellular matrix (ECM) and develop neocartilage only in certain areas of the scaffold [85]. In addition, potential problems may arise due to the lack of physiological properties of flexible materials, material exposure, infections, and other complications [86].

As a matter of fact, currently used materials often do not have sufficient structural and mechanical properties to meet the desired clinical efficacy. Therefore, materials made up by two or more materials in different forms and proportions not only maintain their respective excellent properties, but also obtain comprehensive performance that cannot be achieved by single material through complementation. In particular, the combination of synthetic materials with natural biomaterials can enrich their own biological functions while improving the mechanical properties of the latter [80]. For instance, Visscher et al. [87] developed a hybrid auricular implant model by combining 3D-printed poly-ε-caprolactone (PCL) with alginate. The study demonstrated that the hybrid auricular implant model exhibited enhanced (bio)mechanical properties, biocompatibility, and new cartilage formation. Likewise, Jang et al. [88] constructed a three-dimensional hybrid scaffold using PCL to reinforce the mechanical properties of regenerated auricular cartilage and injected alginate hydrogels containing a combination of adipose-derived stem cells (ASCs) and chondrocytes into the auricular cartilage. The results demonstrated an enhanced trend of chondrogenic differentiation in the particular cartilage. Besides that, Zeng et al. [89] developed gelatin methacryloyl (GelMA) hydrogels reinforced with bacterial nanofibrillar cellulose (BNC), and markedly enhanced the mechanical properties of the hydrogel with better facilitation on the cell migration. Tang et al. [90] loaded chondrocytes into GelMA hydrogels and combined them with 3D-printed PLA scaffolds in order to mimic the biomechanical properties of auricle, which showed great potential in clinical application of auricular regeneration.

2.3. Scaffold modeling techniques

The advancement and wide application of 3D printing and tissue engineering in medicine shows great promise for future innovations and more consistent outcomes for patients with microtia [17]. 3D printing methods can shorten surgical time, avoid donor-area morbidity, produce reproducible results, and reduce rejection rates when compared to autologous rib cartilage grafts [91–93]. In tissue engineering, 3D printing technology can be used to fabricate scaffolds of biomaterials, artificial organs, and tissue models. This technology offers significant advantages in constructing precise anatomical contours because it allows for the precise definition of the spatial distribution of cells and materials [94–96]. Injection modeling has been extensively studied using natural material scaffolds, such as alginate [97], fibrin gel [98], and hydrogel [99–101]. Gel-chondrocyte constructs can be engineered and molded from silicone molds, which can then be injected into the subcutaneous tissue of animal models or used directly as minimally invasive implant materials for further studies. However, the individual construction of such molds is time-consuming, and the quality cannot be precisely controlled and adjusted. The first step in creating an engineered auricle is to precisely design and sculpt a three-dimensional auricle that is unique to the individual. As a result, the use of computer-aided machining methods is becoming more and more crucial [102].

The development of digital technology, specifically computer-aided design/computer-aided manufacturing (CAD/CAM) technology, has made it possible to create a digital ear model by using 3D scanning to obtain the parameters of the healthy ear contour. When combined with 3D printing technology, this model can produce high-fidelity anatomical scaffolds with complex geometries. These scaffolds can be used for preoperative modeling to guide surgery or to create personalized external ear prostheses [103–105]. Scholars have replicated 3D-printed ear molds to sculpt rib cartilage [106], and create ear scaffolds with personalized features. This technique has improved postoperative outcomes and shortened surgical time in ear reconstruction.

In recent years, there has been rapid development in 3D printing technology, which is particularly suitable for reconstructing structurally fine and complex tissue sites in tissue engineering. The use of 3D bioprinting is increasingly common in studies of auricular cartilage tissue engineering [107–109]. The production of cartilage tissue constructs using 3D printing technology relies on advancements in materials science and equipment. The primary focus of research is to find biomaterials that are suitable for printing, as well as for the adsorption and proliferation of chondrocytes. However, a major obstacle to the clinical translation of engineered auricular scaffolds is the significant shrinkage and loss of shape that occurs during maturation of the soft collagenous chondrocyte matrix into elastic cartilage. To overcome this obstacle, Dong et al. [110] developed a system to prevent shrinkage and morphological loss of hydrogel structures. The modified system includes an external disk-shaped and ridged scaffold, which was designed and 3D printed using polylactic acid (PLA). PLA scaffolds, which were 3D printed, were placed around the hydrogel to prevent deformation *in vivo*. The study indicates that custom-designed, 3D-printed, biocompatible external scaffolds can significantly reduce shrinkage of bAuC seed structures and maintain complex morphology. This approach can be further refined and expanded by combining it with construct fabrication using injection molding, which may aid in the development of full-size auricular scaffolds. Although such scaffolds allow constructs to maintain their shape *in vivo*, cages cannot prevent inhomogeneous ECM maturation of these grafts.

In their study, Wang et al. [111] tested pUs that had undergone ultrasonic high-temperature treatment for 30 min (PU-30) or 60 min (PU-60) to better simulate the mechanical properties of auricular cartilage. The results indicated that the compression modulus of PU-30 was 2.21–2.48 mPa, which was similar to that of natural auricular cartilage (2.22–7.23 mPa). Therefore, PU-30 was selected for subsequent experiments. The pores of the treated polyurethane (PU) were filled with a hydrogel made of gelatin and sodium alginate, which was loaded with chondrocytes. *In vivo* analyses, using a rabbit model, confirmed that the implanted PU-30 scaffolds filled with chondrocyte-containing hydrogel successfully fused with normal auricular cartilage. Additionally, new cartilage was generated at the scaffold-tissue interface, as confirmed by histological examination. These findings indicate that the engineered scaffold may be a viable option for clinical repair of auricular cartilage damage.

Otto et al. [30] employed hybrid bioprinting techniques to fabricate ear-shaped structures using previously validated biomaterials, smart scaffold design, and recently identified progenitor cell populations. The scaffolds were primarily made of polycaprolactone (PCL) and were 3D printed using fused deposition modelling techniques. Hydrogels containing human ear cartilage progenitor cells were photocrosslinked within the scaffolds using methacryloyl gelatin (gelMA). The bioengineered structures were cultured in cartilage medium for 30 days *in vitro*. The study found that the fabrication process preserved cell viability and cartilage phenotype. The compressive properties of the hybrid PCL and gel MA auricular structures were comparable to those of native auricular cartilage. Additionally, the biofabricated hybrid auricular structures demonstrated excellent shape fidelity when compared to 3D digital models. Furthermore, cartilage-like matrix deposition was observed in both the peripheral and central regions of the auricular structures. Otto et al. developed a strategy to enhance the anatomical structure of the auricle with suitable mechanical properties. This ensures the contain of auricular shape during dynamic *in vitro* culture, allowing

chondrogenic progenitor cells to produce a significant amount of cartilage-like matrix throughout the auricular structure. The combination of smart scaffold design, 3D bioprinting, and chondrogenic progenitor cells shows promise for developing clinically translatable regenerative medicine strategies for auricular reconstruction.

The first international clinical breakthrough in tissue-engineered auricular reconstruction was achieved based on polymer scaffolds. However, this method has not yet been recognized as a clinically available treatment due to its unsatisfactory clinical efficacy. This is mainly because reconstructed structures are prone to inflammation and deformity. In the study of Jia et al. [112], they proposed a novel strategy that utilizes auricular chondrocytes and a bioactive bioink based on a bionic microporous methacrylate-modified decellularized cartilage matrix (ACMMA) in the form of a polymer scaffold. They integrated a multi-nozzle bioprinting technology, gelatin methacrylate (GelMA), poly(ethylene oxide) (PEO), and polycaprolactone (PCL). Photo-crosslinkable ACMMA was utilized to imitate the complexity of the cartilage-specific microenvironment, promoting active cellular behavior. GelMA, PEO, and PCL were used to balance printability and physical properties, resulting in precise structural stability and mechanical support for higher shape fidelity. The resulting structure is microporous, allowing for unobstructed nutrient exchange. The successful *in vivo* regeneration of mature auricular cartilage-like tissues provides new opportunities and strategies for the preparation and regeneration of patient-specific auricular cartilage.

3. Progress of tissue-engineered auricles

3.1. Natural materials-based tissue-engineered auricles

Cohen et al. [66] used a mold to prepare full-size collagen hydrogels in the shape of the human ear. They then composite calf auricular chondrocytes with collagen hydrogels and transplanted them subcutaneously on the back of nude mice. After 6 months, there was little change in the morphology, and the cartilage was enriched with proteoglycans, which formed an elastin network similar to that of auricular cartilage. However, the drawbacks of hydrogels cannot be ignored, including low strength and difficulty in forming and maintaining complex shapes over time. The researchers used a combination of synthetic and natural materials to address these issues. Visscher et al. [87] utilized polycaprolactone (PCL) to create a high-strength, porous auricular-shaped scaffold using 3D printing. They then added alginate gel-encapsulated auricular chondrocytes inside the scaffold's pores, resulting in an auricular-shaped graft that enhances the scaffold's mechanical properties, maintains its auricular shape, and promotes the proliferation and differentiation of auricular chondrocytes in the hydrogel. Ultimately, this approach leads to the formation of elastic cartilage tissue in the shape of the auricle.

Hydrogels made from decellularised extracellular matrix (dECM) extracted from tissues are commonly used as bioinks for cellular 3D bioprinting. This is due to the presence of tissue-specific ECM components that are essential for cell adhesion, growth and differentiation. Visscher et al. [113] isolated and decellularized porcine ear cartilage tissues, and conducted histological, biochemical, and proteomic characterizations of the resulting decellularized cartilage tissues. The methacrylate reaction was used to chemically modify the decellularized extracellular matrix (ECM) derived from cartilage (cdECM). This modification resulted in cdECM-based structures that maintained their structural integrity after the printing process, as reported in Refs. [114–116] To produce a printable bioink, chondrocytes were mixed with a photocrosslinkable hydrogel (cdECMMA) that was derived from the methacrylated cdECM. The study examined the rheological properties, printability, and *in vitro* biological characterization of the cdECMMA bioink. The results indicated that auricular chondrocytes maintained their viability and proliferation capacity in the printed cdECMMA hydrogel structures. Additionally, the cells produced cartilage extracellular matrix components, such as collagen and glycosaminoglycans (GAG). The use of this cartilage-specific dECMMA bioink has shown potential for cell-based bioprinting as an alternative option for reconstructing auricular cartilage.

Gelatin methacryloyl (GelMA) is a commonly used hydrogel in 3D bioprinting and has been effective for cartilage tissue engineering [117–119]. However, using only GelMA as a scaffold makes it difficult to produce large-scale functional tissue constructs due to its low mechanical properties and limited printing fidelity [109,120]. To enhance the mechanical stability and printability of hydrogels for cartilage bioprinting using natural materials, Zeng et al. [89] investigated the addition of bacterial nanocellulose (BNC) to GelMA. BNC is a naturally occurring high-molecular-weight polymer with good biocompatibility, high Young's modulus, excellent water retention, and good flexibility [121,122]. Furthermore, the material exhibits excellent printability and shear-thinning properties, rendering it ideal for 3D bioprinting [123]. The findings indicate that the 0.375% BNC composite hydrogel possesses superior mechanical properties, as well as enhanced printability and cell migration. Bioprinted structures were constructed using BNC/GelMA hydrogel bioink containing chondrocytes. The resulting cartilage exhibited a higher Young's modulus and glycosaminoglycan content in nude mice. Additionally, ear-shaped structures were bioprinted with the composite hydrogel, successfully regenerating cartilage tissue *in vivo*. While the superiority of composite hydrogels requires further validation in large animal experiments, this study offers valuable insights into constructing precisely shaped cartilage using alternative materials and detailed technical parameters(Fig. 4).

3.2. Synthetic materials-based tissue-engineered auricles

A team of researchers collected autologous residual ear cartilage from microtia patients as a source of seed cells, and then used to prepare tissue-engineered cartilage ear scaffolds with PCL-PGA-PLA as scaffolds. They then performed scaffold-grafted auricular reconstructive surgeries using the skin-expansion method of auricular reconstruction [124]. The study reports the first clinical application of whole ear reconstruction using tissue engineering. The study involved five patients with unilateral microtia. The scaffolds used in the study had PCL as the inner core and PGA/PLA as the outer layer. The scaffolds were prepared using a 3D printed resin model and were trimmed according to the resin ear model. Ear chondrocytes were implanted into the scaffolds, which were then cultured *in vitro*. After 2.5 years of follow-up, the first patient demonstrated satisfactory auricular morphology reconstruction. Histologic examination revealed typical cartilage formation similar to natural cartilage. The inflammatory response of the scaffold material was initially coped with by extending the *in vitro* induction time. This study presents a new strategy for improving the mechanical strength of engineered cartilage and maintaining morphology after implantation. However, residual polymeric materials can cause aseptic inflammation. Additionally, the formation and distribution of chondrocytes and ECM can affect mechanical stability. This remains a major issue to be addressed in the application of synthetic material scaffolds.

To reduce stent-induced complications, such as infections and hematomas due to frame extrusion, Zielinska et al. [125] proposed a tissue-engineered treatment. This treatment ensures sufficient autologous skin to cover the auricular frame. It is based on the use of a bioprinted autologous ear cartilage structure (EarCartilage) in conjunction with a bioengineered human pigmented and pre-vascularized dermal epidermal substitute (EarSkin). These were combined and tested in immunocompromised rats. The results have confirmed that EarSkin's human-engineered capillaries connected to the recipient's vascular system within one week, allowing for rapid blood perfusion and epidermal maturation. The bioengineered eardermis exhibited a stratified epidermis containing mature keratinocytes and melanocytes. The latter reside within the basal layer of the epidermis and effectively restore skin color. Furthermore, the *in vivo* testing revealed excellent

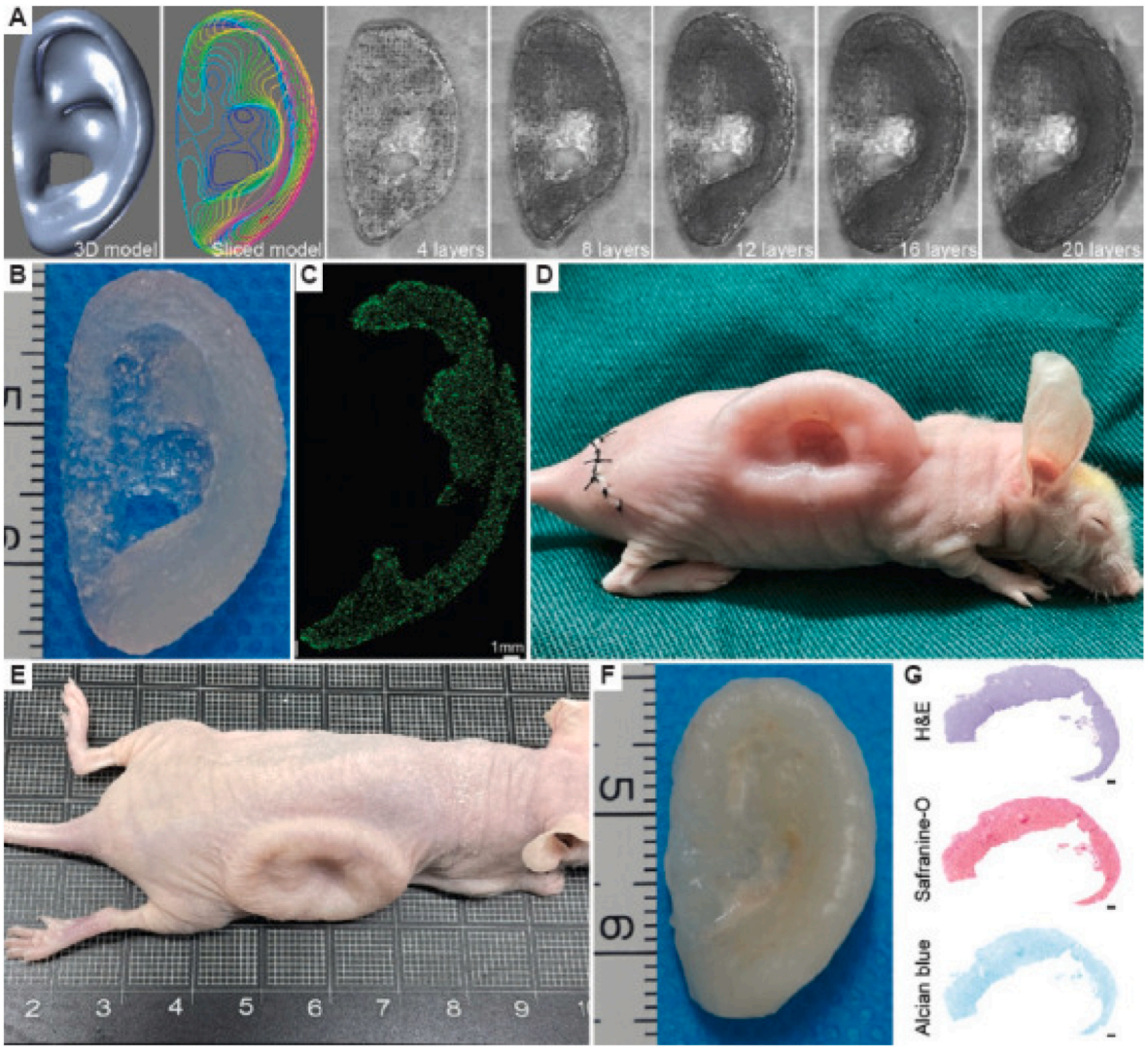

Fig. 4. 3D bioprinting of ear-shaped cartilage using bacterial nanocellulose (BNC)/gelatin methacryloyl (GelMA) bio-ink. (A) 3D-bioprinted ear-shaped scaffold with BNC/GelMA bio-ink. (B) Ear-shaped scaffold laden with chondrocytes before implantation. (C) Calcein AM/PI staining of the ear-shaped scaffold. (D) Ear-shaped scaffold immediately after implantation in nude mice. (e) Ear-shaped scaffold after implantation in nude mice after 24 weeks of culture *in vivo*. (F) Morphology of the ear-shaped scaffold after 24 weeks of culture *in vivo*. (G) H&E staining, Safranine-O, and Alcian blue staining of the scaffold after 24 weeks of culture *in vivo*. Scale bar = 1 mm [89]. Copyright 2023 J Bioprint. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

mechanical stability of the ear cartilage and increased deposition of extracellular matrix. In conclusion, the combination of Ear Cartilage and Ear Skin represents a novel approach to the treatment of microtia. This approach has the potential to overcome existing limitations and improve the aesthetic outcome of microtia reconstruction. By covering the auricle with tissue-engineered skin, complications such as frame exposure can be avoided, and the aesthetic outcome can be effectively improved (Fig. 5).

The primary challenge in tissue engineering auricular cartilage is the presence of dysplastic tissue that contains the cellular scaffold structure. To overcome this issue, Hirano et al. [126] evaluated the effectiveness of bioresorbable scaffolds made of poly-ε-caprolactone (PCL) and polyglycolic acid nanofibers (nanoPGA) by treating them with ethanol prior to inoculation of ear cartilage cellular scaffolds. This treatment is expected to increase the hydrophilicity of the scaffolds and promote cartilage regeneration. Auricular chondrocytes were isolated from canine and human surgical specimens obtained during otoplasty, including microtia reconstruction. Adhesion of ethanol-treated canine chondrocytes was significantly higher ($p \leq 0.05$) than that of untreated scaffold sheets. Ten weeks after implantation, human ear chondrocyte structures inoculated on ethanol-treated scaffolds were covered with smooth cartilage, whereas structures composed of the same cells inoculated on untreated scaffolds showed sparse connective tissue and cartilage regeneration. RT-qPCR analysis of chondrocytes grown on ethanol-treated scaffolds 10 weeks after implantation showed higher expression levels of several cartilage-related genes (Fig. 6). In addition, there was a significant increase in the expression level of SRY box transcription factor 5 (SOX5) and a decrease in the expression level of interleukin 1α (inflammation-related gene) compared to cells cultured on untreated scaffolds ($p \leq 0.05$). After ethanol treatment, the scaffolds produced more cartilage at 20 weeks than the constructs at 10 weeks. Although the study did not directly evaluate the hydrophilicity of the scaffolds, the pooled data suggest that the hydrophilicity of ethanol-treated nanoPGA/PCL scaffolds may be enhanced. This effect may improve chondrocyte adhesion, cellular microenvironment, and cartilage regeneration in tissue-engineered ENT constructs.

Novel scaffolds developed by Nürnberger et al. [127] are the first to be able to use a dense cartilage matrix, repopulate the cartilage matrix through channels, and provide a compact type II collagen environment for the cells. The use of "AuriScaff" (an enzymatically perforated bovine

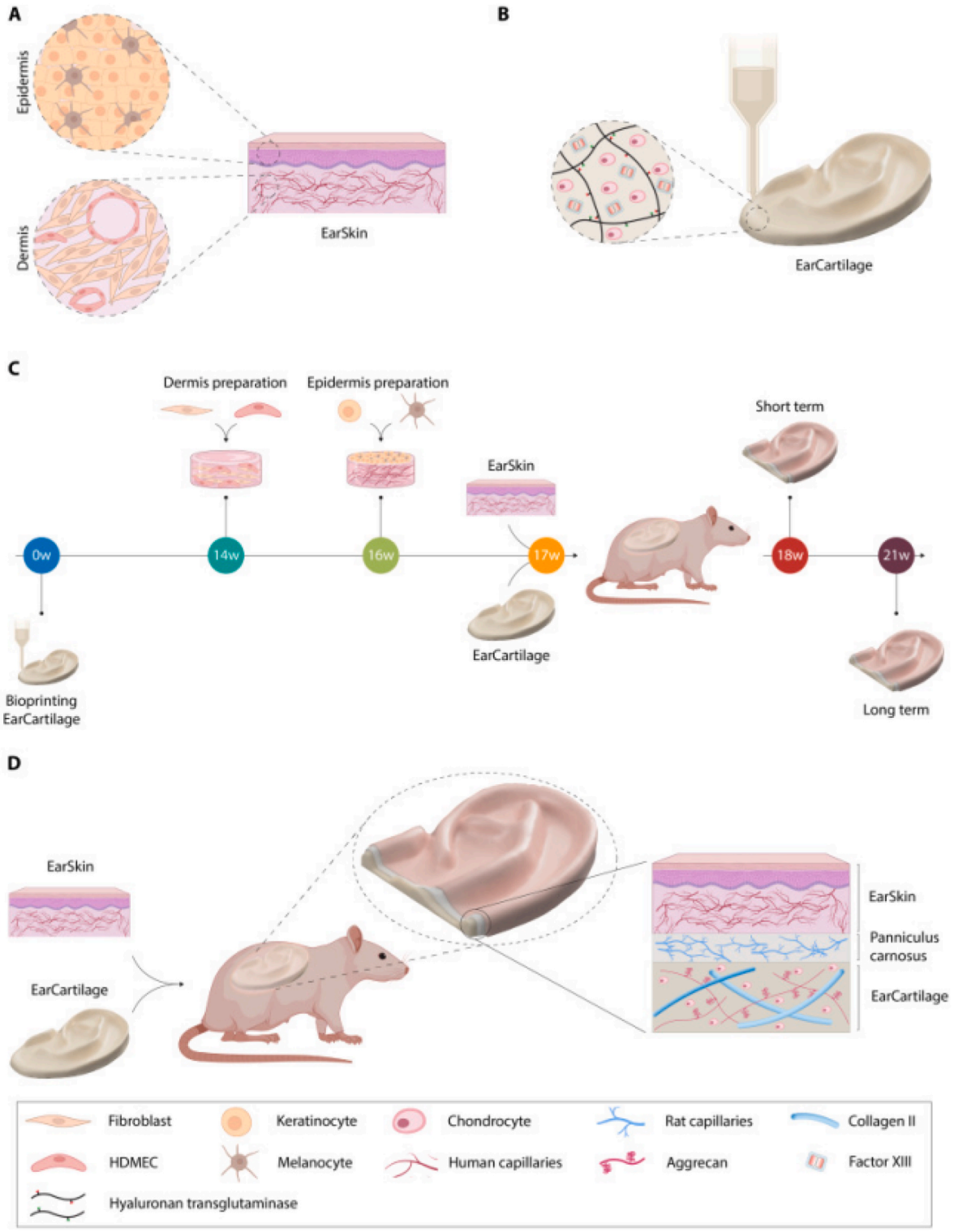

Fig. 5. Schematic of the combination of EarCartilage and EarSkin. (A) EarSkin was prepared by creating a dermal layer fabricated out of human fibroblasts and human dermal microvascular endothelial cells (HDMECs) in a collagen I hydrogel. Once matured, an epidermal layer of human keratinocytes and melanocytes was seeded on top of the dermal part. (B) EarCartilage was fabricated using a hyaluronan transglutaminase (HATG)–based bioink together with primary human auricular chondrocytes. Postprinting constructs can be enzymatically crosslinked using calcium-triggered enzymatic crosslinking of factor XIII (30). (C) Experimental timeline of EarCartilage and EarSkin *in vivo*. EarCartilage was matured for 17 weeks before implantation. EarSkin was created by combining fibroblasts and HDMECs in a collagen I hydrogel and matured for 2 weeks before seeding keratinocytes and melanocytes on top and culturing it for an additional week. Constructs were then implanted together *in vivo* and analyzed after 1 (short term) and 4 (long term) weeks. w, weeks. (D) Human EarSkin (A) and human EarCartilage (B) were combined *in vivo* in an immunocompromized rat model. A subcutaneous pocket below the panniculus carnosus was created along the dorsal midline into which EarCartilage was transplanted. The panniculus carnosus was used to cover the EarCartilage framework and to provide rapid nourishment to EarCartilage and EarSkin. EarSkin was then transplanted on top of the panniculus carnosus [125]. Copyright 2023 Sci Adv.

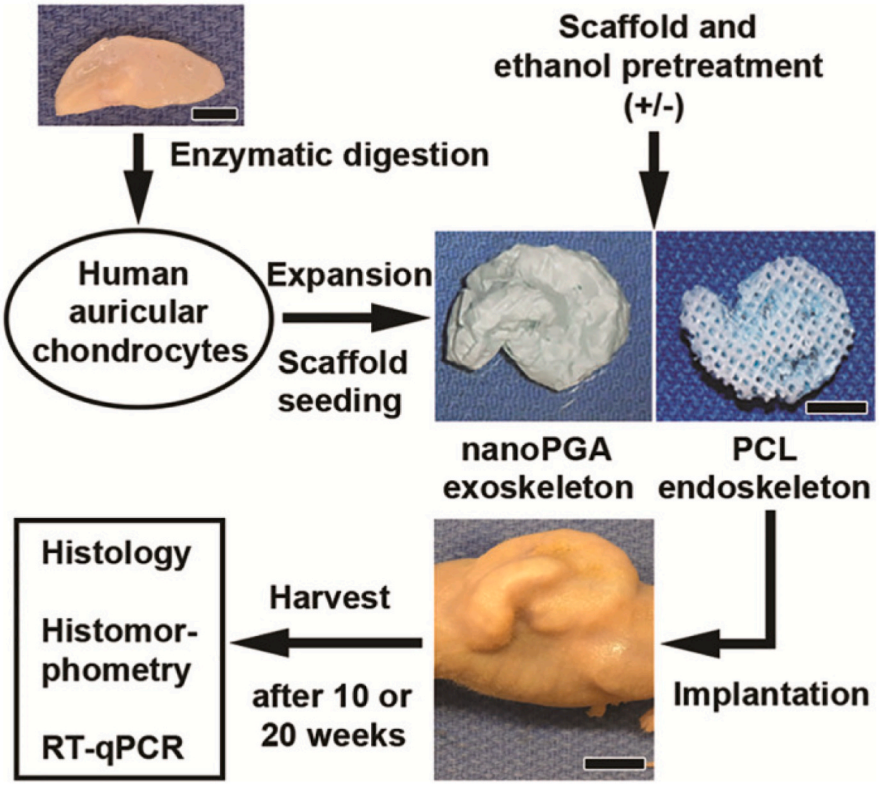

Figure 6A

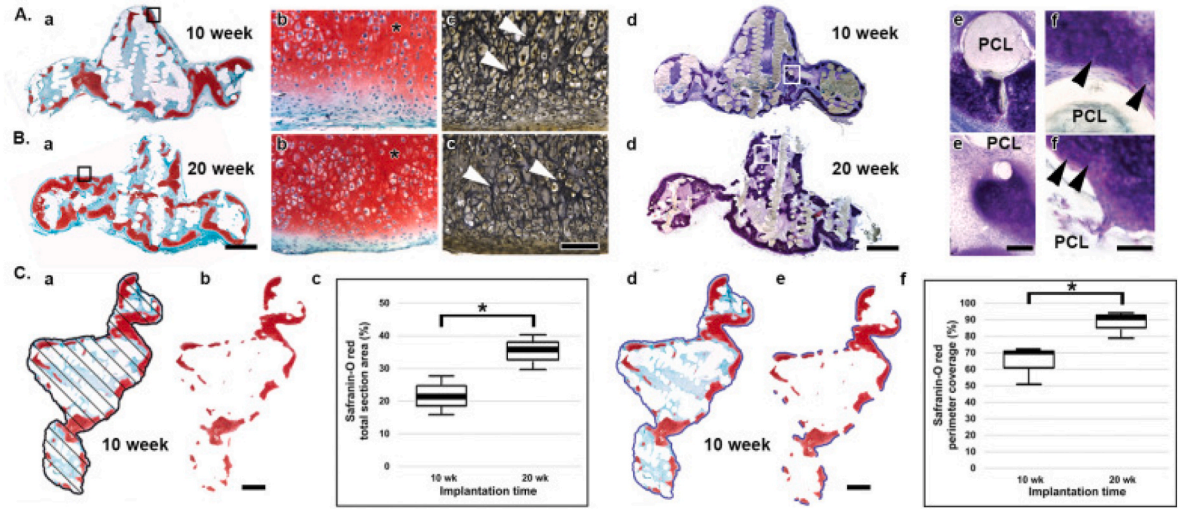

Fig. 6. (A) Experimental design for examination of tissue-engineered auricle-shaped constructs of human auricular chondrocytes seeded onto 3D nanoPGA/PCL composite polymeric scaffolds with or without ethanol treatment. **(**B) Representative histology and histomorphometry of human auricular cartilage regenerated on nanoPGA/PCL composite constructs consisting of ethanol-treated scaffolds and implanted for 10 and 20 weeks in nude mice [126]. Copyright 2021 PLoS One.

ear cartilage scaffold) as a novel biomaterial for regeneration of regenerative cells and formation of high-quality hyaline cartilage. AuriScaff has a network of traversing channels, which is generated by selective depletion of elastin fibers, which allows for the homogenous repopulate the treated cells. In the osteochondral plug model, AuriScaff fills the intact defect with a dense type II collagen matrix and achieves chondrogenic differentiation within the channel. Due to its density, it also offers better mechanical properties than currently used materials in the clinic, and the Auricular Cartilage Scaffold (AuriScaff) has great potential to improve future cartilage regeneration methods.

Taken together, in the field of tissue engineering, the combination of 3D printing technology and composite biomaterials has led to a significant advancement in the manufacturing of artificial auricles (Table 1). The application of 3D printing technology enables the accurate replication of the three-dimensional structure of a patient's normal auricle. Composite biomaterials, comprising synthetic, natural, or hybrid materials, are employed as print inks to fabricate artificial auricular scaffolds with great biocompatibility and tunable mechanical strength [57]. These composite biomaterials typically combine the stability and plasticity of synthetic materials, such as polylactic acid-hydroxyacetic acid copolymer (PLGA) and polycaprolactone (PCL), with the bioactivity and biocompatibility of natural materials, such as collagen and hyaluronic acid. The as-printed scaffolds not only simulate the intricate geometry of the auricle but also provide the requisite

Table 1
Current progress of 3D printing tissue-engineered scaffolds for auricular reconstruction.

Cell source	Materials	Instrument	Progress	Reference
Chondrocytes of bovine ear	Collagen	Stratasys FDM 2000 3D printer (Eden Prairie, MN)	animal experiment	Cohen et al. (2016) [66]
Chondrocytes of human ear	PCL-PGA- PLA	Spectrum 510 3D printer (Z Corporation, USA)	Clinical application	Zhou et al. (2018) [17]
Chondrocytes of goat's ear, fat stem cells	PCL, alginate	3DDiscovery printer (RegenHU, Switzerland)	cell experiment	Visscher et al. (2019) [87]
Chondrocytes of the human ear, articular chondrocytes from rabbit	PCL, alginate	3D multi-nozzle printing (multi-head tissue/ organ building system, MtoBS)	animal experiment	Jang et al. (2020) [88]
Chondrocytes of rabbit ear	GelMA	Fused deposition modeling 3D printer (ANYCUBIC 13 MEGA, China)	animal experiment	Tang et al. (2021) [90]
Chondrocytes of bovine ear	Collagen	Makerbot Replicator 5th Generation (MakerBot Filament, MP05776)	animal experiment	Dong et al. (2021) [110]
Chondrogenic cells of human ear	PCL, GelMA	3DDiscovery printer (RegenHU, Switzerland)	cell experiment	Otto et al. (2021) [30]
Chondrocytes of pig's ear	GelMA	Tissue-organ printing (ITOP) system	cell experiment	Visscher et al. (2021) [113]
Chondrocytes of human and canine ear	PCL, nanoPGA	simple melt technique (KLS Martin SE & Co. KG)	animal experiment	Hirano et al. (2021) [126]
Chondrocytes from pig and rabbit ears	PCL, GelMA	3D-Bioplotter printer (Envision Tec, Germany)	cell experiment	Jiang et al. (2022) [112]
Chondrocytes of rabbit ear	BNC, GelMA	3D-Bioplotter printer (Envision Tec, Germany)	animal experiment	Zeng et al. (2023) [89]

microenvironment for cellular growth and auricular tissue regeneration [87]. The development of 3D printing tissue-engineered scaffolds offers a minimally invasive and personalized treatment option for microtia patients, while also providing new research directions and applications in the field of tissue engineering. Nevertheless, numerous challenges remain before this technology can be applied in a clinical setting.

4. Future development trends and challenges

This review discusses the different aspects of tissue-engineered auricles, including seed cell selection and culture methods, scaffold materials, and molding techniques, as well as the standardization of the ear cartilage preparation process. Currently, auricular reconstruction is primarily treated surgically using autologous rib cartilage sculpted grafts. Significant progress has been made in auricular cartilage tissue engineering in recent years, particularly in cell sampling and culture, scaffold selection and preparation, and functional molding of auricular cartilage. Although clinical applications are currently available, existing methods of constructing auricular cartilage for tissue engineering generally have certain shortcomings.

(1) In terms of cell selection and culture, the maintenance of cell viability and activity during bioprinting still represents a significant challenge. More researches on the structure-activity of hydrogel components to maximally maintain the cell viability through scaffold design are thus urgent and necessary. Moreover, the update of more mild 3D printing technique is also demanding as the current rationale is mainly focused on the photocuring which greatly hamper the synchro print of cells and materials while cell viability of activity is the most critical element to be considered in the whole construction process [128].
(2) In terms of scaffold material and construction, the choice of printing material is of paramount importance. Ideally, the material should exhibit good biocompatibility, low immunogenicity, suitable mechanical properties, and the capacity to support cell growth and differentiation. The current techniques for auricular reconstruction are still seeking superior materials with desirable physiochemical properties to ensure the shape and position of the auricle can be maintained over time. Furthermore, the degradation ratio of the scaffolds orchestrating with the process of new tissue formation to ensure the structural stability and functionality of the auricle is still unsatisfactory [129].

Overall, the optimization and application of future bioprinting technologies is anticipated to provide a robust foundation for further developments in ear cartilage tissue engineering research. This encompasses the investigation and advancement of intricate intercellular connections, as well as the growth, development, metabolism, differentiation and immunology of ear chondrocytes. Reconstruction techniques for the auricle are still in the laboratory and clinical trial stages and have not yet been widely adopted in clinical practice. However, as an emerging technology, 3D printing in healthcare shall undergo a rigorous regulatory process to ensure its safety and efficacy. This process entails a comprehensive assessment of the printing materials, devices, and the final product. Furthermore, cost-benefit analysis is another crucial consideration. Through controlling costs while ensuring high quality and safety standards, 3D printing technology may be more economically viable to cover a wider patient population. Finally, the application of 3D printing technology in the field of tissue engineering also necessitates interdisciplinary collaboration, including the coordinated efforts of bioengineers, materials scientists, cell biologists, and clinicians, to facilitate the advancement and clinical translation of this technology. It is reasonable to posit that 3D printing technology will become an increasingly pivotal tool in the field of auricular reconstruction, as well as other areas of tissue engineering, as a result of the ongoing research and innovation in this field.

CRediT authorship contribution statement

Shuyi Gao: Writing – original draft, Conceptualization. **Tianqi Nie:** Writing – review & editing, Supervision. **Ying Lin:** Writing – review & editing, Supervision. **Linlan Jiang:** Conceptualization. **Liwen Wang:** Conceptualization. **Jun Wu:** Writing – review & editing, Supervision. **Yuenong Jiao:** Writing – review & editing, Supervision, Funding acquisition.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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