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Abstract

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A bioprinting printing approach to regenerate cartilage for microtia treatment

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Abstract

The need to search for biomaterials that can promote tissue regeneration and easy to replicate and manufacture is a major driving force for research and development in the area of reconstructive surgery and regenerative medicine. It is of great importance to otolaryngologists to find alternate solutions that require harvesting large amounts of autologous cartilage in patients needing cartilage grafts. Due to its very limited self-regeneration capacity, cartilage repair and reconstruction is extremely challenging. Microtia is a congenital condition of abnormal development of the outer and/ or the middle ear and can range from mild to complete absence of the ear. Current treatment methods such as autologous, alloplastic and prosthetic reconstruction have limitations such as donor site morbidity, long-term complications and implant failure. 3D printing is an exciting solution to address the challenges of microtia and create customised implants. The ability to deposit cells and biomaterials in a controlled and precise manner, allows the fabrication of implants with complex internal architecture and

functional properties not achievable through traditional manufacturing methods. Despite the ability to mimic native properties and structure of tissue, 3D printed constructs using pristine inks lack the structural integrity and adequate mechanical properties for use *in vivo* or handling. These requirements highlight the importance of ink development and selection, which is a continuing challenge in the bioprinting process. This review will address the current treatment options for patients with microtia and the potential of 3D bioprinting in area of auricular cartilage regeneration. In particular, the use of hybrid printing to better mimic the practical and functional requirements of an ear scaffold will be discussed.

Keywords: 3D bioprinting, cartilage regeneration, microtia, bioink development

1. Introduction

In Otorhinolaryngology, cartilage is of particular interest because of its presence in multiple structures in the ear (pinna), nose (septum and alar) and throat (larynx and trachea). Further it is used as graft material in surgical interventions from cerebrospinal fluid (CSF) leak repairs [1], Ossicular Chain Reconstruction (OCRs) [2], Microtia reconstruction [3] and Laryngotracheal reconstructions [4]. It is therefore a tissue of great importance to Otolaryngologists. Due to its very limited self-regeneration capacity, cartilage repair and reconstruction is extremely challenging.

Microtia is a congenital condition where there is an abnormal development of the outer and/ or the middle ear and can range from mild to complete absence of the ear [5]. Overall, the incidence of microtia is about 1-4 per 10,000 births, but can vary between countries. Of these, 10% of patients will present with bilateral diseases [6]. Treatment is demanding with long hours and numerous stages of surgery. In addition, complications present in the donor site and the receiver site includes failure, scarring, and most of all, donor site morbidity.

Bioprinting is an exciting solution to address the challenges of microtia and create customised implants, but research in this area around the world has not yet translated into meaningful results of clinical relevance. In this review, the various treatment options for microtia will be discussed and the potential of 3D bioprinting in the area of cartilage regeneration will be highlighted. In particular, the use of 3D printing to satisfy the mechanical, biological and printing requirement of the entire construct will be discussed. The range of printable biomaterials and cell sources relevant to auricular reconstruction will also be reviewed.

2. Pathology

Ear deformity can have a significant social and psychological impact on the individual. It can occur as a result of trauma (67,000 patients have ear deformities caused by burns/year in the US), burns, cancer (malignant lesion of the ear account for approximately 13% of all head and neck melanomas), scars, or congenital abnormalities [7].

The external ear consists of the auricle and the external ear canal. Microtia is a developmental malformation of the external ear, characterised by a small abnormally shaped auricle or pinna. The disorder can be either a genetic or environmental factor, an isolated condition or part of a spectrum of abnormalities. Several possibilities for the cause is suggested but still poorly understood. These include vertebrae abnormalities [8], medications [9, 10], genetic disorders [11], and even illness during pregnancy [12]. There are two forms of microtia, unilateral or bilateral. Unilateral occurs in 79-93% of the cases and the right side is more often affected [6, 13]. In individuals that have unilateral microtia, the speech and language developments are normal, due to the normal functioning collateral ear. Figure 1 presents different grades of microtia, ranging from mild structural abnormalities to complete loss of the ear. The classification system was first developed by Hermann Marx in 1926 and was later modified by Meurman [5]. Grade I refers to individuals having all features of the normal auricle, but the pinna is smaller than normal. In grade II, only some features are present. Grade III is most commonly seen in patients that undergo ear reconstruction as only a portion of soft tissue is present, resulting in a peanut-shaped ear. In grade IV (or anotia), there is no external ear and auditory canal. This is often associated with hearing loss and requires treatment for hearing impairment and surgical ear reconstruction. Another classification also in use separates microtic ear deformities into lobular and conchal types [3, 11].

Figure 1 Ear classified by grades I to IV and as lobular or conchal types

3. Treatment options for microtia

3.1 Autologous reconstruction

Autologous reconstruction remains the first treatment option for patients with classical microtia and no prior surgery, as this approach leaves options open for allografts or Osseo integrated prosthesis if it fails. The reconstruction of microtia can be broken down into 2 main steps. First is to mimic the contours of the normal ear by “sculpturing” a framework from autogenous cartilage. The second step is the coverage of the framework with skin grafts or adjacent skin [3]. It has been suggested that reconstruction of the ear can begin as early as 5 to 6 years of age to prevent social and psychological stress from peers at school. Many have reported the benefits of delaying the surgery until 9 to 10 years old [14-17] , and when the chest circumference has grown to at least 60cm so that there is sufficient cartilage for harvesting [5]. The benefits of autologous reconstruction include long-term stability, limited rejection and minimises the risk of extrusion of the implant. The grafts also possess the potential to grow with the patient as they mature [18].

3.1.1 Techniques

The anatomy of the ear consists of eminences and depressions, shown in Figure 2. A significant breakthrough in the field of ear reconstruction was introduced by Tanzer in the late 1950s [19]. It involves a 4-stage reconstruction process from carving a solid block of autogenous costal cartilage. Costal cartilage (rib cartilage) has proven to be the source of cartilage that provided an adequate quantity and integrity [20, 21]. Modifications of this technique are still regarded as the 'gold standard' for patients with microtia and other ear deformities [22]. Brent mastered a three-stage reconstruction building from this technique, while Nagata and Firmin further refined the technique to allow early positioning of the lobular segment of the vestige and constructing in 2 stages [14, 17]. A summary of the different techniques to reconstruct microtia is shown in Table 1, all aiming to further improve aesthetics and decrease complication rates [23].

Figure 2 Illustration of the ear anatomy

Table 1.Summary of techniques to reconstruct microtia

Surgeon	Ages	Stages	Technique
Tanzer [19, 24]	6-7+	4	I. Rotation of Lobule II. Fabrication of the costal cartilage framework III. Elevation of the framework IV. Construction of tragus and conchal cavity
Brent[21]	6-8+	3	I. Fabrication of the costal cartilage framework II. Lobule transposition III. Elevation of framework IV. Tragus reconstruction
Nagata [14-16, 25, 26]	10+	2	I. Fabrication of the costal cartilage framework. Include tragus, conchal cavity and lobule II. Elevation of the framework after 4 months. Covered with skin graft
Park [27]	10+	3	I. Insertion of tissue expander. Continues for 5 months II. Fabrication of costal cartilage framework and inserted between skin flaps III. Skin incisions to create tragus, conchal and external auditory canal
Firmin [17]	9-10+	2	I. Framework placed under skin pocket for 6 months II. Sulcus created using additional cartilage graft for projection

3.1.2 Limitations of autologous reconstruction

Donor site morbidity is a constant concern reported in several case studies because of the need to harvest multiple sections of the costal rib, mainly 6th, 7th and 8th, to make just the auricular body framework and 9th for the helical component [28]. Besides pain, there is also a potential for lung collapse or chest wall depression. As the costal ribs are harvested, the stability of the anterior ends of the rib against the sternum is much less and the rib bends inwards through growth and negative respiratory pressure [29]. This is some of the reasons why several studies prefer Brent's technique over Nagata's as it requires less cartilage, but also delaying the surgery till the patient is much older [23, 30].

A method to prevent chest wall deformity include using cartilage from cadavers (allogeneic reconstruction). The use of cadaver cartilage has also shown to be a promising alternative to autologous cartilage. Studies conducted by Go *et al.* [31] on 42 patients demonstrated that patients having their empty perichondrial space filled with irradiated cadaver cartilage after harvesting are equally effective in preventing chest wall depression as ones filled with autologous cartilage. Another study used irradiated homograft costal cartilage directly to perform auricular reconstruction on 19 patients. The reports showed no short-term and long-term complications after 36 months and 90% of subjects were satisfied with the outcome [32]. Limitations for using cadaver cartilage is most certainly the possibility of graft infection and surgical cost [31]. Nevertheless, this approach can eliminate the need for graft harvesting, hence donor site morbidity.

Aesthetically, some of the limitations include cartilage resorption and scarring. Costal cartilage is still reported as the best substitute for auricular reconstruction [20, 21]. However, the costal cartilage has no elastic fibres and lacks the flexibility of a normal ear, giving it an overall rigid appearance [28, 33]. There are also reports on calcification in using costal cartilages for auricular reconstruction [34]. Finally, it also resorbs rapidly and as the skin contracts heavily during the healing process, scarring and skin necrosis can occur. Table 2 summarises the types of complications after costal cartilage rib harvesting and are usually indicators of success and failure in auricular reconstructive surgery [20] .

Table 2. Percentage of complications after autologous auricular reconstruction

Complications	Incidence	Total number of patients in study	Reference
<u>Donor-site morbidity</u>			
Infection	5 (19%)	27	[11]
Chest deformities	5 (6%)	88	[35]
	3(7%)	42	[36]
Thoracic scoliosis	4(25%)	16	[29]
Cartilage Resorption	3 (11%)	27	[11]
Pleural Tear	1 (4%)	27	[11]
	19 (22%)	88	[35]
Necrosis	1 (4%)	27	[11]
	1 (0.3%)	350	[37]
Atelectasis	1 (7%)	15	[3]
	7 (8%)	88	[35]
<u>Aesthetics</u>			
Under/oversized framework	3 (11%)	27	[11]
Scarring	3 (20%)	15	[3]
	12 (14%)	88	[35]
	21 (6%)	350	[37]
Exposure	2 (5%)	40	[3]
	4 (1.2%)	350	[37]

3.2 Alloplastic reconstruction

Alloplastic reconstruction can offer several advantages over autologous reconstruction, such as avoiding donor site morbidity, shorter surgery time, improved size and contour matching,

and less demanding learning curve for the surgeon. The ideal shaped ear can be produced in advance and several materials such as nylon mesh, silicone, polyethylene, acrylic and polyester have been introduced over the years as potential candidates [20, 38]. As with all other foreign body implants, there are potential risks associated with this method, such as infection, necrosis and implant exposure. Another difficulty in manufacturing alloplastic implants is to accommodate all the variations in size and shape required to mimic the patient's normal ear. The implants are not customised and often the surgeon would have to compromise for one that is the closest match. This is not the case in autologous reconstruction where the surgeons can carve the cartilage to obtain what is required. Interestingly, around 10% of patients still have either a under or oversized framework, showing the complexity in shaping the harvested cartilage and matching the contralateral ear either by eye or imaging technologies [11].

Silicone framework for auricular reconstruction was first introduced by Cronin in the 1960s [39, 40] and later by Ohmori *et al.* [41, 42]. These materials showed good initial results, with flexibility mimicking the native cartilage, excellent aesthetic outcomes and donor site morbidity was not a concern. Follow up results however, found that implant exposure was a real concern and minor trauma or abrasion can cause implant failure.

Polyethylene (PE) was first described for use as a framework in auricular reconstruction by Berghaus in 1983[43]. It is a non-reactive material that is available in high-density porous configurations with sizes of around 100-200µm. The porosity provides good anchorage for tissues compared to the smooth silicone as described by Cronin, thus allowing vascular and soft tissue in-growth [38]. The current commercially available implant, Medpor® (Porex Surgical Inc, Stryker, GA), is a porous and flexible polyethylene framework pioneered by John Reinisch that comes in two parts, a helical rim and an ear base, allowing better sizing and positioning [44]. Despite improvements to operative techniques, the majority of complications reported are still a result of framework protrusions and implant exposures rather than infection. Reinisch *et al* [45] reported the largest series of reconstruction using PE framework on 786 ears and reported an exposure rate of 7% with no major infection during a 12 year follow up. Other studies with follow-ups up to 11 years have also reported implant exposure being the most common issue [46, 47]. Long-term studies are still required to assess the implant performance over a lifetime, but early results have allowed Medpor® to become the most favourable alloplastic material for surgeons when performing a total ear reconstruction. Since Medpor® is relatively infection resistant; exposures can be managed quite effectively through complete flap coverage [48]. By not having to wait for cartilage to grow and mature, reconstruction can

be done before the child enters school [49]. In addition, ear reconstruction can be performed at the same time as canal atresia (absence of an ear canal) surgery allowing children to have completely functional and aesthetically good looking ears as early as three to four years of age [38].

3.3 Prosthetic reconstruction

Prosthetic reconstruction is a cost effective method to cover areas of defect for patients who are unable or in need to postpone surgical reconstruction. Surgeons would usually consider an auricular prosthesis in situations such as [44, 50]:

- 1) Traumatic loss of the auricle or surrounding tissue
- 2) Failed autologous reconstruction
- 3) Underlying medical conditions
- 4) Age
- 5) Following tumour resection with possibility of recurrence
- 6) Inability to undergo multiple surgeries

The alloplastic ear is generally made from silicone and created as a mirrored contralateral ear through solution casting or more recently, additive manufacturing [51, 52]. There are various methods of attaching an auricular prosthesis to the skin, by either adhesives or an osseointegrated approach [53-55]. With adhesives, no surgery is needed and there is lower possibility of infections and inflammations around the skin. However, long-term contact with adhesives may result in allergic contact dermatitis and are especially difficult for patients who have limited vision to accurately put on. The latter approach allows the patient to accurately wear the prosthesis without a mirror and can withstand larger mechanical load during activities [56].

The concept of osseointegration as reported by Branemark in the 1960s could be considered as one of the most significant advances in prosthetic surgery over the past 20 years [50, 57-59]. Using titanium screws as bone anchored fixtures, these implants can be connected to load bearing prosthetics with high success rates [54, 55, 60, 61]. The bone implants are intended to last a lifetime and prosthesis can be replaced every 2 to 5 years depending on wear [60].

4. 3D bioprinting

3D printing have become increasingly popular in reconstructive surgery as this technology enables fabrication of patient-specific models for pre-operative planning, surgical guides, education purposes or implants. Computed tomography (CT) and magnetic resonance imaging (MRI) can be used to provide information regarding tissue geometries and the function at cellular level of the tissue [62, 63]. These raw data are then converted into a computer-aided design (CAD) model, which are often used as a stereolithography (STL) file. STL files can be imported into the 3D printer by slicing them into thin horizontal 2D layers, containing exact geometrical information of the 3D model. The slicing thickness of the layers and filament spacing is referred to as the “resolution” of a printer and depends on the ink and the nozzle tip of the syringe [62-64]. Finally, the accurate 3D-printed model is then built bottom-up layer by layer from the 2D slices.

Bioprinting combines the technologies of 3D printing and molecular biology. It enables geometrically precise scaffolds to be fabricated easily and to act as a platform for living cells to grow and mature either *in vitro* or *in vivo*. Eventually the scaffold will degrade over time leaving native functional tissue in the predetermined shape, arrangement and location. Furthermore, the ability to control the delivery of “bioink” facilitates accurate and homogeneous distribution of cells in the scaffold. This is particularly beneficial in fabricating scaffolds for cartilage repair when the turnover of cartilage is very slow and nutrient transfer relies heavily on diffusion. A study conducted by Levenson *et al.* [65, 66] noted that non-uniform distribution of cells led to overcrowding on the scaffold surface and that a thorough distribution of cells within the structure plays a vital part in extracellular matrix (ECM) production. The internal architecture could also be important in chondrogenesis and vascularisation, which could be incorporated into the print design to induce specific biological responses, such as vascular loops [67]. Tissue maturation and specific tissue matrix deposition are also found to be related to matrix stiffness and porosity, both of which can be easily controlled by printing parameters[68].

4.1 Types of Biofabrication techniques

There are two 3D bioprinting techniques widely adopted in scaffold fabrication for cartilage regeneration: inkjet based printing and extrusion based printing systems (Figure 3). Inkjet

printing operates on either thermal or piezoelectric actuators, which produce pressure pulses to form ink droplets (Figure 3a). The electrical heater uses air-pressure for the pulses to form droplets from the nozzle tip [63]. Both techniques are non-contact processes and are well known for the fast printing speed and high accuracy. Risk associated with fast printing speeds and small diameter sized nozzles are tip clogging, preventing the use of high viscosity inks and high cell densities in this method [62]

Figure 3 Bioprinting systems A) ink-jet based printing and; B) extrusion-based printing techniques

Extrusion printers on the other hand deposit the ink into a continuous filament rather than micro droplets by a pneumatic air-pressure or mechanical plunger based delivery system. A minimum filament diameter of $\sim 100\mu\text{m}$ results in structures with lower resolution compared to the above-mentioned techniques (Figure 3b). This fabrication technique however, is ideal for high viscosity inks and allows the printing of cell-laden hydrogels carrying high cell-density [62, 63, 69]. The advantage of extrusion-based printer towards cell viability after the printing process is studied by Park *et al.*[69]. They were able to prove that cells encapsulated in the hydrogel showed higher cell viability and attachment to the synthetic framework than cells seeded onto the prefabricated framework[69].

4.2 Hybrid extrusion printing

Biofabrication of the ear cartilage for example, requires a scaffold that matches the shape of the contralateral ear and is able to incorporate autologous chondrocytes or stem cells. It also needs to be strong enough to withstand contractive forces of the skin and natural bending, as well as degrading slowly for neo-cartilage formation. These requirements highlight the importance of ink development and selection, a component most challenging in the bioprinting process as it should satisfy both the biological needs of cell, but also the physical and mechanical needs of the printing process itself [70]

Hybrid printing, which is the printing of constructs using one or more inks, has been explored as a promising strategy for engineering tissue constructs where simply one type of bioink cannot satisfy the mechanical, biological and printing requirement of the entire construct. The core element of hybrid printing involves co-deposition of two or more elements in a spatial controlled manner. For instance, one structure could be cell-free, providing structural integrity and mechanical stability of the 3D printed construct, while the other being cell-laden, provides the conducive environment enabling cell growth and differentiation. An example of a hybrid printing process is where polycaprolactone (PCL) is used as a structural material and printed in between chondrocyte-impregnated alginate strands [71]. In most instances, constructs with complex shapes are in need of a sacrificial material to account for angulation and orientation.

The combination of a synthetic polymer as a framework and a cell-laden hydrogel enable the possibility to fabricate cartilage-like tissues as a 3D printed construct. This method is a promising alternative to single material inks for the reconstruction of the auricle and to produce patient-specific, large-volume tissues [28, 69, 72-74]. The final 3D printed construct can be divided into three main components as shown in Figure 4: 1) sacrificial material, 2) structural framework, and 3) cell supporting material. Each component has specific requirements necessary to build up a functional 3D construct and will be discussed in the following section.

Figure 4 Hybrid printing steps: A) Bioprinting a sacrificial material; B) addition of structural support for mechanical integrity; C) printing cell supporting material on top or in between strands; D) removal of sacrificial material to obtain final scaffold

4.2.1 Sacrificial materials

'Sacrificial materials' are required to achieve optimal shape fidelity and to improve the accuracy of 3D printed constructs. Therefore, the most important requirement of this material is the ability to be removed easily after the printing procedure. In addition, extra materials must be printable under similar conditions to other materials and they should be biocompatible. Because of these special properties, there are only a limited number of sacrificial materials available to select from as listed in Table 3[62, 63].

Table 3: Overview of the printable sacrificial materials used for auricular cartilage regeneration

Material	Scaffold design	Sacrificial procedure	Reference
Pluronic (F-127)	Ear shape	Sacrificed with water	[72]
PVA	Ear shape	Sacrificed with aqueous solution	[75]
PEG	Ear shape	Sacrificed with distilled water or cell culture media	[73, 74]

PVA= Poly (vinyl alcohol); PEG= Poly (ethylene-glycol)

Pluronics ® (Lutrol F127) are a class of commercially available thermosensitive hydrogels that have been widely reported as delivery systems for drugs and proteins [76]. These triblock copolymers of PEO-PPO-PEO (poly(ethylene oxide)-poly(propylene oxide)-poly(ethylene oxide)) can exhibit gel-like behaviour at concentrations between 15-30% w/w, and is a reversible process that depends on physiological temperature, pH and the critical micellar concentration (CMC) [77]. One major drawback is the rapid dissolution of Pluronics in aqueous medium, thus limiting its use in long-term drug release or tissue encapsulation applications [78, 79]. This drawback in drug delivery however, is advantageous when considering it as a sacrificial support material for bioprinting. Kang *et al.* [72], used Pluronic F-127 as sacrificial support for the cell-laden hydrogel mixture to guarantee the printed ear shape prior to crosslinking. Afterwards, the un-crosslinked Pluronic F-127 was easily washed out with water[72].

Poly (vinyl alcohol) (PVA) is a water-soluble synthetic polymer with high swelling properties. It can also be easily degraded by biological organisms through hydrolysis of its hydroxyl groups on the carbon backbone [80]. The complete decomposition of PVA in water can be achieved by immersing it in ~100°C for 30 minutes. Visser *et al.* [75], printed the anatomical structure of the human ear based on PCL with PVA as a support. The PVA was easily sacrificed through washing in hot water without compromising the quality of the target structure [75].

Poly(ethylene-glycol) (PEG) is a water soluble polyether that is non-immunogenic, non-toxic and possess useful properties such as protein and cellular resistivity [81]. PEG was used as

sacrificial material to support the fabrication of ear scaffolds in the work conducted by Lee *et al.* [73, 74]. PCL and PEG polymers were deposited layer-by-layer into a porous framework and the PEG components were easily removed with distilled water or cell culture media once printing was completed [73, 74].

4.2.2 Structural framework

To ensure the integrity of the scaffold after printing, polymers with sufficient mechanical strength can be incorporated into the design, herein referred to as the “structural framework”. When printed alongside other bio-inks, these materials can give rise to the overall rigidity of the scaffold, thereby improving the ease of handling for surgeons during implantation and help to maintain shape fidelity in post printing cleaning stages. In the case of hybrid printing, the presence of a structural framework within a scaffold design can provide temporary support for the weaker hydrogels and maintain their shape before crosslinking [82]. In the long run, this framework also protects the cell laden hydrogels from external forces to give time for cells to proliferate, differentiate and mature over time, such as skin contractions during healing in auricular cartilage reconstruction[71, 72, 74].

A list of printable polymers used as structural frameworks in scaffolds for auricular cartilage regeneration is listed in Table 4. Polylactic acid (PLA) is an organic biodegradable polyester, which is biocompatible and immunologically inert. Additionally, PLA has a melting temperature of 170 - 180 °C and is easy printable, making it interesting for 3D printing methods[83].

Polycaprolactone is a Food and Drug Administration (FDA) approved bioresorbable polyester and is used for medical devices since the 1980s [84]. It is one of the most commonly investigated biocompatible polymers for 3D printing because of the low melting point of 60°C and a slow degradation time (~1.5 to 2 years) [85, 86]. The low melting temperature enables PCL to be printed easily and in hybrid printing- alongside cell-laden hydrogels [87]. In addition, the synthetic polymer is well-known for excellent cell attachment, cell proliferation and a rapid cooling after extrusion, which minimises cell damage from heat transfer and provides long-term structural stability [72, 88-91].

PCL framework as support for the mechanical strength of a scaffold is one of the most commonly seen applications for 3D-printed biomaterials. Visser *et al.* [75], printed a uniform PCL scaffold in the shape of an outer ear with an auger screw driven melt extruder. This fabrication method showed promising results in the shape resolution of the print with overhangs and pertinent human dimensions [75]. Many research groups support their hybrid scaffolds with the synthetic material, which allows control of the mechanical properties and provides the shape [69, 72]. An alternate approach to use PCL as structural support in a scaffold was to design a cage-like structure surrounding the cell-laden hydrogel [82]. The cage was designed to prevent the cell-laden hydrogel from *in vitro* scaffold contraction while the tissue matures. This study showed that the framework maintains the shape during maturation and is a promising technique to withstand contractions and deformations [82].

Table 4: Printable polymers as structural materials used for auricular cartilage scaffolds

Material	Scaffold design	MW	Pattern design [#]	Mechanical testing	References
PCL	Ear shape	M _n 45000; M _w 48000–90000	d = 0.80- 1.80 mm	-	[75]
PCL	Ear shape	-	d = 300 µm f = 130 µm	Bending tests: ΔLoad%	[72]
PLA	Ear model and components	Filament (Builder Premium 3D Printer)	Slice resolution 200 µm	-	[92]
PCL	Cylindrical scaffold	(Polysciences, Warrington, PA)	d = 1000 µm f = 200 µm	Tensile tests: Elastic modulus tensile stress	[69]
PCL	Cylindrical cage	Medical grade (Purasorb; Purac Biomaterials)	-	Microindenter: Young's modulus	[82]

PCL= Polycaprolactone; PLA= Polylactic acid; d = strand spacing; f = filament width

4.2.3 Cell supporting material

For the encapsulation of cells in a hydrogel, natural polymers (biopolymers) such as collagen, gelatin, alginate or fibrin are generally more suitable than synthetic polymers (Table 5). Hydrogels are known for their high-water retention, which protects the cells from drying during the printing process. Biopolymers interact positively with cells and have high biocompatibility but are limited by low stiffness and strength [93-95]. The hydrogel can provide the required environment for the cells to maintain their migration, production of extracellular molecules and proliferation[64].

Hyaluronic acid (HA) is a natural polymer often used for cartilage regeneration. HA is an anionic, non-sulphated glycosaminoglycan (GAG) and is one of the main components of the ECM in the body. It is well-known as a support material for cells to facilitate cell adhesion, migration and proliferation [96, 97]. The weak mechanical properties and viscous nature however, require HA to be blended with other hydrogels or chemically modified (attachment of a methacrylate group) so that it can be photo-crosslinked to provide stability after printing [98].

Collagens constitutes a family of proteins present in the ECM of connective tissues. It comprises of three polypeptide chains that form a triple-helix structure and arranged in fibrils found in tissues such as tendon, bone, cartilage and skin [99]. Type II collagen is the predominant protein found in elastic cartilage with a small amount of type I collagen. Collagen is easily degraded and resorbed by the body and allows good attachment to cells. However, its mechanical properties are very low making it difficult to stack vertically when 3D printed and the rapid resorption by the body can affect the scaffold stability and maturation [100].

Fibrin is a protein, which is part of the clotting process in blood. Fibrin hydrogels seeded with chondrocytes can increase the production of GAG, which influences the production of ECM. Kang *et al.* [72], used a mixture of fibrinogen, gelatin, HA and glycerol as chondrocytes-laden hydrogel for auricular cartilage regeneration. Fibrinogen was cross-linked with thrombin, which converts it to fibrin. The mixture of the hydrogel maintained the structure after printing without cross-linking because of its high viscosity. Additionally, the hydrogel protected the cells during the printing process and produced microchannels for nutrient diffusion after the

sacrificial procedure [72]. Limitations for fibrin includes degrading prematurely prior to adding cell culture media.

Gelatin is an irreversibly hydrolysed form of collagen and mostly extracted from bones or skins of animals. It is often used for 3D cell printing applications due to its similarity to the extracellular matrix. The low stiffness of gelatin is a disadvantage for many applications; however, it is possible to overcome these limitations with chemical modifications of the gelatin hydrogels, as well as for biological properties [101]. GelMA is gelatin functionalized with methacrylate groups, where the hydrogel can become chemically crosslinked through presence of ultraviolet radiation [102]. Melchels *et al.* [103], printed the bioink consisting of 5% w/w GelMA and equine chondrocytes and assessed their viability. The cell were unaffected by the printing process, showing with an overall viability of 90% after 14 days.

Alginate is one of the most commonly used cell-laden hydrogels for 3D printed tissue constructs. However, many studies have limited the study to chondrocyte, as alginate does not have the necessary cell-adhesion motifs for cell-ECM interactions. Therefore, for adherent cell types, alginates are commonly modified with RGD containing peptides to promote adhesion and proliferation [104]. The anionic polysaccharide is commonly received from brown algae and is well known as a supportive material for cells in cartilage tissues. An alginate chondrocyte-laden hydrogel to repair cartilage defects of rabbit ear models was studied by Park *et al.* [69]. The 3D printed scaffolds with the cell-laden alginate hydrogel showed complete cartilage regeneration after 3 month of *in vivo* studies. In addition, the cell printed structures were completely integrated into the surrounded native auricular cartilage of the rabbits and formed round aggregates [69].

Lee and co-workers on the other hand, designed a large 3D printed ear construct using PCL as a structural framework and two different cell-laden hydrogels [74]. The study printed alginate encapsulated with human auricular chondrocytes, and porcine ear cartilage-derived decellularised extracellular matrix (ear-cdECM) onto the pores of the framework. After 8 weeks, they were able to show that the cells underwent chondrogenesis and were not affected by the long printing process with the hybrid printing approach [74]. The study by Kundu *et al.* [105] went even further and used a synthetic framework with three different compositions of the alginate hydrogel (**I** - alginate hydrogel without chondrocytes; **II** - alginate with chondrocytes; and **III** - cell-laden hydrogel with TGF- β as a growth factor). The combination

of a cell-laden hydrogel with a growth factor showed promising results for applications in auricular cartilage regeneration [105].

Table 1: Overview of the printable cell supporting materials used for auricular cartilage regeneration

Material	Scaffold design	Cells	Printing method	Reference
Fibrinogen	Ear shape	Rabbit Chondrocytes	Extrusion	[72]
Gelatin	Ear shape	Equine Chondrocytes	Extrusion	[103]
Alginate	Cylindrical scaffold	Rabbit Chondrocytes	Extrusion	[69]
Nanocellulose – Alginate	Ear shape	Human Chondrocytes	Extrusion	[106]
Alginate-ECM	Ear shape	Human chondrocytes	Extrusion	[74]
Alginate-TGF β	Cubic scaffold	Human Chondrocytes	Extrusion	[105]
Fibrinogen - Collagen	Cubic scaffold	Rabbit Chondrocytes	Inkjet	[107]
Fibrinogen-Hyaluronic acid	Cylindrical scaffold	Goat Mesenchymal Stem cells	Extrusion	[82]
Chitosan-Hyaluronic acid	Nanofilms	Rabbit Mesenchymal Stem cells	Inkjet	[108]

4.3 Cell sources

4.3.1 Primary Chondrocytes

Auricular cartilage is a type of elastic cartilage that is composed predominantly of ECM with relatively few cells. The distinction between elastic cartilage and the other subtypes; hyaline and fibrocartilage, is in the collagen, elastic fibre and tissue morphology [28]. The tissue itself has a very slow turnover at cellular and molecular level, so there is limited capacity for self-renewal and repair. Considerable success has been accomplished in a bio-engineered ear

cartilage using isolated chondrocytes [33, 109, 110]. Primary chondrocytes isolated from human or animal sources can be mixed within hydrogel matrices to create “bio-inks” suitable for printing [105, 111]. One major drawback in obtaining the initial seeding density of primary chondrocytes is that they can de-differentiate upon multiple expansions to fibroblast, thus are unable to generate appropriate matrix the same way as normal cartilage. Furthermore, as a consequence of the de-differentiation process, it switches from collagen type II synthesis to collagen type I. Most studies do not recommend chondrocytes to be used after the third passage. This leads to difficulty in expanding chondrocytes *in vitro* and limiting its use [112]. Acquiring large amounts of human cartilage to expand and stimulate is also not practical, due to limitation in availability and morbidity of donor sites in the body [7]. Therefore, there is a need to search for alternate chondrogenic cell sources and culture methods to minimise the number of chondrocytes needed.

4.3.2 Mesenchymal Stem cells

A promising cell source in the field of cartilage repair is the mesenchymal stem cells (MSC). MSCs are adult stem cells that have the potential to differentiate into tissues including bone, fat or cartilage [113]. They can be isolated from various human tissues, including bone marrow, umbilical cord, and adipose tissue. MSCs hold great promise in the field of cartilage regeneration due to their chondrogenic differentiation capability and their non-immunogenic nature [114, 115]. This key characteristic of MSCs, where they express low to intermediate levels of human leukocyte antigen (HLA) class I and are negative for cell surface expression of HLA class II molecules, makes them suitable for allogeneic therapy [116]. Interestingly, chondrocytes, adipocytes and osteocytes differentiated from human MSCs were also shown to be non-immunogenic in nature [112, 116]. Furthermore, the initial cell numbers required to culture and straightforward *in vitro* expansion protocols are an attractive cell source for tissue engineering. These all indicated that MSCs could be used as off-shelf product for allogeneic application for cartilage repair.

More recently, there are emerging studies suggesting the benefits of using co-cultures of chondrocytes and MSCs in cartilage regeneration. Studies have shown that co-culturing not only leads to increased chondrogenic gene expressions and ECM deposition [117-119], but could also solve the problem of hypertrophy and calcification in MSCs due to extensive chondrogenic induction [120-122]. A study by Meretoja *et al.* [120] also found that the trophic

effect of MSCs may increase the chondrogenic potentials of chondrocytes, thus reducing problems associated with primary chondrocyte harvesting and expansion. The exact molecular mechanism resulting in the stimulatory effect on the production of cartilage-like ECM when both cell types are co-cultured are yet to be fully understood. But there are studies suggesting that soluble factors such as transforming growth factor- β (TGF- β 2), insulin-like growth factors (IGF-1), and bone morphogenetic protein (BMP-2), secreted by chondrocytes provide the initial chondrogenic signals [117, 123], while other studies demonstrated that cartilage specific matrices produced by chondrocytes are the key in differentiation of MSCs [124, 125]. One thing for certain is the synergistic effect between MSC and chondrocytes, where MSCs induce chondrocyte proliferation and chondrocytes stimulate MSC chondrogenesis, thus eliminating the need for constant stimulation of MSCs by growth factors into chondrocytes.

4.3.3 Chondroprogenitor

Chondroprogenitor cells or cartilage stem/progenitors cells in the perichondral layer of the auricular cartilage may serve as an alternate source of cells for elastic cartilage reconstruction [126]. Progenitor cells are very similar to stem cells and have the ability to differentiate into a specific cell type. However, they are more specific than a stem cell and are only able to differentiate into its target cell-type. These cartilage progenitor cells have been identified to have the ability to differentiate into elastic cartilage, but also to regenerate both the perichondrium and chondrium layers, which may be beneficial for long term stability of any implant [126, 127].

The advantage of using autologous auricular progenitor cells as compared to harvesting primary chondrocytes is that it is a minimally invasive procedure that can be obtained from a thin fibrous layer of the auricle, even from microtia patients. From the perichondrial tissue, they can be isolated, cultured and guided TGF- β 2 and IGF-1 [128]. The chondrogenic potential in these types of cells are also found to be age related with younger donors demonstrating five times higher in glycosaminoglycan (GAG) synthesis and collagen type II formation than older donors [113, 128]. In addition, they are highly proliferative and can therefore shorten the *in vitro* culture period [129]. Reports of using perichondrial grafts with different biomaterials to enhance chondrogenesis have also been investigated. Collagen sponge wrapped in perichondrium implanted into rabbit models after 7 weeks showed accelerated cartilage formation than materials containing perichondrium alone [130]. This was also the case when ear perichondrium was wrapped around a piece of demineralised bone matrix and implanted

[131]. These results indicated that not only the cartilage progenitor cells could produce collagenous components, but are also able to maintain a non-calcified phenotype in the reconstructed cartilage. They represent a promising cell source for cartilage regeneration but longer *in vivo* studies are required.

5. Conclusion

The best surgical treatment for microtia, either autologous, alloplastic or prosthetic for auricular reconstruction is determined by various factors. These are mainly patient related factors (age, medication, and allergies), pathology of the residual ear, existing scars, and condition of surrounding tissue. The decision of whether the patient should undergo reconstruction via the autologous or alloplastic option also depends on the surgeon's experience and expertise. One of the limitations in traditional alloplastic frameworks is to accommodate individual patient's reconstructive needs. Autologous reconstruction was better in this aspect in that there is no size or shape limitations as this can be modified during surgery, but comes with various degrees of complications. The use of prosthetics are also reserved for special cases and usually considered when the previous two options have failed.

The combination of tissue engineering and additive manufacturing represents a new era to create custom-made tissue engineering solution to reconstructive surgery, which is heavily reliant on donor grafts and synthetic biomaterials. Compared to other organs, the cartilage is less complicated because of its avascular, aneural and alymphatic nature due to the dense ECM. Nevertheless, the combination of biomaterials, cells and biofabrication techniques to mimic the human ear still requires careful selection of appropriate structural, sacrificial, cell supporting materials and cell source. The choice between chondrocytes, stem cells, chondroprogenitor cells or co-culturing of cells, can all influence the final tissue morphology and composition as it matures over time.

6. Current challenges and future trend

Cartilage tissue engineering through bioprinting provided an alternative to currently used techniques that suffer from various limitations, but auricular cartilage regeneration is still at its infancy [132]. Longer *in vivo* and *in vitro* evaluation and efficacy studies are required. In addition, an ongoing aspect of bioprinting is stem cell harvesting and culturing for use in

bioinks. Without an economically viable, efficient and reproducible method, bioprinting for cartilage regeneration cannot progress onto clinical trials. The use of pre-differentiated stem cells still meant that it requires at least 4-6 weeks of culture *in vitro* and for autologous procedures, the speed of cell processing is critical and requires more attention. Bioreactors may provide a vital role in maturation during *in vitro* culture, as reports have shown that the use of bioreactors speeds up the tissue maturation process and distribute nutrients uniformly across the scaffold. This resulted in better deposition of ECM [133].

Although auricular reconstruction is an aesthetic practice that requires a customised approach, the ongoing challenge is also in the performance and integration of the scaffold longer term. Bioprinting has already simplified the solution to fabricate an exact replica of the contralateral ear cartilage and the challenge now is to provide the required internal architecture or biological cues to better mimic the native cartilage and allow integration/vascularisation [134]. Studies now are leaning towards depositing multiple tissue types or materials into a single construct, as well as using multiple fabrication techniques to produce the scaffold [135, 136]. The use of chondrocytes alone cannot provide the sufficient cell numbers for a full size scaffold and more focus is on the use of MSC or adipose stem cell (ADSC) as they offer larger number of cells with high chondrogenic potential and readily accessible. Another challenge is to regenerate not just the cartilage but the skin and earlobe, which consist of fat cells. Cartilage and fat tissue induced simultaneously through co-culture has been shown possible by studies conducted by Lee *et al.* [137] and can fully utilise the capability of biofabrication technology. In addition, as the direction is heading towards better and more precise deposition of biological materials, laser based bioprinting techniques such as laser induced forward transfer (LIFT) offer several advantages over inkjet and extrusion based printing systems. The spot size resolution is close to the inkjet resolution of 40-100 μm and the printing speed is faster than extrusion. Since this method is nozzle-free, the viscosity (1-300 mPa/s) and cell density (1×10^8 cells/ml) achievable by this technique are much higher than inkjet [138]. However, a viscous material also suggests that cell distribution may not be uniform and the side effects of extended or intense laser exposure on the cell are yet to be well understood [139]. Further studies into the complementary use of different fabrication techniques, such as melt electro writing (MEW) [136, 140], inkjet, laser and extrusion based printing systems may open new doors where the scaffold itself can direct cell growth or maturation through internal architecture and variations in mechanical properties.

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