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Regenerative therapies for tympanic membrane

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ABSTRACT

It is estimated that by 2050 one in every ten people will be suffering from disabling hearing loss. Perforated tympanic membranes (TMs) are the most common injury to the human ear, resulting in a partial or complete hearing loss due to inept sound conduction. Commonly known as the eardrum, the TM is a thin, concave tissue of the middle ear that captures sound pressure waves from the environment and transmits them as mechanical vibrations to the inner ear. Microsurgical placement of autologous tissue graft has been the "gold standard" for treating damaged TMs; however, the incongruent structural and mechanical properties of these autografts often impair an optimal hearing restoration following recovery. Moreover, given the lack of available tissues for transplantations, regenerative medicine has emerged as a promising alternative. Several tissue engineered approaches applying bio-instructive scaffolds and stimuli have been reported for the TM regeneration, which can be broadly classified into TM repair and TM reconstruction. This review evaluates the current advantages and challenges of both strategies with a special focus on the use of recent biofabrication technologies for advancing TM tissue engineering.

1. Introduction

1.1. Tympanic membrane anatomy and function

The tympanic membrane (TM), commonly known as the eardrum, is a thin, concave tissue located at the end of the ear canal, marking the separation between the outer and middle ear compartments (Fig. 1**A**). The main function of the TM is to capture sound pressure waves from the environment and transform them into mechanical motion [1]. The generated acoustic vibrations are transmitted to the ossicular chain composed of the malleus, incus and stapes bones, which passes them on to the electro-mechanical sensory system of the inner ear.

The human TM has a complex three-dimensional (3D) spatial arrangement consisting primarily of two regions – *pars tensa* that occupies the majority of TM and *pars flaccida*, a relatively slack, triangular sub-membrane that lies only in the superior region. Furthermore, the TM is a tri-laminar structure, where the layers of *pars tensa* include mucosal epithelial on the inner, *lamina propria* in the middle and an outer epidermal layer [2]. The *lamina propria* is a thin sheet of connective tissue, composed of an intricate collagenous network with specific spatial orientations – radial, circumferential and parabolic (Fig. 1**B**) [3]. The TM is affixed to the ossicular chain through the *manubrium* (that is, handle) of the malleus, which extends from its neck up to the center of the TM, termed

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as the umbo [4].

Under physiological conditions, the human TM has a concave architecture with an oval layout. The apex of this concave structure is located centrally at an angle of 132–137° and depth of 1.42–2.00 mm, directed towards the inner ear [5]. The horizontal diameter of the oval measures between 8 mm and 9 mm, whereas the slightly longer vertical axis varies in the range of 8.5 mm to 10 mm [2,3]. In a healthy TM, the mucosal epithelium has been reported to be the thinnest (1–10 μm), followed by the outer epidermal layer (5–12 μm). The bulk of the thickness is contributed by the collagen fibrous layer of *lamina propria*, which usually is around 30–50 μm [6]. The thickness has been regarded as one of the critical factors controlling the vibratory response of TM, however, owing to its complex and non-homogeneous distribution even across the same specimen, its precise estimation has been difficult [7]. Researchers in the past have suggested up to 10 thickness zones with varying Young's moduli for each [5,8,9], although an average value around 74 μm is widely accepted [10,11].

1.2. TM injuries and conventional therapies

According to the latest statistics released by the World Health Organization, 466 million people around the globe suffer from disabling hearing loss, which is estimated to rise to over 900 million people by 2050 [12]. Tympanic membrane perforations (TMPs) are the most common damage to the human ear, resulting in a partial or complete hearing disability due to inept sound transmission. The typical TMP etiologies include microbial infections, acoustic traumas and insertion of foreign bodies [13]. Among them, chronic otitis media represents one of the most widespread infectious diseases, especially in young children [14,15]. The infection begins with the middle ear inflammation, which in majority of the cases, is accompanied by a spontaneous discharge of infected secretions through eustachian tube into the nasopharynx. In patients with an obstructed eustachian tube, the infected pus and blood is released as an aural discharge, leading to a perforated TM [16]. For children with recurrent ear infections and persistent fluid build-up, insertion of tiny, cylindrical tympanostomy tubes is the common surgical practice to keep the middle ear ventilated [17]. However, a substantial number of TMPs result following the tympanostomy procedure, especially in cases where these tubes are not extruded spontaneously and a residual hole persists upon removal [18,19].

Most acute perforations usually heal without an external stimulus. The inherent regenerative capacity of the TM has been attributed to the presence of latent progenitor cells within its epithelial layers [20]. However, unlike the acute TMPs, the chronic ones lack a tangible support to facilitate the necessary cell migration and proliferation. They are, therefore, often considered for a surgical repair based on the risk of subsequent complications [16]. Unsafe TMPs often trigger conductive hearing loss or cholesteatoma formation, which is a destructive expansion of squamous epithelium in the middle ear [21]. The first attempts to repair a perforated TM was made in 1640, when a segment of pig bladder was used to close the perforation [22]. Since then, the standard surgical operations for treating TMPs have been tympanoplasty and myringoplasty. In general, tympanoplasty has been defined as the surgical technique to restore a defected TM with or without reconstruction of the middle ear hearing mechanism. Historically, it has been classified into five types depending on the extent of reconstruction required [23]. Among the different categories, myringoplasty has been defined as type I tympanoplasty, which involves only the restoration of the perforated TM when the ossicular chain remains unaffected and the connection between the malleus and the TM is preserved [24]. The other types II–V include at least some forms of ossicular reconstruction, which is beyond the scope of this Review.

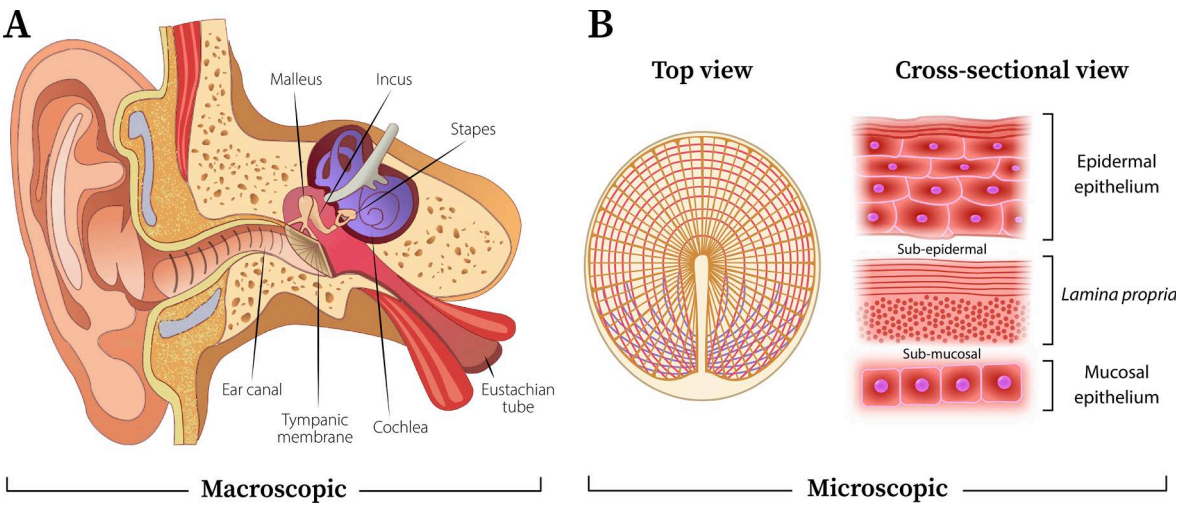


Fig. 1. Anatomy of the human ear and TM. (A) Frontal section of the ear depicting the key anatomical elements, such as the ear canal, TM, ossicular chain (malleus, incus, and stapes), cochlea and the Eustachian tube. **(B)** A closer look at the construction of the human TM – top view: collagen fibrils arranged within the *lamina propria* in a radial (orange), circumferential (pink) and parabolic (purple) pattern; cross-sectional view: illustrating the tri-laminar structure of the TM with three distinct cellular layers. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

The "gold standard" for treating chronic TMPs has been the microsurgical placement of autologous tissue grafts from the temporalis fascia and perichondrium [25]. Two classical approaches of myringoplasty have been developed for precise graft placement, which are the underlay and the overlay techniques [26]. The underlay myringoplasty technique, being the simpler of the two, is normally applied to smaller and easily visualized perforations, where the graft is placed medial to the remaining TM. On the other hand, the overlay technique implements the more challenging lateral positioning. In general, both the surgical procedures have been extremely effective in successful closure of TMPs, but recurrent perforations are still common due to persistent middle ear pressure differences, thereby leading to multiple revision surgeries [27]. Given the limited availability of autologous graft tissues, the surgeon is often left without sufficient materials for the successive tympanoplasties. The other limitations include high capital investment, specialized microsurgical skills for precise tissue carving, donor site morbidity in autografts and risk of infections in homo- and xenografts [28,29]. Moreover, tissue grafts harvested from other anatomical sites often lack the requisite mechanical properties for an effective vibro-acoustic transmission. Therefore, small perforations located centrally on the *pars tensa* have conventionally been sealed with absorbable scaffolding materials, such as gel foams and paper-patches for guiding the migration of epithelial cells from the borders of the perforation [30]. However, these primitive grafts have demonstrated a poor success rate, due to their easy detachment and inadequate resistance to subsequent infections [31]. Besides, in larger perforations, even if the outer epithelial layers manage to migrate onto the implanted patches, they usually fail to regenerate the collagen fibrillar network of the *lamina propria* [31,32]. Thus, resulting in creation of a weak tissue deprived of the essential structural features for an optimal sound conduction.

Owing to the existing shortcomings of the current surgical techniques, there has been an increasing interest towards developing novel tissue engineering (TE) therapies for the construction of TM scaffolds with functional material properties and 3D geometry. The following subsections provide a brief overview on this subject.

1.3. Tissue engineering approaches towards TM restoration

TE is an evolving field of research with immense potentiality for treatment of damaged or dysfunctional tissues. Considering its growing relevance in medical science, several TE approaches have been introduced for an optimal restoration of TM and its functionalities. Based on the regeneration mechanism, the current strategies can be broadly classified as TM repair and TM reconstruction.

Surgical approaches towards TM repair are predominantly an offshoot of the conventional myringoplasty technique, where autologous tissue grafts have been replaced by biomaterial-based artificial patches. Reasons, such as scarcity of suitable grafting tissues, donor site morbidity, lack of tunable mechanical properties, and reduction of surgical time have prompted the rise of TE as a supporting tool, to guide and enhance the inherent regenerative capabilities of the perforated TM [33]. Some of these works are described in section 2, where considering the recent progress in this field, we have focused primarily on the repair surgeries reported to be successful in clinical trials. Several *in vitro* and animal studies have also been reported for the repair of TMPs. For more information on these, the reader is encouraged to refer to the reviews by Teh *et al.* [33], Hong *et al.* [31], and Villar-Fernandez *et al.* [34].

Myringoplasty trials both with and without tissue engineered grafts have demonstrated a higher and faster closure rate; however, restoration of the full hearing capacity has remained a challenge [35]. This has been ascribed to the loss of precise alignment of the collagen fibers and the associated vibro-mechanical behavior, which are never fully recovered with the contemporary grafting

Table 1
Resume table with the current biofabrication studies reported for the reconstruction of the human tympanic membrane. The techniques applied can be broadly classified into: [§]additive manufacturing, and [†]nanofiber spinning. The list of abbreviations used (in order): poly(caprolactone) (PCL), silk fibroin (SF), poly(lactic-*co*-glycolic acid) (PLGA), poly(ethylene oxide terephthalate)/poly(butylene terephthalate (PEOT/PBT), poly(dimethylsiloxane) (PDMS), poly-L-lactic acid (PLLA), gelatin methacrylate (GelMA), growth factor (GF), hyaluronic acid (HA).

Literature	Techniques	Biomaterials	Characterizations
Lee *et al.*, 2014 [45]	† Solution electrospinning	PCL, SF	Mechanical, *in vitro*, *in vivo*, auditory
Mota *et al.*, 2015 [38]	† Solution electrospinning § Melt extrusion	PLGA, PEOT/PBT	*In vitro*
Danti *et al.*, 2015 [46]	† Solution electrospinning	PEOT/PBT	*In vitro*
Kuru *et al.*, 2016 [44]	§ Selective laser sintering/ molding	Silicone rubber	Acoustical, biomechanical
Kozin *et al.*, 2016 [40]	§ Solution extrusion	PDMS, PLA, PCL, collagen	Mechanical, acoustical
Jang *et al.*, 2017 [42]	† Melt centrifugal spinning § 3D bioprinting	PCL, collagen, alginate	Mechanical, *in vitro*, *in vivo*, auditory
Immich *et al.*, 2017 [47]	† Solution electrospinning	PLLA, PLGA	Surface, *in vitro*, *in vivo*
Kuo *et al.*, 2018 [43]	§ 3D bioprinting	GelMA	Mechanical, *in vitro*, *in vivo*
Li *et al.*, 2018 [48]	† Solution electrospinning	Gelatin, genipin	Surface, mechanical, *in vitro*
Seonwoo *et al.*, 2019 [49]	† Solution electrospinning	PCL	GF release, *in vitro*, *in vivo*
Moscato *et al.*, 2020 [50]	† Solution electrospinning	PCL, HA	Mechanical, *in vitro*
Anand *et al.*, 2021 [39]	† Solution electrospinning § Melt extrusion	PEOT/PBT	Mechanical, acoustical, *in vitro*
Ilhan *et al.*, 2021 [41]	§ Solution extrusion	PLA, chitosan, alginate	Thermal, mechanical, *in vitro*
Witzleben *et al.*, 2021 [51]	§ Melt electrowriting	PCL, collagen	Mechanical, acoustical, *in vitro*

technologies. The intricate 3D architecture and material properties of the TM have been deemed crucial for an efficient sound transmission [36]. Therefore, in the last few years, technologically-enabled TE approaches have been introduced to develop novel strategies for replicating the exact fiber arrangement and properties of the native collagen layers. The current regenerative therapies, especially for chronic perforations are witnessing a gradual shift towards a partial or full reconstruction of the damaged TM to improve the post-treatment auditory response. This review presents a critical assessment of all the relevant efforts made in this direction, along with their existing challenges and outlook on future improvements.

1.4. Biofabrication: a reconstruction toolkit

Biofabrication has evolved as a powerful toolkit for TE, owing to the multitude of techniques available for manufacturing 3D scaffolds with complex architectures and broad range of mechanical characteristics [37]. Scaffolds with hierarchical or smart surface properties capable of steering cell activity have been developed with biofabrication techniques, such as additive manufacturing (AM) and electrospinning (ES). Table 1 offers a concise compilation of the current approaches employed for a partial or full reconstruction of the TM, ranging from AM technologies, such as melt or solution extrusion [38–41], 3D bioprinting [42,43], and selective laser sintering (SLS) [44] to melt or solution ES [38,39,45–51]. Additionally, the biomaterials employed for each study have been highlighted along with the respective characterizations conducted. The working principles of all these strategies including their key strengths and weaknesses have been further discussed in section 4 to evaluate their applicability in fabricating functional TM scaffolds.

2. Regenerative therapies for TM: *The TE approach*

The techniques of tissue and organ transplantations have been regarded as a revolutionary breakthrough in medicine. However, the factual possibility of applying them to millions of patients across the globe is still limited by some inevitable constraints. Among them, the lack of available donor tissues and organs remains to be the most pressing one. Statistics suggest that over 114,000 patients have been currently placed on the transplant waiting lists in the United States, where 12,548 of them lost their lives or became too sick to receive a transplantation in 2017 alone [52]. Other challenges include the immune response, thereby leading to chronic rejection of the transplanted tissue or organ over time. Therefore, an alternative to organ transplantations is deemed necessary.

TE has emerged as a promising approach towards tackling the unavailability of sufficient implantable tissues and organs. Conceived in the late 1980s, TE has been defined as *"an interdisciplinary field that applies the principles of engineering and the life sciences toward the development of biological substitutes that restore, maintain, or improve tissue function"* [53]. Three essential pillars have been identified for the creation of these artificial yet biologically functional tissues; these are (a) supporting scaffolds, (b) tissue-inducing bioactive stimuli, and (c) living cells (Fig. 2). The tissue-inducing bioactive molecules provide a suitable biochemical microenvironment for the proliferation, migration, and differentiation of the living cells over the supporting scaffold. In the subsequent subsections, the three pillars will be discussed in depth for TE approaches for the TM.

2.1. TM scaffolds

Scaffolds in TE are defined as a 3D arrangement of porous biomaterials, acting as a template for cell adhesion, growth and subsequent tissue formation. Numerous biomaterial and biofabrication strategies have been devised for mimicking the fundamental properties and architectures of native tissues, within these artificially constructed templates. They are often intended as temporary

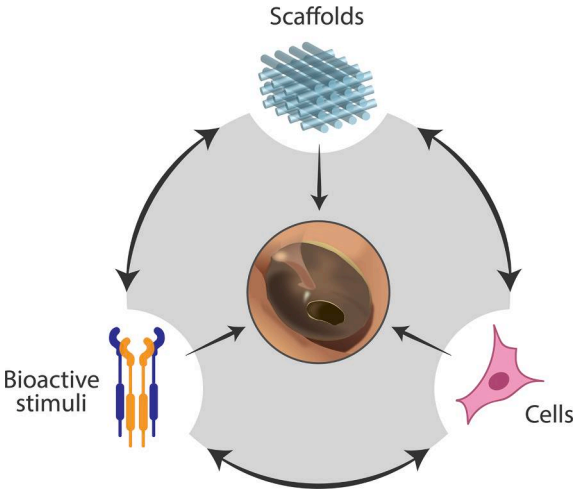


Fig. 2. Regenerative therapies for the TM. Schematic representation of the three pillars of TE applied in the context of perforated eardrums. The double headed arrows represent their growing inter-dependence of each other for an effective regeneration strategy.

implants that are gradually replaced by new extracellular matrix (ECM) deposited by the cells. Some of the key factors to be taken into account while designing a tissue engineered scaffold include its biocompatibility, biodegradability, mechanical properties, porosity and ease of fabrication [54]. An interconnected pore structure is highly desirable to facilitate cell infiltration and diffusion of nutrients within the tissue construct. Therefore, a balance between the scaffold porosity and its mechanical properties has been deemed crucial.

Several bioresorbable materials have been explored for the reconstruction of a damaged TM [55]. These can be broadly classified into natural and synthetic, based on their origin. Among the naturally derived substrates, decellularized tissues have received a special attention in the recent years. The decellularization process preserves the functional proteins and structural features of the native tissue, thereby mimicking the inherent ECM microenvironment when engineered as a tissue scaffold [56]. Some of the key studies manipulating decellularized tissue for TM repair have used acellular dermis [57], porcine urinary bladder [58], porcine dermis and dura mater [59], and human cadaver skin (commercially available as AlloDerm®) [60]. However, these tissues often present batch variability and biosafety concerns over the presence of residual antigenic components in the donor grafts, that can induce inflammatory and immunogenic reactions, ultimately leading to acute or chronic rejections [61].

Other biomaterials applied for TM reconstruction include naturally derived polymers such as silk fibroin (SF) [29,62], chitosan [63,64], hyaluronic acid (HA) [65,66], alginate [19], and gelatin [43,67]. The use of polymers offers a superior control over the structural and mechanical properties, which has been further explored by the introduction of synthetic biomaterials into TE. Poly (glycerol sebacate) [68], poly(dimethylsiloxane) (PDMS) [40], poly(lactic acid) (PLA) [40,47], poly(caprolactone) (PCL) [40,45,49,50], poly(lactic-*co*-glycolic acid) (PLGA) [38,47], and poly(ethylene oxide terephthalate)/poly(butylene terephthalate) (PEOT/PBT) [38,46] are some of the synthetic polymers that have been utilized so far for the TM reconstruction. The applications of these materials and the biofabrication strategies applied have been covered extensively in sections 3 and 4.

2.2. Bioactive stimuli for TM regeneration

With the evolution of advanced cell culture techniques for deriving tissue-specific cell types, the application of bioactive stimuli has become crucial to modulate the essential cellular behaviors, such as, adhesion, proliferation, migration, differentiation, and maturation for tissue regeneration [69]. One of the frequently used bioactive stimuli are growth factors (GFs) in the form of cytokines, hormones and proteins that are known to instruct the cells during their physiological development. In the context of TE, these cell-signaling molecules have been identified to induce three prime activities – mitosis, proliferation and morphogenesis [70]. They achieve this by binding to specific cellular receptors and activating relevant signaling pathways to regulate gene transcription within the nucleus and stimulate desired response. The efficacy in transmitting the appropriate message does not only depend on the choice of GFs and their diffusion through the ECM, but is also determined by their corresponding receptors, target cell number and intracellular signal transduction. The same GF can transmit completely distinct instructions to two different receptors or cell types that it binds to [70]. Some of the common GFs applied for tissue regeneration include epidermal growth factor (EGF), basic fibroblast growth factor (bFGF), hepatocyte growth factor (HGF), nerve growth factor (NGF), platelet-derived growth factor (PDGF), transforming growth factor (TGF), vascular endothelial growth factor (VEGF), and insulin-like growth factor (IGF) [71–73]. Among them EGF, bFGF, TGF and PDGF have demonstrated promising results for the regeneration of the human TM [31,74].

As early as in 1995, EGF and bFGF were identified to be expressed during the healing of traumatic TMPs in guinea-pigs [75]. Owing to the recent advancement in recombinant DNA technology, these GFs along with a few others have been evaluated and demonstrated to accelerate the wound healing in TMPs [74]. In general, two key strategies have been explored so far for loading them into the biomaterial substrates – (1) physical encapsulation, and (2) chemical immobilization [70]. Both the approaches have been applied widely for TM repair; however, initial studies have reported even direct one-time application of these GFs to be equally effective in treating acute perforations [76]. In a series of clinical studies performed by Lou *et al.*, the efficiency of bFGF and EGF in promoting TMP closures was thoroughly investigated [77–83]. For large perforations (>50% of TM area), closure rates of 100% and 97% were reported in case of groups treated with direct bFGF application and bFGF with Gelfoam®, respectively, whereas the control group with no medical intervention resulted in only 55% of successful closures after 3 months [77]. Patients treated with bFGF also demonstrated an accelerated closure time, with a four-fold improvement in their average rates as compared to the non-treated ones. Identical results were also obtained by Kanemaru *et al.*, where they used an additional fibrin glue over the sponge as a sealant [84]. To further highlight the effectiveness of bFGF in closing large TMPs, a comparative study for patients with curled perforations was conducted [79]. It has been hypothesized that curled perforations, in particular the ones with inverted edges, may induce abnormal epithelium migration, and develop middle ear cholesteatoma. The need for approximating these folded edges to improve the TM restoration was investigated by Lou *et al.* [67,79], where it was reported that edge approximation alone yielded a perforation closure rate of only 60% as compared to 100% success of direct bFGF application.

In another clinical trial performed by Lou *et al.*, the healing rates at the end of one month were correlated with the time when a patient with traumatic TMP was admitted into the hospital [78]. All patients were equally treated with gelatin sponge loaded with bFGF, but at different time intervals: 0–3 days, 4–7 days, 8–14 days, and 14–28 days. The results indicated that bFGF does not have much role to play during the initial inflammatory phase of TM regeneration, and becomes significant only later during the proliferation stage. A similar experiment conducted with direct application of bFGF further corroborated that the supplementary factor is most effective only when applied three days after the injury, that is after the inflammatory phase [80]. As a continuation of this work, a comparative study between bFGF and EGF was reported, where it was demonstrated that both GFs were equally effective in healing traumatic TMPs [83]. Both the GFs exhibited higher closure rates and lower closure times with respect to the control group. The work revealed that, although EGF and bFGF were both active during the proliferation phase, they targeted different responses – EGF stimulated the proliferation and migration of the epithelium, whereas bFGF induced a faster proliferation of the fibroblasts in the

fibrous tissue. More recently, Lou *et al.* proposed the topical application of GFs with no scaffold material as a cheaper and safer alternative for the restoration of traumatic TMPs [82]. The study found that the topical treatment with EGF significantly shortened the recovery time as compared to the patients who did not receive any treatment. However, no results were presented to compare their performance with respect to the conventional patches loaded with EGF.

TGF, a family of proteins structurally and functionally similar to EGF, has also been explored as a potential agent to promote wound healing in persistent TMPs [85]. However, there is a lack of investigation verifying its effectiveness in humans. PDGF is another growth factor reported to stimulate tissue regeneration within ruptured TMs [86]. It is known to induce mitosis of connective tissue cells and fibroblasts accompanied by an increased collagen production [87]. After a couple of successful animal studies in chinchilla [88] and rat models [89], Röösli *el al.* conducted the first clinical trial using PDGF for treating patients with chronic suppurative otitis media [90]. However, the application of PDGF was found to be of no added advantage as compared to a placebo.

2.3. Cells in TE approaches for TM

Incorporation of cells, the third pillar of TE approaches, constitutes a promising alternative to scaffold-only strategies. An expansion and culture of cells in a 3D environment is usually necessary before applying them as tissue implants. The cellular microenvironment, composed of scaffolds and bioactive stimuli provide the favorable biochemical and biophysical cues for the growth of cells into the desired tissue. Currently, there are three major sources for procuring tissue-specific cell types – primary tissues, cell lines, and stem cells. Owing to the innate regenerative capabilities of the TM not many cell-based studies have been conducted. Researchers have predominantly relied on "guided tissue regeneration", where an acellular scaffold is designed to promote tissue restoration solely by cells surrounding the implantation site [91]. The outer epidermis is the first layer to migrate during the TM healing process, followed by the proliferation of stratified squamous epithelium along the excoriated edges of the perforated TM. However, in case of chronic perforations, the epithelial cells alone are inadequate in repairing the damage [32]. Several studies have reported the need of a mechanical support to initiate their migration and subsequent proliferation, thereby advocating an acellular approach as long as a TM remnant exists with progenitor cells for regeneration [33]. The area around the umbo and manubrium of malleus is believed to host progenitor cells that induce the epithelium migration [92]. On the other hand, there are some cellular studies as well that have demonstrated an enhancement in TMP healing using embryonic or mesenchymal stem cells [93,94].

In 2003, Unge *et al.* conducted a pilot study to investigate the influence of embryonic stem cells (ESCs) over the healing of TMPs [93]. Applying the principles of moiré interferometry, the mechanical stiffness of ESC-treated TMs were compared with the control group. The ESCs were reported to enhance the healing process. However, further studies would be required to ensure the efficacy and safety in applying these cells for clinical use. Later, in the subsequent work by Rahman *et al.* on Sprague–Dawley rats, a detailed assessment of stem cell treatment was performed for acute [95] and chronic TMPs models [94]. No significant difference was observed between ESC-treated and control ears in the case of acute perforations. All the TMs (treated or untreated) were closed by day 14, and demonstrated an identical pressure tolerance and stiffness after 6 months [95]. However, the ESC-treated TMs were found to be slightly thicker than their control group at the site of the perforation (36 μm versus 28 μm). This localized thickening was even more profound in the case of chronic perforation in the same Sprague-Dawley rat model, where a five-fold increase was reported with mesenchymal stromal cells (MSCs) [94]. This increment in thickness of the *lamina propria* was attributed to the invading fibroblasts and subsequent ECM deposition. The overproduction of these fiber-like structures was hypothesized to compensate for the lack of any desired fiber orientation. Furthermore, the thickening of the TM allowed to withstand pressure gradients mimicking sneezing and coughing. The MSC-treated ears demonstrated a higher rate of perforation closures as compared to the control (40% versus 10%), although no statistical analysis was presented owing to the small number of specimens tested. The MSCs are known to secrete a variety of surface molecules, trophic factors and immunomodulators relevant for the TM regeneration, although the exact mechanism of the MSC-regulated healing for TMPs is still under investigation [96]. Several studies have been initiated in this direction using the recent biofabrication technologies [38,42,46,50], which are covered in section 4 of this review.

In the recent years, there has been a growing interest towards harvesting TM-derived cells for TE applications. Deng *et al.* isolated fibroblasts from guinea pig TM and cultured them on porcine acellular dermis and dura mater scaffolds for treating chronic TMPs [59]. Similar to the previous studies, an initial thickening of the TM was observed, which gradually became thinner and transparent. Although no statistical differences were highlighted, the healing rate of scaffolds without cells was found to be lower than the ones seeded with cells. Furthermore, culture of eardrum keratinocytes, harvested from human explants, have also been reported and investigated for TE applications [29,97]. Ghassemifar *et al.* isolated human TM cells from the *pars tensa*, and cultured them on *Bombyx mori* SF membrane. After 15 days in culture, the TM-derived cells were found to be expressing protein markers related to the keratinocyte phenotype, adhesion and proliferation [29]. However, the applicability of using terminally differentiated keratinocytes for repairing damaged TMs was questioned justifiably by Liew *et al.* [98]. Instead, they advocated the isolation and culture of regenerative cells, localized in the umbo and annulus regions of the TM. In a previously reported work, Kim *et al.* had demonstrated the isolation of these latent progenitor or stem cells, and studied their role as potential regulators of TM regeneration [20]. Applying a rat TM model in both these studies, the presence and successful harvest of potential epidermal stem cells was confirmed, based on putative stem cell markers including CK 19 and integrin β1 [20,98]. Keratinized epithelium of the TM has been recognized analogous to the skin epidermis [99], therefore, epidermal cell markers were used for identifying the TM epithelial stem cells. Not much was reported on the regenerative mechanisms for the connective tissue, although an outgrowth of mixed population of fibroblasts and epidermal cells was observed at 24 h in perforated TM explants [98]. Other cell-based therapies investigated in combination with different biofabrication approaches have been further discussed in section 4.

3. Biomaterials employed for TM regeneration

Biomaterials is a growing field of research for a wide range of biomedical applications, such as, therapeutic treatments, TE, regenerative medicine, diagnostics and drug delivery [100]. The exact definition of a biomaterial has been under a constant debate ever since its first usage decades ago. However, a widely accepted definition describes biomaterials as *"a nonviable material used in a medical device, intended to interact with biological systems"* [101]. This simplistic interpretation avoids the inclusion of transplanted organs as biomaterials, while embracing decellularized tissues and artificial scaffolds, eventually repopulated with cells.

Traditionally, biomaterials have been manipulated for three key approaches in TE – (1) as supporting structures to induce cellular migration and tissue regeneration [19,64,102], (2) as immunoprotective systems for encapsulation and transplantation of cells [103,104], and (3) as scaffolding matrices promoting cell growth for tissue reconstruction [38,105,106]. All the three strategies have been applied extensively for different TE applications. However, considering the regenerative capabilities of the TM, the cell-based approaches have not received enough attention. Most of the earlier works have focused on transplanting acellular grafts to stimulate migration and proliferation of the native epithelium [33]. Only recently with the advent of latest biofabrication techniques, researchers have initiated attempts to reconstruct the TM for a complete replacement. Based on section 2.1, the biomaterials employed for both these approaches have been categorized into natural and synthetic polymers, which have been further discussed in the following sections.

3.1. Natural polymers

Several naturally derived polymers have been applied as grafting materials to improve TM wound healing. Among which, HA has emerged as a promising candidate with excellent results. HA is known to regulate the orientation of fibroblasts and collagen fibers during closure of the *lamina propria*, thereby providing additional ECM support [107]. Clinical trials with exogenous HA has been conducted both in the form of direct application [107–109], and while compounded with graft materials [66,110–112]. For the latter, Saliba *et al.* introduced the use of a commercial biomaterial composed of HA ester, EpiDisc® otologic lamina (Medtronic Xomed, Jacksonville, Florida, USA) [110]. Through a series of myringoplasty trials, the procedure was reported to be simple and inexpensive, with closure rates comparable to that of conventional techniques [66,110,111].

More recently, a transparent silk patch derived from the fibroin protein of silkworms was investigated for treatment of traumatic TMPs in humans [113]. Backed by several *in vitro* [62,114] and animal studies [115], the clinical trial confirmed a faster closure time with SF as compared to the paper patch myringoplasty. This was attributed to its superior elastic, adhesive and keratinization inducing properties. Another latest addition to the list of grafting materials tested for treating TMPs is bacterial cellulose (BC) [116,117]. Formed by random interwoven cellulose microfibrils, BC is obtained as a translucent gelatinous biocompatible film with mechanical characteristics analogous to that of the soft tissues [118]. A comparative study demonstrated an enhanced healing and hearing response in patients treated with BC graft as compared to the ones operated with fat graft and temporalis fascia [117]. Chitosan is another natural biomaterial derived from the shell of crustaceous that has been studied extensively for the manufacturing of patches for TM repair. *In vivo* studies performed on rats with mechanically perforated TMs were treated with chitosan patches with [119,120] and without EGF [64,121], showing a closure rate from 90% (2 weeks) to 57% (10 weeks). Despite the high variability of the obtained results in the rat models, a study performed in patients with small and medium TM perforations treated with chitosan patches revealed a closure rate that varied from 74% to 61% respectively [122]. Therefore, with the current outcomes achieved with the chitosan patches, further improvements are necessary to ensure an optimal closure and repair of the perforated eardrums.

Among the other naturally-derived polymers, alginate and gelatin are two examples, which have been manipulated for biofabricated TM grafts. Predominantly extracted from brown seaweeds, alginate has been widely investigated for several biomedical applications. With desirable properties such as good biocompatibility, low cost of production, and facile gelation, alginate-based hydrogels have emerged as a promising biomaterial for TE [123]. Weber *et al.* employed it for treating chronic TMPs in a chinchilla model [19]. Calcium alginate-based TM grafts were constructed using injection molding technology, which has been further discussed in section 4. Gelatin, on the other hand, is a protein derived from collagen hydrolysis that has been well explored for TM repair in the form of an absorbable sponge scaffold [78,84,124,125]. Commercially available as Gelfoam®, gelatin sponges are a widely applied grafting material, that has been reported to be effective both with [78,84,125] and without the application of external GFs for treating TMPs [124]. Moreover, owing to its favorable qualities as that of alginate, gelatin has also been extensively applied for the biofabrication of tissue engineered scaffolds [126]. Some of these approaches reported for constructing TM grafts have been carefully reviewed under section 4.

3.2. Synthetic polymers

Unlike natural polymers, which are usually applied directly as films and sponges, synthetic polymers often involve several processing steps. The only notable exception found in literature is that of poly(glycerol sebacate) (PGS) [68], a bioresorbable polymer synthesized by the polycondensation of glycerol and sebacic acid [127]. PGS plugs were tested for perforation closure and graft neovascularization in a chinchilla model. The elastic properties of the polymer allowed an easy insertion. Furthermore, a minimal host inflammatory response induced by the implanted PGS facilitated a successful healing [68].

Other synthetic biomaterials investigated for TM reconstruction include PDMS, PLA, PCL, polyamide and PEOT/PBT [38,40,44,46,49]. With careful application of the latest biofabrication technologies, these polymers have been well exploited to construct tissue grafts with precise shape and architecture. These have been covered in depth in the following section.

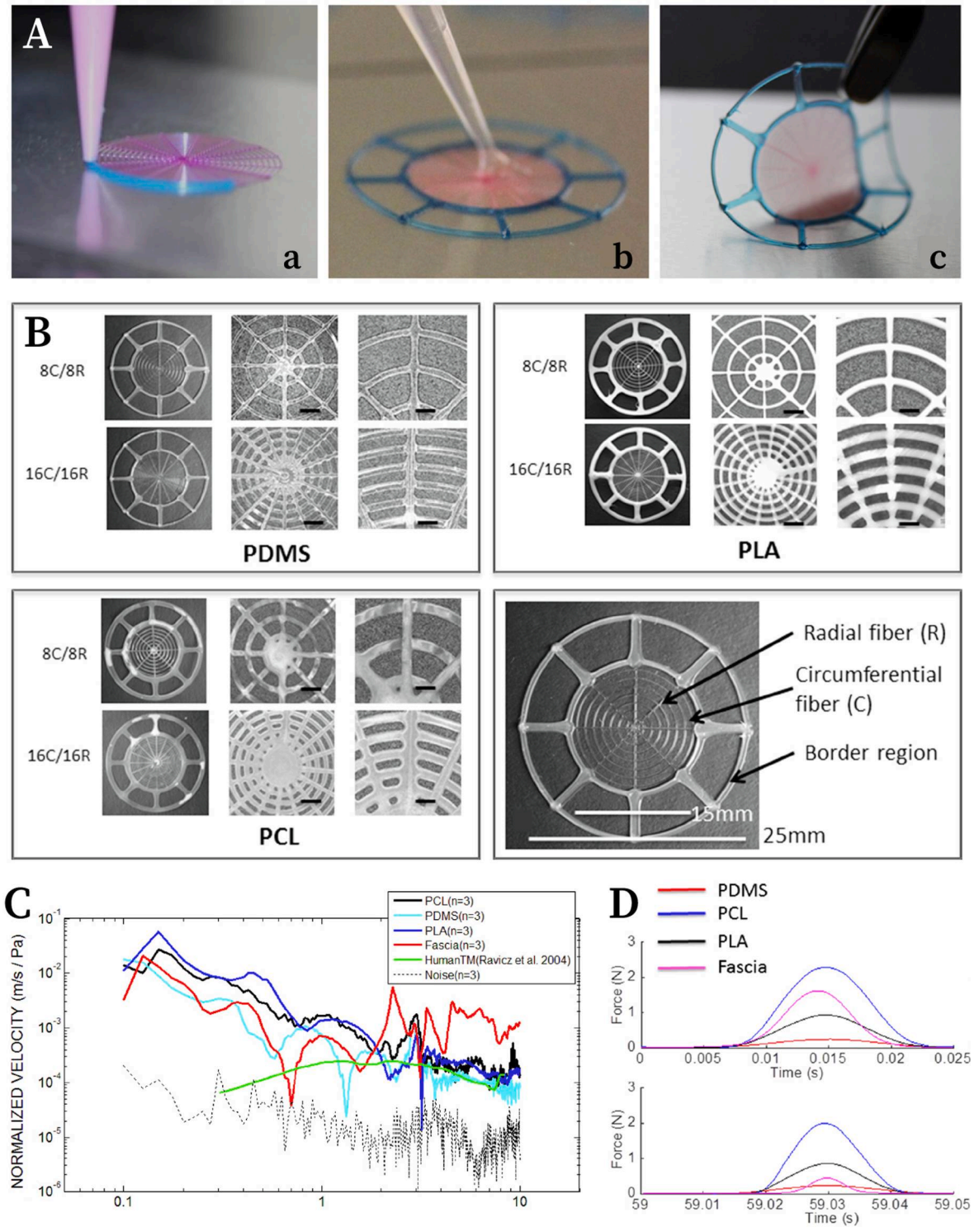


Fig. 3. AM based approaches for TM reconstruction. **(A)** 3D printing of TM grafts: **(a)** layered deposition of circumferential and radial filaments; **(b)** infilling the printed scaffold with fibrin/collagen hydrogel matrix; **(c)** final composite TM graft. **(B)** Printed TM scaffolds with different materials and designs (8 or 16 circumferential (C) + radial (R) fibers). **(C)** Normalized velocity measured using LDV for three samples each of 8C/8R TM composite grafts, fascia and native human TMs. **(D)** Representative cycles of the dynamic mechanical analysis performed to compare different graft materials with fascia. Reproduced with permission from [40].

Beside the polymers that have been manipulated for a direct repair or reconstruction of the TM, poly(glycolic acid) (PGA) was introduced as an indirect reinforcing material in the form of sheets to support conventional fascia grafts [128]. PGA sheets detached from the fascia within 3 weeks demonstrating a successful closure of the perforations in all the patients. The application of PGA sheets was in this case a promising alternative to the fibrin glue protecting the fascia implant while re-epithelialization of the implant occurred.

4. Biofabrication approaches towards TM reconstruction

Biofabrication has emerged as an essential toolkit for modern-day TE and regenerative medicine. It has been defined as the *"the automated generation of biologically functional products with structural organization from living cells, bioactive molecules, biomaterials, cell aggregates such as micro-tissues, or hybrid cell-material constructs, through bioprinting or bioassembly and subsequent tissue maturation processes"* [37,129]. Different biofabrication strategies have been implemented to guide the cellular activity by replicating the complex microenvironment of the native tissue. All these techniques at their core can use cells and biomaterials as the building blocks for an automated fabrication of artificial tissues and organs. To classify these technologies in terms of their fabrication efficiency, Moroni *et al.* introduced the concept of spatial resolution/time for manufacturing (RTM) ratio [37]. Some of the commonly used techniques with high RTM ratio include 3D fiber deposition (3DF) [130–132], SLS [133–135], digital light processing [136]; followed by 3D bioprinting [137–140] and ES [141,142] in the middle range, and spheroid-based approaches [143,144] on the lower end of the spectrum.

4.1. Additive manufacturing

AM technologies have been witnessing a growing significance in TE due to its innate ability to fabricate intricate 3D architectures, mimicking the native tissue microstructure. The construction of artificial tissues with such precise geometry and porosity was not possible with conventional fabrication techniques, such as, injection molding or subtracting techniques [145]. Therefore, with the ongoing research on developing biomaterials for AM, a bottom-up approach has been deemed crucial for replicating complex geometries and porosity [139,146]. The following sub-sections cover some of the most relevant AM technologies that have been applied for the production of scaffold for TM reconstruction.

4.1.1. Melt extrusion

Melt extrusion or fused deposition modeling (FDM) is an AM technique where molten thermoplastic polymer is extruded as fibers and these are deposited in a layer-by-layer fashion, guided by a computer-aided design (CAD) software. The approaches of 3DF and bioextrusion have evolved as an extension to the FDM technology, where the thermoplastic character of biocompatible polymers is utilized for the construction of tissue engineered scaffolds [147]. These extrusion-based techniques have been credited with several advantages over its counterparts, such as a high RTM ratio, non-existent post-processing steps and availability of surface modification strategies for a direct incorporation of bioactive entities [37,148]. In an innovative work by Mota *et al.*, a hybrid fabrication approach based on FDM and ES was developed to produce TM multiscale scaffolds using a copolymer of PEOT/PBT [38]. The reported strategy was further optimized recently to investigate the significance of scaffold geometry in eardrum TE [39]. Both these studies have been discussed in greater depth under hybrid biofabrication strategies (section 4.3).

4.1.2. Solution extrusion

Another important class of AM techniques include the solution extrusion based approaches, where instead of applying high temperatures to melt the polymer, a suitable solvent system is used to generate the printing ink [147]. Similar to the FDM technologies, they allow a controlled deposition of the polymer solution in predesigned 3D architectures. Moreover, by avoiding high temperatures, the solution based strategies prevent any possibility of thermal degradation, which is one of the major drawbacks of melt extrusion [149]. Although an additional post-processing step is required to remove the solvent from the deposited fibers and to ensure the scaffold biocompatibility. Depending on the solvent, this could be achieved either through evaporation at room temperature [40,41] or freeze-drying [150].

Kozin *et al.* demonstrated the successful fabrication of TM grafts using a solution extrusion based AM technique, along with their *in vitro* acoustic characterization [40]. An extrusion-based direct ink writing technique was employed to print PDMS, PLA and PCL (Fig. 3). Inspired by the scanning and transmission electron microscopy images of the native TM, a radial and circumferential fibrous arrangement was chosen for constructing the graft scaffold. The 3D printed filamentary skeleton was then infilled with a fibrin-collagen hydrogel to facilitate an environment that could mimic the natural ECM of human TM. The acoustic properties of the fabricated scaffolds were measured using digital opto-electronic holography and laser Doppler vibrometry (LDV), which was compared with fresh cadaveric TMs and cadaveric temporalis fascia. The sound induced surface motion and velocity results of 3D printed scaffolds were found to be more consistent and similar to the human TM in comparison to the temporalis fascia. Furthermore, the grafts were also found to be more resilient over time during the dynamic mechanical analysis – showing a drop of only 10–15% of its load bearing capability, in contrast to the 70% loss observed in case of human fascia.

Recently, another study investigated solution extrusion for producing PLA-based patches for treating ruptured eardrums [41]. Different concentrations of chitosan and alginate were tested in combination with PLA to obtain printable compositions possessing the optimal viscosity and surface tension. The addition of natural polymers such as chitosan and alginate was envisaged to enhance the biocompatibility of the fabricated scaffolds, which was confirmed by an increased metabolic activity of human MSCs after 7 days of

culture on PLA/chitosan and PLA/alginate patches as compared to PLA alone. Furthermore, the biodegradability assessment of these PLA scaffolds also demonstrated a positive influence of the naturally-derived additives, especially in the initial days. Differential scanning calorimetry revealed no significant differences in the melting temperatures of the chosen compositions; although on the contrary, a three- and a four-fold decline in the tensile strength of the printed constructs was observed with the inclusion of chitosan and alginate, respectively. Finally, the PLA-based patches were manufactured with a thickness within the range of the native tissue to ensure a consistent functionality of these TM patches. However, no efforts were reported for mimicking the collagen alignment within the human eardrum.

4.1.3. 3D bioprinting

3D bioprinting is one of the most recent additions to the current list of biofabrication strategies. It has been defined as the application of AM technology for a direct layer-by-layer deposition of cells, usually encapsulated within biomaterial inks in the form of hydrogels [137–140]. The complex spatial arrangement of cells within a tissue has been deemed crucial for maintaining its micro-architecture and biological functions [151]. With conventional AM techniques, a posterior cell-seeding step often follows the manufacturing of the scaffolds, which limits their ability to accurately position cells within the constructed scaffold. Conversely, the recent advances in 3D bioprinting allows the deposition of multiple cell types, simultaneously or sequentially, with or without encapsulation in a hydrogel [152–154]. Based on the dispensing principles, bioprinting can be broadly classified as (1) extrusion based (also known as bioplotting), (2) inkjet based (also known as droplet-on-demand), or (3) laser-assisted [147,153].

Kuo *et al.* in their relatively recent work [43] proposed the use of 3D bioprinting for the fabrication of patient-specific gelatin methacrylate (GelMA) grafts for the treatment of TMPs. Preliminary wound healing effects of GelMA and EGF were investigated *in vitro* showing cell proliferation and migration from the periphery of the scaffolds to the center, where an enhanced invasion rate and matrix metalloproteinase activity was reported with EGF. Led by these results, customized acellular grafts were manufactured with respect to the endoscopic images obtained for the perforation sites in a chinchilla model. A "butterfly" structure was designed to match the TM defect and allow an easy insertion without the use of any patches, sutures or glues. The bioprinted grafts with and without EGF were implanted *in vivo* and demonstrated a higher regenerative capacity as compared to the spontaneous healing in untreated TMPs with a higher success rate for GF-containing cases. Finally, micro-computed tomography (μCT) and histological analyses were conducted to investigate the dimensions and integration of the regenerated TM with the host tissue, thus, confirming an optimal repair corresponding to the native membrane.

4.1.4. Selective laser sintering

SLS is an AM technology that uses a computer-controlled scanning laser beam to sequentially fuse regions in a powder-bed. The local heat generated by the laser fuses the powder together in desired patterns and assembles the objects layer-by-layer [155,156]. Powder biomaterials have been developed for SLS that fuse with reduced thermal degradation when exposed to a laser irradiation.

In 2016, Kuru *et al.* used the SLS technology to become the first group to manufacture a real size middle ear model [44]. Essential functional elements of the human ear (ossicles, TM, ear canal, and inner ear simulating compartment) were identified by segmenting micro-computer tomography (μCT) data obtained from the temporal bone. Polyamide powder was processed with SLS to obtain bony anatomical structures, such as the ossicles and ear canal, along with 3D molds for casting soft tissues, like the TM. The ultra-thin air-tight properties of the TM was mimicked by vulcanizing two-component silicone rubbers on the surface of the fabricated molds. To assess the auditory performance of the model, tympanometry was conducted and stapes' footplate response to sound was measured using LDV. The tympanogram results demonstrated a compliance range comparable to that of a healthy human middle ear. Moreover, it was reported that the acoustic response was largely dependent on the material used for the soft tissue simulation. This was also confirmed by the LDV measurements of stapes' vibrations, where the transmission and cut-off frequency of the transfer function showed high correlation with the material properties. Overall, the artificial model presented in this study displayed favorable acoustic and biomechanical performance for testing potential prosthetic devices. However, the application of SLS technology to produce TM scaffolds has remained limited due to its restrictions on the materials that can be processed, and its manufacturing resolution.

4.2. Electrospinning

ES is a widely used technique for manufacturing polymeric fibers in the range of several micrometers down to a few nanometers [157–159]. With an ability to fabricate nanofibrous scaffolds with dimensions resembling the ECM collagen fibrils, ES has emerged as a promising biofabrication approach for TE [142,160]. Current ES strategies can be broadly classified into solvent-based solution ES and heat-based melt ES. Both the techniques have demonstrated a growing potentiality toward the manufacturing of scaffolds for TM reconstruction. The subsequent paragraphs discuss some of these approaches in depth.

4.2.1. Solution electrospinning

The electrohydrodynamic phenomenon governing the solution ES is induced by applying a high voltage at the tip of the polymer solution emerging from a cylindrical nozzle or needle. The charging of the fluid results in a conical deformation of the droplet, followed by ejection of a fiber jet towards the counter electrode or collector [161]. The repulsive electrical forces overcome the surface tension and viscoelastic stresses experienced at the tip of the spinneret, thereby culminating in the stretching of the jet, and formation of a solid nanofiber while the solvent evaporates. The stretching of fibers continues as long as a sufficient electric field is maintained between the nozzle and collector; however, beyond a few hundreds of microns, the collected electrospun mesh gradually insulates the collector, influencing the subsequent fiber deposition and thickness [162]. Owing to this operating limitation, ES is commonly used for the

fabrication of thin scaffolds, that can be applied in multiple soft tissues, such as skin, blood vessel, cardiac patch and tendon/ligament [163].

In 2014, Lee *et al.* demonstrated the applicability of ES to fabricate tissue grafts for the closure of subacute TMPs – a condition between chronic and acute injuries [45]. A composite of PCL and SF combined with human umbilical cord serum (UCS) was electrospun, and evaluated for TM regeneration (Fig. 4**E–F**). *In vitro* culture of human dermal fibroblasts on PCL/SF/UCS nanofibrous scaffolds showed an enhanced cell viability and proliferation, which was attributed to the presence of basic amino acids in SF and growth factors such as EGF, TGF-β and NGF in UCS. Following which, the regenerative capability of the fabricated acellular nanofibrous scaffolds was assessed *in vivo* using a guinea pig model. Twenty one days after the surgery, a 100% closure rate was observed for the PCL/SF/UCS meshes in contrast to the 40% of paper patch control group. Finally, *in vivo* LDV and *ex vivo* optical coherence tomography (OCT) were used to analyze the acousto-mechanical response of the regenerated TM. The OCT revealed a thickened fibrous

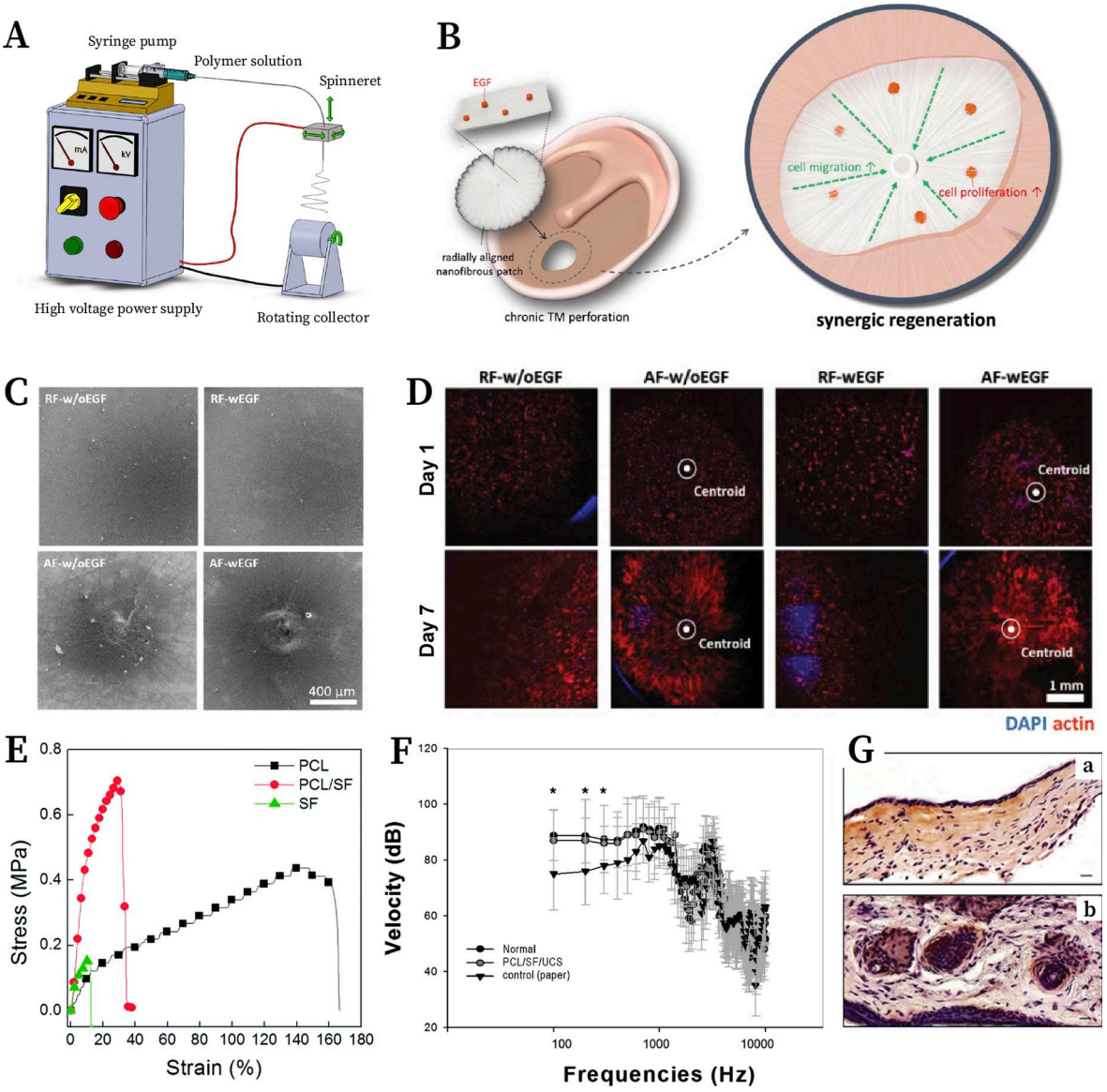


Fig. 4. ES based approaches for TM reconstruction. **(A)** Schematic representation of a typical ES setup. **(B)** Fabrication of EGF-releasing radially aligned nanofibrous patches for treating perforated TM. **(C)** SEM images of random fibers with (RF-wEGF) and without EGF (RF-w/oEGF), and radially aligned fibers with (AF-wEGF) and without EGF (AF-w/oEGF). **(D)** Immunocytochemistry results confirming the alignment and proliferation of TM-derived cells along the centroid of AF patches at day 7. **(E)** Stress–strain curves for tensile measurements performed on electrospun meshes of PCL, SF and PCL/SF. **(F)** LDV results demonstrating significant recovery of nano-vibration below 1 kHz in PCL/SF/UCS nanofibrous scaffolds as compared to the paper patch and native TM. **(G)** Immunostaining for cytokeratin 14, performed after a month of recovery in Sprague-Dawley rats: **(a)** spontaneous regeneration without any graft; **(b)** regeneration after implantation of an ES scaffold with epithelial equivalent tissue. Reproduced with permission from [46] (A), [49] (B–D), [45] (E–F), and [47] (G).

layer indicating desired tissue regeneration, whereas the LDV confirmed significant recovery of TM vibration at lower frequency range.

Danti *et al.* proposed the use of ES alone for manufacturing functional TM scaffolds with the PEOT/PBT copolymer (Fig. 4**A**) [46]. Human MSCs and TM keratinocytes were chosen in this study as the suitable cellular models, along with a custom-made bioreactor to enhance their infiltration within the electrospun mesh. The morpho-functional analysis of samples under the dynamic conditions confirmed the capabilities of ES in developing TM substitutes using TE approaches. As a follow-up study, Moscato *et al.* recently reported an enhanced collagen expression in dynamically cultured stromal cells [50]. Star-branched PCL loaded with nano-hydroxyapatite was used to fabricate electrospun nonwoven membranes with tunable mechanical properties. Human MSCs were cultured on these membranes, and differentiated toward the TM fibroblastic phenotype under static and dynamic conditions. The oscillatory culture environment generated by a customized bioreactor for TM applications was found to promote the expression of fibroblast surface marker and collagen type II, thereby highlighting its superiority over the conventional static cell culture techniques.

In another study on developing electrospun bioabsorbable polymers as artificial tissue grafts for myringoplastic surgeries, Immich *et al.* investigated PLA and PLGA based scaffolds for the co-culture of fibroblasts and keratinocytes [47]. A combination of the two cell types was used for the fabrication of epithelial-equivalent tissue constructs, which were implanted in Sprague-Dawley rats. The artificial tissue grafts were reported to be biologically compatible, durable and effective in minimizing the potential side effects, such as cholesteatomas and granulomas that can develop post-myringoplasty. This final improvement observed in the tissue engineered scaffolds was attributed to their lower immune response as compared to the spontaneous healing (Fig. 4**G**). However, no functional analyses were conducted to confirm the requisite biomechanical and acoustic response of the regenerated TM. Li *et al.* in 2018 proposed the use of natural polymers for the fabrication of electrospun TM patches [48]. A series of gelatin and genipin blends were evaluated with respect to their surface and mechanical properties. Genipin was used as a natural crosslinking agent for gelatin, where an improvement in the overall scaffold characteristics was reported with an optimization of the gelatin/genipin ratio. Preliminary *in vitro* studies performed with human umbilical vein endothelial cells and skin fibroblasts confirmed no apparent cytotoxicity in the chosen electrospun patches. However, in absence of any relevant biological and acousto-mechanical analysis, the investigation remained incomplete with respect to the TM regeneration.

Considering the significance of nanotopography in TM, Seonwoo *et al.* recently developed radially aligned nanofibrous patches for chronic TMPs (Fig. 4**B–D**) [49]. A custom-designed collector was used to manufacture PCL electrospun scaffolds loaded with EGF. The radial alignment was hypothesized to promote cell migration, whereas the release of EGF was considered to enhance cell proliferation. Preliminary *in vitro* study conducted with TM cells isolated from 4 week old Sprague-Dawley rats demonstrated a radial cell distribution at day 7, along with an enhanced mRNA expression for proliferation and angiogenesis markers in EGF-releasing scaffolds. Moreover, the cell migration studies also reported superior wound healing capabilities with the inclusion of GF, specifically in the case of aligned fibers. Similar trend was later confirmed by *in vivo* studies performed in a rat model, where the combination of EGF-loaded radially aligned meshes resulted in faster regeneration rates when compared with random ES meshes and untreated controls.

4.2.2. Melt electrospinning

Removal of toxic organic solvents is one of the key challenges of conventional solution ES. This has been regarded as the principal hurdle towards clinical translation of electrospun tissue engineered scaffolds. The recent years has witnessed a gradual shift from the polymer solution to a molten-based ES, also known as melt ES [164]. Apart from the potential to completely avoid expensive organic solvents, melt ES has also been gaining popularity due to its direct writing abilities. The direct melt writing approach offers a precise control over the filament placement, thereby facilitating construction of complex 3D architectures with high resolution and remarkably ordered porosity [165,166]. Moreover, the pneumatic dispensing technology of melt electrowriting (MEW) offers the possibility to integrate multi-modal diameters into a single fabrication process [164]. Owing to its exceptional resolution and control, MEW has emerged as a promising candidate for creating thin tissue scaffolds with controlled pore architecture. Despite that the porosity obtained in these scaffolds in the X-Y plane can be accurately controlled, the porosity in Z is normally less controlled as it largely depends on the quenching or recrystallization velocity of the polymer [167].

Recently, von Witzleben *et al.* reported the first work on applying MEW for manufacturing biomimetic TM replacements [51]. The study utilized the precise fiber deposition of MEW for creating PCL-based scaffolds with a strand diameter as low as 10 μm. However, this was accompanied by large pore sizes between the adjacent strands, which were sealed using collagen as an infilling layer. Furthermore, 3D constructs mimicking the concavity and collagen alignment of the native TM were presented as a proof of concept (Fig. 6**G–H**), although the subsequent characterizations were performed on conventional grid patterns with layer-to-layer orientations of 45° and 90°. Special attention was paid toward the scaffold thickness, where thicker samples were associated with a higher bending stiffness. The vibrational measurements were noted to be affected by unintentional curling of the fabricated scaffolds, but in general, an acceptable acousto-mechanical behavior was recorded with respect to the human TM. Finally, the *in vitro* investigations conducted with human keratinocytes suggested a faster integration of collagen-coated TM implants into the body, yet future studies will be preferred with non-coated samples to induce some cellular alignment. A critical assessment of the current state of the art indicates that with its distinct advantages, MEW scaffolds can be important candidates for eardrum reconstruction in the coming years.

4.3. Hybrid biofabrication strategies

Even with all the advantages of AM and ES techniques, attaining scaffolds with the optimum pore resolution coupled with suitable mechanical and acoustic properties remains a challenge. The pore size of AM scaffolds is considerably larger than the dimensions of an average cell. Therefore, a higher cell density is required to produce enough ECM for the complete tissue regeneration – which is not always feasible. One alternative suggested to tackle this issue is the integration of 3DF or FDM with ES [168,169]. Moroni *et al.* used the

copolymer of PEOT/PBT for the fabrication of an integrated scaffold, consisting of 3DF periodical macrofiber and random ES microfiber networks. This was achieved by ES a fibrous network of the copolymer for every two layers of 3DF layers. The combination of the two biofabrication strategies exhibited enhanced cell entrapment and tissue regeneration due to simultaneous presence of structural (macro scale) and topographical cues (micro/nano scale).

Based on a similar approach, Mota *et al.* manufactured an artificial TM scaffold [38], resembling its anatomic features and collagen fiber arrangement of the human tissue. The integration of the two techniques was exploited to produce PEOT/PBT-based multiscale scaffolds with AM-generated radial and circular microfiber patterns, coupled with electrospun meshes (Fig. 5**A–E**). The AM patterns on an ultrathin electrospun membrane were considered crucial for achieving a zonal growth of the seeded cells while providing the requisite structural stability to the scaffold. The biomimetic micro-patterns were reported to guide the attachment and proliferation of human MSCs along the architectural directions, thereby demonstrating the potentiality to fabricate functional TM replacements applying TE. In a recent work by Anand *et al.*, an identical fabrication technique was adopted and optimized for manufacturing 3D hierarchical constructs within the anatomical dimensions of the human eardrum [39]. The study highlighted the significance of geometry in tissue engineered TM scaffolds by investigating a combination of theoretical and experimental characterizations. Simplified geometrical designs were chosen to decouple the key micro-anatomical features of the TM, namely radial and circumferential collagen fiber alignment. The mechano-acoustical response evaluated using indentation and LDV measurements confirmed favorable results in comparison to the native tissue (Fig. 5**I**). Furthermore, among the two fiber arrangements, the radial ones were reported to have a stronger influence on the Young's modulus, whereas the circumferential fibers were critical for the structural stability. Although the LDV comparisons on the different conditions was inconclusive. Finally, biological studies conducted with human dermal fibroblasts and human mesenchymal stromal cells exhibited the role of 3D hierarchy in steering cellular orientation and extracellular collagen deposition (Fig. 5**F–H**).

Another hybrid strategy for TM regeneration was introduced by Jang *et al.* [42], where 3D bioprinting was implemented in combination with melt centrifugal spinning – a technique that applies centrifugal force to drive out molten polymer in the form of solidified nanofibers [170]. In this study, cellular constructs were manufactured by bioprinting human MSCs-laden alginate over melt-spun fibrous PCL coated with collagen type I (Fig. 5**J**). The fabricated scaffolds were investigated for the treatment of subacute TMPs in a Sprague–Dawley rat model by comparing their rates of healing, acousto-mechanical properties, and regenerated morphologies with that of their acellular counterparts. A 100% closure rate was reported for the MSCs-laden scaffolds, where only 72% was achieved with the ones without cells (Fig. 5**K**). Auditory brainstem response and single-point LDV measurements of the regenerated tissues demonstrated an acousto-vibrational recovery analogous to that of the native TM (Fig. 5**L**). Moreover, OCT and light microscopic examination revealed a regeneration with optimal thicknesses in the PCL/collagen/alginate-MSC based hybrid scaffolds.

5. Challenges and future outlook

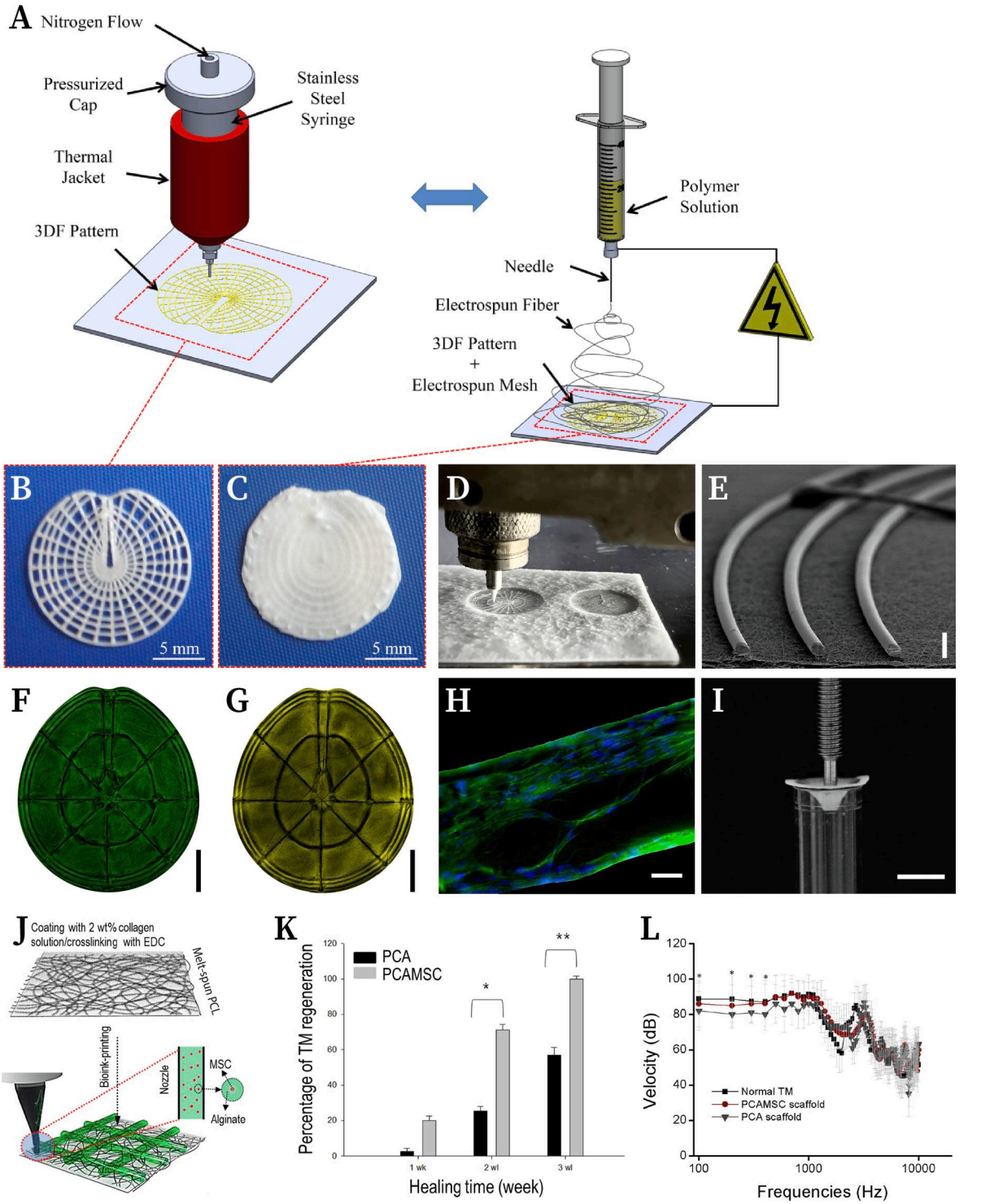
5.1. Repair versus reconstruction

The treatment of injured TMs have predominantly relied on applying tissue implants and patches to seal the perforation. Autologous grafts from the temporalis fascia and perichondrium have been the gold standard to treat TMPs, followed by the more recent biomaterial based cellular and acellular patches. With the emergence of biofabrication techniques within TE, a partial or total reconstruction of the TM with suitable geometry is already possible to some extent. A careful reconstruction of the TM offers several advantages over the traditional grafting approach, such as, improved hearing, reduced re-perforations, and relatively simpler surgeries.

The successful sound conduction by the TM has been attributed to its distinct 3D architecture, where the precise arrangement of its collagen fibrils within a concave-shaped structure has been reported critical for optimal mechanical and acoustic response [171–173]. The lack of these key anatomical features and mechanical properties in tissue grafts result in suboptimal hearing restoration along with higher chances of re-perforations [36]. Moreover, the delicate carving of autologous tissue grafts demands specialized micro-surgical skills, which can become relatively easier with pre-fabricated TM scaffolds, manufactured using the appropriate dimensions. However, despite the clear advantages of reconstruction over repair, there are several other patient-specific factors that influence the surgical strategy, such as the size and location of the perforation, the presence of infected tissues or other pathological complications in the middle ear. A smaller perforation results in comparatively minor losses to the geometry and mechanical response, which can be compensated for, just by applying suitable grafting materials. Furthermore, owing to the inherent regenerative capacity of latent progenitor cells localized in the umbo and annulus regions of the TM [20], smaller perforations in these sections can be sealed with absorbable patches that guide the migration of epithelial cells from the borders of the perforation [30]. Therefore, the choice of repair versus reconstruction depends on the need and feasibility of individual patient case.

5.2. Technical and functional challenges

The human TM is a thin layer of tissue with an anisotropic 3D geometry. Composed of three distinct cellular regions, all within a few micrometers of thickness, the TM has been a difficult tissue to replicate. The precise arrangement of its collagen fibrils along with an overall conical architecture further adds to the challenge. Several reconstruction strategies in the past have attempted the incorporation of these key geometrical and biological features. However, the development of an optimal TM scaffold with the requisite thickness, collagen alignment and cellular components is yet to be fulfilled. Looking at the ES based approaches for TM regeneration – with the exception of the radially-aligned nanofibrous patches reported by Seonwoo *et al.* [49] and Mota *et al.* [38], the deposition of



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polymeric fibers in desired 3D patterns have remained unexplored. On the contrary, AM based technologies allow an accurate representation of complex geometries but in the past were limited by their ability to construct TM scaffolds within a thickness range of 100 μm [38,40]. Latest advancements in the manufacturing technologies have addressed this issue, and now with appropriate optimizations the fabrication of eardrum replacements within the anatomical dimensions of the native tissue has been achieved

Fig. 5. Hybrid fabrication strategies for TM reconstruction. **(A)** Schematic representation of combining AM with ES. FDM-printed TM scaffold before **(B)** and after **(C)** coating it with an electrospun mesh. **(D)** Deposition of 50 μm PEOT/PBT fibers in radial and circumferential patterns over prefabricated electrospun membranes. **(E)** Scanning electron micrograph highlighting the 3D hierarchy of the hybrid constructs; scale bar = 100 μm. **(F)** Phalloidin labelled F-actin (green) at day 7 for human dermal fibroblasts (NHDFs) cultured on the TM scaffolds; scale bar = 2 mm. **(G)** CNA35-FITC staining at day 14 highlighting the production of collagen (yellow) by the cultured NHDFs; scale bar = 2 mm. **(H)** Higher magnification image depicting the gradual growth of NHDFs on the FDM fibers (F-actin in green; nuclei in blue); scale bar = 50 μm. **(I)** Indentation approach implemented for evaluating the mechanical response of the fabricated TM scaffolds; scale bar = 10 mm. **(J)** Schematic representation for fabricating PCL/collagen/alginate/mesenchymal stromal cells (PCAMSC) hybrid scaffolds. **(K)** Regenerative abilities of the fabricated scaffolds in Sprague-Dawley rats. **(L)** LDV results demonstrating significant recovery of nano-vibration with respect to the native human TM. Reproduced with permission from [38] (A–C), [39] (D–I), and [42] (J–L). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

[39,41,51].

Most of the current state-of-the-art studies on TM reconstruction have focused their investigation on flat scaffolds, where the native membrane is a conical structure with a depth of 1.42–2.00 mm [2]. Moreover, finite-element models of the middle ear have demonstrated an improvement in sound transmission with increasing concavity [172,173]. This is attributed to an enhancement in the force transferred to the ossicles at higher frequencies. Therefore, taking these apparent advantages into consideration, the present biofabrication techniques should be further expanded for manufacturing concave-shaped TM scaffolds instead of the usual flat ones. The possibility to do so was highlighted in the recent studies of Anand *et al.* [39] and von Witzleben *et al.* [51] (Fig. 6); however, no characterizations were performed to corroborate the significance of the reported concavity. Furthermore, as discussed, the internal alignment of the collagen fibrils has also been reported crucial for replicating the required acousto-mechanical response [171]. A comparative investigation conducted on different TM patterns revealed a strong influence of the micro-anatomical morphology of the

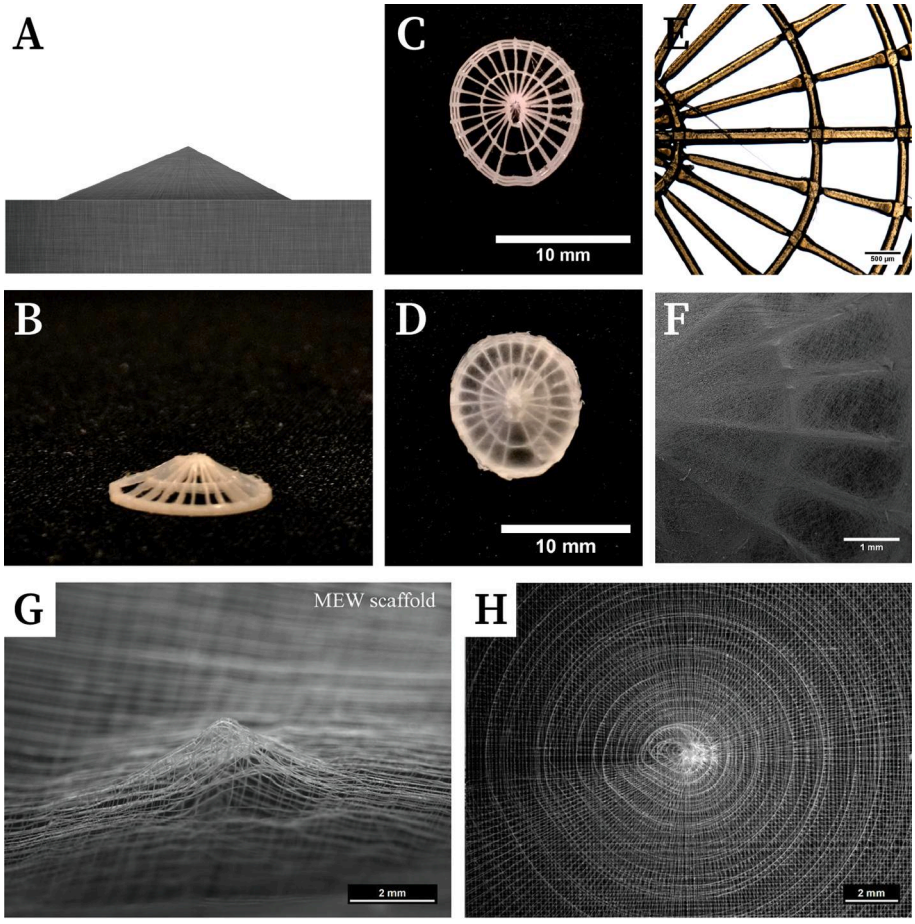

Fig. 6. TM scaffolds manufactured with a concavity. **(A)** 3D mold used as the support for depositing PEOT/PBT fibers. **(B)** An FDM printed conical structure. Top view of FDM scaffolds with concavity, before **(C)** and after **(D)** electrospinning. Microscopy images of the TM scaffolds, before **(E)** and after **(F)** electrospinning. **(G)** Melt electrowritten PCL scaffolds fabricated with a concavity. **(H)** Radial and circumferential arrangement of the PCL fibers deposited with MEW. Reproduced with permission from [39] (A–F) and [51] (G–H).

eardrum [39]. Thus, future efforts in this direction should focus on applying functionally relevant combinations of these radial and circumferential arrangements to obtain the desired results.

TMPs have often been treated topically with antimicrobial drugs to prevent onsite infections that may arise during the process of wound healing [174]. This in particular has been crucial for preventing subsequent infections in the ear injuries caused due to acute and chronic otitis media [175]. However, the current biofabrication strategies employed for the TM repair and reconstruction are yet to make a significant progress toward topical delivery of drugs at the site of implantation. With the exception of the recent work by Seonwoo *et al.,* where the release of GF from electrospun scaffolds was presented as a drug delivery model [49], no major efforts have been made in this direction with respect to the TM. On the other hand, several biofabrication approaches have already been reported for a direct integration and release of antimicrobial drugs from the fabricated scaffolds [176,177]. Therefore, application of similar strategies also for TM reconstruction will be critical for manufacturing clinically relevant tissue scaffolds.

6. Concluding remarks

TE has advanced as a promising alternative for the treatment of injured and defective tissues. Recent progress in the field has relied on the development of several inter-disciplinary approaches to promote tissue growth and regeneration in the human body. Among which, biofabrication has been recognized as an essential toolkit for the replication of intricate 3D tissue geometries. The precise spatiotemporal arrangement facilitated by these manufacturing techniques, has been regarded critical for mimicking the native microenvironment and functionality of the actual tissue. The human TM is one of such tissues, where the distinct arrangement of its collagen fibrils has been accredited for the successful transmission of the incoming sound waves.

This review summarizes the various TE strategies applied for the repair and reconstruction of the TM, with a special focus on the ones employing biofabrication technologies. Conventional myringoplastic surgeries have predominantly focused on the local repairing of the perforated membrane. Several grafting materials have been reported in this regard with high success rates in humans. However, the lack of requisite geometry in these tissue patches have remained a challenge, resulting in a suboptimal hearing restoration post-recovery. Therefore, there has been a growing interest toward investigating biofabrication based approaches for a partial or full reconstruction of the damaged TM. All the current attempts made in this direction were reviewed in this article by discussing their key objectives and achievements. Furthermore, potential advantages and limitations of the biofabricated TM scaffolds were compared with those of the traditional repair techniques, which led to the conclusion that the appropriate treatment strategy is subject to the patient's clinical case and subsequent feasibility. Finally, the present challenges in biofabrication of functional TMs were highlighted, where it was advocated that future efforts should focus on manufacturing concave-shaped scaffolds with precise collagen alignment and drug-loading capacities. The recent progress in tissue engineered approaches for human TM regeneration confirms that with careful application of the available biofabrication technologies, it will soon be possible to achieve clinically relevant reconstructions.

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.

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