Development of Biocompatible Materials for Use in Myringoplasty

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This thesis is presented for the degree of Doctor of Philosophy of
The University of Western Australia

The work presented in this thesis was performed in the
Ear Sciences Centre, School of Surgery
The University of Western Australia

2014
Declaration

This is to certify that this thesis does not incorporate, without acknowledgement, any material previously submitted for a degree or diploma from any university and that, to the best of my knowledge and belief, does not contain any material previously published or written by another person except where due reference is made in the text.

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Abstract

Tympanic membrane (TM) perforations lead to significant hearing loss and may promote infection of the middle ear (ME). Myringoplasty is commonly performed to repair chronic perforations, with the primary goals of perforation closure and hearing restoration. Although various grafts and materials have been used to promote TM regeneration, all have associated limitations. Recent experimental studies have shown the potential of silk fibroin scaffold (SFS) and porcine-derived acellular collagen type I/III scaffold (ACS) as onlay graft materials for TM perforation repair. The aims of this study were to evaluate the in vivo safety and efficacy of SFS and ACS, compared with two commonly used graft materials (paper patch and Gelfoam®) for the promotion of TM regeneration.

In this thesis, a general overview of different types of biological and synthetic scaffolds for TM regeneration was provided, and evidence of scaffolds’ function in TM wound healing was described. Furthermore, a brief review of ME packing agents and a discussion on the potential benefits of no packing also was supplied.

Firstly, the in vivo biocompatibility of SFS and ACS were characterised and compared with Gelfoam and paper in a rat model. The scaffolds were implanted in subcutaneous (SC) tissue and ME cavity followed by histological and otoscopic evaluation for up to 26 weeks. Our results revealed that SFS and ACS were well tolerated and compatible in rat SC and ME
tissues throughout the study. The tissue response adjacent to the implants evaluated by histology and otoscopy showed that SFS and ACS have a milder tissue response with minimal inflammation as compared to paper control. Gelfoam gave similar results to SFS and ACS after SC implantation, but it was found to be associated with pronounced fibrosis and osteoneogenesis after ME implantation. It is concluded that SFS and ACS both were biocompatible and could serve as scaffolds for tissue engineering in the ear.

In an acute TM perforation rat model, these scaffolds were implanted using onlay myringoplasty. Surface morphology of the scaffolds was observed prior to implantation with scanning electron microscopy (SEM). The morphology of the TM was assessed at various time points postimplantation using otoscopy, light and electron microscopy, and functional outcomes by auditory brainstem responses (ABR). We found that SFS and ACS significantly accelerated the TM perforation closure, produced optimal TM thickness, and resulted in better trilaminar morphology with well-organised collagen fibres and early restoration of hearing. By contrast, paper and Gelfoam lost their scaffold function in the early stages and showed an inflammatory response, which may have contributed to delayed healing. This study indicates that compared with paper and Gelfoam, SFS and ACS are more effective in promoting an early TM regeneration and an improved hearing.
In addition, the efficacy of SFS and ACS for the repair of TM was further confirmed using a guinea pig acute TM perforation model. The perforations were repaired with SFS, ACS, and paper using onlay myringoplasty, or they were allowed to heal spontaneously (control). TM structural healing was evaluated by otomicroscopy and histology, and functional hearing was analysed by ABR. Prior to the study, mechanical properties of SFS and ACS were also investigated. Tensile strength and elasticity of SFS and ACS were within the known range for human TM. Based on otologic and histologic evaluation, TMs treated with SFS or ACS showed complete closure of the perforation at an earlier stage, with a trilaminar structure and more uniform thickness compared to paper and control treated groups. ABR assessment demonstrated that SFS or ACS treatment facilitated a faster restoration of hearing function compared to paper and control groups. The results of this study substantiate that SFS and ACS are effective graft materials for myringoplasty and may be utilised as alternatives to current grafts for TM repair.

In summary, the results in this thesis indicate that compared to paper and Gelfoam, SFS and ACS are safe and effective in promoting early TM regeneration and improved hearing, suggesting that these novel scaffolds may be potential substitutes for clinical use.
Acknowledgements

I would like to thank the Ear Sciences Centre of The University of Western Australia, the Ear Science Institute Australia, and Ningbo Lihuili Hospital (Ningbo Medical Centre) of the People’s Republic of China for providing such a precious opportunity to study in Perth, and supporting me to complete this project.

I also would like to acknowledge the Scholarship for International Research Fees (SIRF), University International Stipend (UIS) and UIS Safety-net Top-up Scholarship offered by The University of Western Australia for the funding of my PhD degree.

Special thanks to my supervisors Dr Robert Marano, Winthrop Professor Ming-Hao Zheng, Adjunct Professor Robert Eikelboom and Winthrop Professor Marcus Atlas. I would like to thank all of you for the valuable guidance, ongoing enthusiasm, remarkable support and encouragement throughout this project. I appreciate your wisdom and advice which will certainly be beneficial for the rest of my career. Also, a big thanks to Adjunct Associate Professor Rodney Dilley for your scientific recommendations and generous support. Your inspirational guidance has encouraged me along the way.

I wish to extend many thanks to the senior researcher Ms Sharon Redmond for her excellent lab assistance and technical support, Dr Rangam Rajkhowa
for the manufacturing of silk fibroin (SF) membranes, Ms Charley Budgeon for the statistical advice and analysis, and my fellow colleague Dr Bing Teh and Dr Allen Yu-Yu Wang for their full support and assistance with enthusiasm and constructive advice throughout the years. I express my gratitude to Professor Shen Yan, Professor Lin Zhou, and Dr Yan Wang from the Key Laboratory of Combined Multi-organ Transplantation, Ministry of Public Health of China, for providing the facilities and technical support during the animal experiments.

I also would like to acknowledge the facilities, scientific and technical assistance rendered by the Australian Microscopy and Microanalysis Research Facility at the Centre for Microscopy, Characterisation, and Analysis, The University of Western Australia.

Last but not least, an immense appreciation to my loving family, especially my wife Qiong He, for your endless support and understanding throughout the years. To my beloved parents-in-law, Dr Yi-Tian He and Ms Hui-Jun Liang, thank you for taking care of my son Jia-He Shen while I studied abroad in Australia. To my dear parents, Mr Wei-Sheng Shen and Ms Cai-Feng Wu, thanks for your continuing encouragement allowing me to chase and fulfil my dreams. I would not have achieved this degree without your constant support. To my uncle, Professor Jian-Qing Zhou, I appreciate your invaluable advice and ongoing assistance. I hope that you are all proud of me, and wish to achieve even more academically in the near future.
Statement of candidate contribution

All experimental and written work in this thesis was performed by Dr Yi Shen, except:

The drafts for Figures 1.1 to 3 were designed by Dr Yi Shen with the final format created by Dr Shu-Yang Zhou and Mr Jun-Jie Gao.

Candidate’s contribution in these publications (statements attesting to this have been received by the coordinating supervisor from all co-authors):

   Contribution: design of experiments, conduct of all experiments, sample harvest, data collection and analysis, writing of manuscript and correction of manuscript

   Contribution: design of experiments, conduct of all experiments, sample harvest, data collection and analysis, writing of manuscript and correction of manuscript

*Contribution*: design of experiments, conduct of all experiments, sample harvest, data collection and analysis, writing of manuscript and correction of manuscript.


*Contribution*: critical review of literature, analysis of data, design of figures, writing of manuscript, manuscript adjustments and corrections.
Publications associated with this project


Awards arising from thesis

1. **Scholarship for International Research Fees (SIRF)** offered by The University of Western Australia (2011-2014)

2. **University International Stipend (UIS) and UIS Safety-net Top-up Scholarship** offered by The University of Western Australia (2011-2014)

3. **The Valerie Alder Research Prize 2013** awarded by the Ear Science Institute Australia (ESIA) (2013)


5. **Best Poster Award** (2012)
   

6. **Best Poster Award** (2012)
   
   Abstract entitled: “Comparison of Four Different Scaffolds for Tympanic Membrane Regeneration in a Rat Model”. Shen Y, Redmond
7. Graduate Research Student Travel Award awarded by The University of Western Australia (2012)


8. Graduate Research Student Travel Award awarded by The University of Western Australia (2013)

Presentations arising from thesis

Poster presentations

1. “Middle Ear Packing: If, What and How?” Poster presentation at the Australian Society of Otolaryngology Head and Neck Surgery Annual Scientific Meeting, Melbourne, Australia (April 2011).

2. “Comparison of Four Different Scaffolds for Tympanic Membrane Regeneration in a Rat Model”. Poster presentation at the School of Surgery Research Symposium, Perth, Australia (December 2011).

3. “Comparison of Four Different Scaffolds for Tympanic Membrane Regeneration in a Rat Model”. Poster presentation at the Australian Society of Otolaryngology Head and Neck Surgery Annual Scientific Meeting, Adelaide, Australia (April 2012). * Best Poster Award


Oral presentations


2. “Comparison of Four Different Scaffolds for Tympanic Membrane Regeneration in a Rat Model”. Oral presentation at the School of Medicine Zhejiang University Research Symposium, Hangzhou, China (January 2012).


Myringoplasty
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<th>Description</th>
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<tbody>
<tr>
<td>3D</td>
<td>Three-dimensional</td>
</tr>
<tr>
<td>ABG</td>
<td>Air-bone gap</td>
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<tr>
<td>ABR</td>
<td>Auditory brainstem responses</td>
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<tr>
<td>ACS</td>
<td>Acellular collagen scaffold</td>
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<tr>
<td>ANOVA</td>
<td>Analysis of variance</td>
</tr>
<tr>
<td>BAPC</td>
<td>Bisphenol-A polycarbonate</td>
</tr>
<tr>
<td>C</td>
<td>Celsius</td>
</tr>
<tr>
<td>CMC</td>
<td>Carboxymethylcellulose</td>
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<tr>
<td>CT</td>
<td>Connective tissue</td>
</tr>
<tr>
<td>DAB</td>
<td>3,3’-Diaminobenzidine tetrahydrochloride</td>
</tr>
<tr>
<td>dB</td>
<td>Decibel</td>
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<tr>
<td>dH2O</td>
<td>Distilled water</td>
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<tr>
<td>D,L-PLA</td>
<td>Poly(D,L-lactic acid)</td>
</tr>
<tr>
<td>DNA</td>
<td>Deoxyribonucleic acid</td>
</tr>
<tr>
<td>EAC</td>
<td>External auditory canal</td>
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<tr>
<td>ECM</td>
<td>Extracellular matrix</td>
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<tr>
<td>EDTA</td>
<td>Ethylenediaminetetraacetic acid</td>
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<tr>
<td>FDA</td>
<td>Food and Drug Administration</td>
</tr>
<tr>
<td>FGF</td>
<td>Fibroblast growth factor</td>
</tr>
<tr>
<td>g</td>
<td>Gram</td>
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<tr>
<td>h</td>
<td>Hour</td>
</tr>
<tr>
<td>HA</td>
<td>Hyaluronic acid</td>
</tr>
<tr>
<td>HCl</td>
<td>Hydrochloric acid</td>
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<tr>
<td>H&amp;E</td>
<td>Haematoxylin and eosin</td>
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<tr>
<td>HIV</td>
<td>Human Immunodeficiency Virus</td>
</tr>
<tr>
<td>HRP</td>
<td>Horseradish peroxidase</td>
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<tr>
<td>Hz</td>
<td>Hertz</td>
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<tr>
<td>IHC</td>
<td>Immunohistochemistry</td>
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<tr>
<td>ISO</td>
<td>International Organisation for Standardisation</td>
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<tr>
<td>Symbol</td>
<td>Abbreviation</td>
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<td>--------</td>
<td>------------------------------------</td>
</tr>
<tr>
<td>kg</td>
<td>Kilogram</td>
</tr>
<tr>
<td>KGF</td>
<td>Keratinocyte growth factor</td>
</tr>
<tr>
<td>L</td>
<td>Litre</td>
</tr>
<tr>
<td>LDV</td>
<td>Laser doppler vibrometry</td>
</tr>
<tr>
<td>LiCl</td>
<td>Lithium chloride</td>
</tr>
<tr>
<td>LiSCN</td>
<td>Lithium thiocyanate</td>
</tr>
<tr>
<td>Ltd</td>
<td>Limited</td>
</tr>
<tr>
<td>M</td>
<td>Handle of malleus</td>
</tr>
<tr>
<td>ME</td>
<td>Middle ear</td>
</tr>
<tr>
<td>MEPA</td>
<td>Middle ear packing agent</td>
</tr>
<tr>
<td>mg</td>
<td>Milligram</td>
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<tr>
<td>min</td>
<td>Minutes</td>
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<td>Millilitre</td>
</tr>
<tr>
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<td>Millimetre</td>
</tr>
<tr>
<td>MPA</td>
<td>Megapascal</td>
</tr>
<tr>
<td>ms</td>
<td>Millisecond</td>
</tr>
<tr>
<td>n</td>
<td>Number</td>
</tr>
<tr>
<td>NaCl</td>
<td>Sodium chloride</td>
</tr>
<tr>
<td>Na$_2$CO$_3$</td>
<td>Sodium carbonate</td>
</tr>
<tr>
<td>NaOH</td>
<td>Sodium hydroxide</td>
</tr>
<tr>
<td>nm</td>
<td>Nanometre</td>
</tr>
<tr>
<td>OM</td>
<td>Otitis media</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate buffered saline</td>
</tr>
<tr>
<td>PBzAL</td>
<td>Poly(β-benzyl-L-aspartate-co-L-leucine)50/50</td>
</tr>
<tr>
<td>PDMS</td>
<td>Polydimethylsiloxane</td>
</tr>
<tr>
<td>PF</td>
<td>Pars flaccida</td>
</tr>
<tr>
<td>PGA</td>
<td>Poly(glycolic acid)</td>
</tr>
<tr>
<td>PGS</td>
<td>Poly(glycerol sebacate)</td>
</tr>
<tr>
<td>PT</td>
<td>Pars tensa</td>
</tr>
<tr>
<td>PTA</td>
<td>Pure tone average</td>
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<tr>
<td>PTFE</td>
<td>Polytetrafluoroethylene</td>
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<tr>
<td>Abbreviation</td>
<td>Description</td>
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<tr>
<td>--------------</td>
<td>-------------</td>
</tr>
<tr>
<td>RCT</td>
<td>Randomised clinical trial</td>
</tr>
<tr>
<td>rpm</td>
<td>Revolutions per minute</td>
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<tr>
<td>RT</td>
<td>Room temperature</td>
</tr>
<tr>
<td>SC</td>
<td>Subcutaneous</td>
</tr>
<tr>
<td>SDS</td>
<td>Sodium dodecyl sulphate</td>
</tr>
<tr>
<td>SEM</td>
<td>Scanning electron microscopy</td>
</tr>
<tr>
<td>sem</td>
<td>Standard error of the mean</td>
</tr>
<tr>
<td>SF</td>
<td>Silk fibroin</td>
</tr>
<tr>
<td>SFS</td>
<td>Silk fibroin scaffold</td>
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<td>SIS</td>
<td>Small intestine submucosa</td>
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<td>SPL</td>
<td>Sound pressure level</td>
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<td>SRT</td>
<td>Speech reception threshold</td>
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<td>STWS</td>
<td>Scott’s Tap Water Substitute</td>
</tr>
<tr>
<td>TEM</td>
<td>Transmission electron microscopy</td>
</tr>
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<td>TM</td>
<td>Tympanic membrane</td>
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<tr>
<td>TMP</td>
<td>Tympanic membrane perforation</td>
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<tr>
<td>UBM</td>
<td>Urinary bladder matrix</td>
</tr>
<tr>
<td>UK</td>
<td>United Kingdom</td>
</tr>
<tr>
<td>US</td>
<td>The United States</td>
</tr>
<tr>
<td>V</td>
<td>Volt</td>
</tr>
<tr>
<td>VEGF</td>
<td>Vascular endothelial growth factor</td>
</tr>
<tr>
<td>VT</td>
<td>Ventilation tube</td>
</tr>
<tr>
<td>w/v</td>
<td>Weight per volume</td>
</tr>
<tr>
<td>WHO</td>
<td>World Health Organisation</td>
</tr>
<tr>
<td>µg</td>
<td>Microgram</td>
</tr>
<tr>
<td>µl</td>
<td>Microlitre</td>
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<tr>
<td>µm</td>
<td>Micrometre</td>
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</table>
1. Introduction and literature review
1.1. TM perforation

The TM is a thin, transparent structure that separates the external ear from the ME and plays a key role in the tympano-ossicular system for sound transmission (Figure 1.1). TM perforation is defined as a rupture of the eardrum (Figure 1.2 B) occurring mainly as a result of infection (e.g., otitis media (OM)) or trauma. Moreover, iatrogenic causes are not infrequent and may also predispose a TM to perforations. It is a ubiquitous and potentially severe condition. In the United States, it is estimated that 1-3% of the population have experienced a TM perforation (Gladstone et al. 1995) while other reports have shown the prevalence as high as 28-43% in Indigenous Australians (Access-Economics 2009).

The majority of TM perforations undergo spontaneous closure, with healing rates up to 80% (Kristensen 1992; Farrior 1983; Lindeman et al. 1987). However, large perforations and those developing from recurrent OM or iatrogenic injury, such as tympanostomy tube insertion, may fail to heal and result in chronic perforations (Griffin 1979; Kristensen 1992). There are considerable morbidities associated with chronic perforations if left untreated, such as conductive hearing loss, which is the most common sequela. The World Health Organisation (WHO) estimates the global burden of hearing impairment overall as the most frequent sensory deficit in human populations, with over 250 million people affected (Mathers et al. 2000). Other complications include chronic infection, recurrent aural discharge, tinnitus, acquired cholesteatoma, facial nerve palsy, labyrinthitis and
subperiosteal abscess (Verhoeff et al. 2006; Osma et al. 2000; Dubey & Larawin 2007). The WHO reports that chronic TM perforations with OM involves 65-330 million individuals worldwide (Acuin 2004), with over 650,000 individuals in Australia as of 2008 (Access-Economics 2009). The cost of treatment for this condition in Australia was estimated to be approximately AU $400 million (Access-Economics 2009).

Figure 1.1 Overview of the anatomy of the human ear
The TM separates the external ear from the ME.

Figure 1.2 Diagrammatic representations of a normal (A) and perforated TM (B)
A normal TM consists of the pars tensa (PT) and pars flaccida (PF). M indicates handle of malleus, and asterisk indicates perforation.
1.2. **Myringoplasty: the solution for TM perforation**

Myringoplasty, also known as type 1 tympanoplasty, is the most commonly used surgical treatment to repair chronic perforations in the TM. The ultimate goals of a myringoplasty are to reinstate the integrity of the TM and to restore audiometric function for an ongoing period of time (Aggarwal et al. 2006). Since first described by Berthold in 1878 (Berthold 1878), this procedure is still considered the most effective therapy for chronic TM perforations (Levin et al. 2009). However, the associated limitations such as defective donor sites, high cost, complex surgical skills, aseptic procedure, and need for general anaesthesia and hospitalisation are the major concerns (Laidlaw et al. 2001; Kim et al. 2010b). Although initial perforation closure rates are high, the long-term healing results become significantly decreased due to reperforation, especially for paediatric patients (Jurovitzki & Sade 1988; Kumar et al. 2010; Yung et al. 2007). In addition, restoration of hearing to normal levels (air-bone gap (ABG)<10dB) occurs in only 37-80% of reported cases (Packer et al. 1982; Levin et al. 2009). Thus, further endeavours are warranted to optimise the therapeutic outcomes of this method.

Choice of graft material has been shown to be an essential factor in TM perforation repair (Gibb & Chang 1982). Currently, the most commonly utilised graft materials in clinical settings are autografts, which include temporalis fascia (gold standard), tragal cartilage, perichondrium and fat. These materials have the advantages of minimum rejection rates and ease of handling. However, their disadvantages such as donor site morbidity,
additional incisions for harvest, longer operation times and lack of availability for revision cases have limited their clinical applications (Levin et al. 2009). In light of these limitations, various biological and synthetic scaffolds have been developed and explored as potential alternatives. In the recent years, many of these materials are becoming increasingly popular for TM repair owing to the encouraging outcomes reported (Teh et al. 2013). However, no universal consensus exists in terms of the type of grafting materials or scaffolds. This chapter aims to examine evidence of scaffolds’ function in TM wound healing, to clarify the requirements of an ideal scaffold, and to provide an overview of different types of biological and synthetic scaffolds for TM regeneration.

1.3. TM healing and the role of scaffolds

The TM possesses two distinct regions: the pars tensa (PT) and pars flaccida (PF) (Figure 1.2 A). The former plays a key role in the tympano-ossicular system for sound transmission and is where the majority of perforations occur; by contrast, the latter’s main function is to protect against sudden and loud sounds by its dampening effects (Stenfors et al. 1984). Microscopically, a TM consists of three different layers: an outer epidermal layer, a lamina propria, and an inner mucosal epithelial layer (Figure 1.3 A). Specifically, the epidermal layer is made of a keratinising epithelium similar to the epidermis of the skin, whereas the mucosal layer is composed of a single-lined cuboidal or flat epithelium depending on species (Lim 1970). The lamina propria in
the PT consists of subepidermal and submucosal connective tissue (CT) layers, and a middle fibrous layer, which contains mainly radial and circular collagen fibrils (Lim 1995). In humans, type II collagen is the major collagen constituent of the PT, whilst collagen types I and III have been found to increase in the early phases of TM repair (Lim 1995; Knutsson et al. 2009; Stenfeldt et al. 2006).

For an acute perforation, TM wound healing undergoes several sequential and overlapping stages, including haemostasis, inflammation, cellular migration, cellular proliferation, and remodelling (Gladstone et al. 1995; Teh et al. 2013; Santa Maria et al. 2010) (Figure 1.3 B-F). Following TM injury, the initial phases of haemostasis and inflammation are similar to the healing processes of other tissues such as dermal skin. During this period, a crust layer consisting of interstitial fluid, lymph and blood, forms at the perforation margin to provide a matrix for cellular migration and to protect against dehydration of the underlying tissue (Güneri et al. 2003). Unlike dermal wound healing, the migratory phase precedes the proliferative phase which is unique to the TM (Gladstone et al. 1995). The squamous epithelium migrates across the wound to primarily bridge the perforation. The initial keratin layer produced by this hyperplastic epithelium first closes the perforation and serves as a template for further cellular migration by underlying cells from the epithelial and fibrous layers (Govaerts et al. 1988; Spandow et al. 1996; Stenfors et al. 1980; Makino et al. 1990). The fibrous layer is the last to migrate across a perforation. Failure of cellular migration from the fibrous
layer results in a dimeric neomembrane containing an epithelial and mucosal layer with a few disorganised fibres between them (Yamashita 1985). The remodelling stage is the final phase after perforation closure. It features prominent thinning of the regenerated TM mainly due to the structural rearrangement of the fibrous layer (Santa Maria et al. 2010).

![Figure 1.3](image)

**Figure 1.3 Microstructure of the TM (A) and the healing process of acute TM perforations (B-F)**

(A) A normal TM consists of three different layers: an outer epidermal layer, a lamina propria, and an inner mucosal epithelial layer. (B) Retraction of the perforation margin is seen with the formation of a crust layer above. (C) The keratin layer produced by the hyperplastic epithelium first closes the perforation and serves as a template for cellular migration. (D) The epidermal layer migrates across the wound to primarily bridge the perforation. (E) The fibrous layer (consisting of the collagen fibrils) of the lamina propria is the last to migrate across a perforation. (F) The remodelling stage is the final phase, featured by thinning and structural rearrangement of the regenerated TM.

Most perforations heal spontaneously within 7 to 14 days and are classified as acute. However, a perforation that fails of to heal within 3 months is termed chronic. The mechanism of chronicity is unknown but may be due to the establishment of a muco-epithelial junction at the perforation margin,
whereby the squamous epithelial layer grows over the perforation edge to meet the inner mucosal layer, and by contact inhibition preventing further spontaneous healing (Gladstone et al. 1995). Another reason may be failure to activate expression of extracellular matrix (ECM) genes or various growth factors during healing, which diminishes the fibrous TM’s regenerative capacity leading to persistence of the perforation (Spandow et al. 1996). While these hypotheses are widely accepted, further studies are warranted to fully understand the mechanism of chronicity. In addition, the trilaminar structure is essential for the healed TM, particularly the middle fibrous layer, since it consists of collagenous fibres which play vital roles in maintaining the strength and vibroacoustics of a TM for sound transmission (Merwin & Boies 1980; O'Connor et al. 2008). An atypical neomembrane with a dimeric structure and a deficient fibrous layer is associated with various complications, including suboptimal hearing, retraction pockets, atelectatic TM, reperforation and cholesteatoma (O'Connor et al. 2008; Forsen 2000; Cassano & Cassano 2010). In certain circumstances, surgical interventions may be required.

Scaffolds are considered beneficial for the repair of chronic TM perforations. Since it has been proven that the TM healing process is initiated and dominated by epithelial migration, scaffolds may enhance healing by providing structural support for epithelial migration to close the defect (Gladstone et al. 1995). Moreover, scaffold materials can supply a suitable environment for mesenchymal cell infiltration, angiogenesis and CT
synthesis, thus resulting in fibrous layer repair. Various scaffolds have been shown to regulate the healing pattern of the fibrous layer by preventing dehydration of the TM remnant and reinforcing attachment to the perforation margins (Kim et al. 2010a; Shen et al. 2013b). This facilitates the alignment of fibroblasts and collagen fibres in an orderly and stable fashion, leading to a neomembrane with a well-organised fibrous structure and minimal scarring. Given that the TM is suspended in air without underlying tissue, the use of scaffolds may be valuable for TM repair by supporting the migrating epithelium and regenerating neovessels during healing (Santa Maria et al. 2010). The mechanism by which scaffolds themselves remodel in the TM environment is considered to be primarily dependent on their biodegradability and absorbability, although this mechanism is poorly understood. Postimplantation, biodegradable scaffolds may be incorporated into the neomembranes allowing the ingrowth of host cellular and CT, which will eventually lead to replacement of the scaffold by neotissue (Szabó 2006). In contrast, synthetic nonbiodegradable scaffolds may remain in the reconstructed TM and function as a permanent reinforcement, especially in the absence of a midfibrous layer (Feenstra et al. 1984).

1.4. Biological scaffolds

Since the introduction of tympanoplasty in the 1950s (Golz et al. 2003), there have been various differing types of scaffolds developed and applied as grafting materials for the repair of TM perforations, which include allografts,
xenografts and synthetic substances. Based on the source of origin, these can be broadly classified into biological and synthetic materials. Some of these are commercial scaffolds and their component and manufacture information are summarised in Table 1.1. Scaffolds constructed from biological materials have been used successfully in both animal studies and human clinical trials. These biological scaffolds include predominantly decellularised matrices, animal based biomaterials (e.g. silk, collagen, gelatine, chitosan, and hyaluronic acid), and plant based biomaterials (e.g. paper and alginate) (Tables 1.2 and 1.3).
### Table 1.1 General information of commercial scaffolds for TM regeneration

<table>
<thead>
<tr>
<th>Trade name</th>
<th>Texture</th>
<th>Components</th>
<th>Manufacturer (location)</th>
</tr>
</thead>
<tbody>
<tr>
<td>AlloDerm®</td>
<td>Biology</td>
<td>Human cadaver dermis</td>
<td>Life Cell Corporation (NJ, USA)</td>
</tr>
<tr>
<td>Tutoplast®</td>
<td>Biology</td>
<td>Human cadaver dura mater</td>
<td>Biodynamics Inc. (Erlingen, Germany)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Human cadaver temporal fascia</td>
<td>Tutogen Medical, Inc. (NJ, USA)</td>
</tr>
<tr>
<td>Zenoderm®</td>
<td>Biology</td>
<td>Porcine dermis</td>
<td>Ethicon Ltd. (Edinburgh, Scotland)</td>
</tr>
<tr>
<td>Surgisis®</td>
<td>Biology</td>
<td>Porcine SIS</td>
<td>Cook Inc. (Bloomington, IN, USA)</td>
</tr>
<tr>
<td>Parmatym®</td>
<td>Biology</td>
<td>Bovine jugular vein</td>
<td>Xomed (Lapperre, Belgium)</td>
</tr>
<tr>
<td>Gelfoam®</td>
<td>Biology</td>
<td>Porcine dermis</td>
<td>Pharmacia &amp; Upjohn Inc. (MI, USA)</td>
</tr>
<tr>
<td>Gelfilm®</td>
<td>Biology</td>
<td>Porcine dermis</td>
<td>Pharmacia &amp; Upjohn Inc. (MI, USA)</td>
</tr>
<tr>
<td>Seprafilm®</td>
<td>Biology</td>
<td>HA and CMC</td>
<td>Genzyme Corporation (MA, USA)</td>
</tr>
<tr>
<td>MeroGel®</td>
<td>Biology</td>
<td>HA</td>
<td>Medtronic Xomed Inc. (FL, USA)</td>
</tr>
<tr>
<td>EpiFilm®</td>
<td>Biology</td>
<td>HA</td>
<td>Medtronic Xomed Inc. (MN, USA)</td>
</tr>
<tr>
<td>EpiDisc®</td>
<td>Biology</td>
<td>HA</td>
<td>Medtronic Xomed Inc. (FL, USA)</td>
</tr>
<tr>
<td>Steri-Strip™</td>
<td>Biology</td>
<td>Synthetic paper coated with hypoallergenic adhesive</td>
<td>3M Corporation (MN, USA)</td>
</tr>
<tr>
<td>Micropore™</td>
<td>Biology</td>
<td>Paper tape</td>
<td>3M Corporation (MN, USA)</td>
</tr>
<tr>
<td>Tympanic Membrane Patcher</td>
<td>Synthetic</td>
<td>Silicone</td>
<td>Medtronic Xomed Inc. (FL, USA)</td>
</tr>
</tbody>
</table>

SIS: Small intestine submucosa; HA: Hyaluronic acid; CMC: Carboxymethylcellulose.
Table 1.2  Animal experiments of biological scaffolds for TM regeneration

<table>
<thead>
<tr>
<th>Animal model</th>
<th>Year</th>
<th>Scaffold materials</th>
<th>Perforation type</th>
<th>Cases</th>
<th>Follow-up (months)</th>
<th>Healing rates (perforation closure)</th>
<th>Hearing outcome</th>
<th>References</th>
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</thead>
<tbody>
<tr>
<td>Guinea pig</td>
<td>2012</td>
<td>AlloDerm</td>
<td>Acute</td>
<td>50</td>
<td>2</td>
<td>90.9% (underlay); 89.3% (onlay)</td>
<td>NA</td>
<td>Qin et al. 2012</td>
</tr>
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<td>2007</td>
<td>Formalin-fixed and non-formalin-fixed AlloDerm</td>
<td>Acute</td>
<td>15</td>
<td>1.5</td>
<td>Both 85%</td>
<td>NA</td>
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<td>Chronic</td>
<td>17</td>
<td>2.5</td>
<td>90%</td>
<td>NA</td>
<td>Downey et al. 2003</td>
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<td>Chronic</td>
<td>28</td>
<td>2</td>
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<td>NA</td>
<td>Laidlaw et al. 2001</td>
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<td>10</td>
<td>2</td>
<td>80%</td>
<td>NA</td>
<td>McFeely et al. 2000</td>
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<td>Other acellular ECM</td>
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<td>Porcine acellular UBM</td>
<td>Chronic</td>
<td>14</td>
<td>3</td>
<td>100%</td>
<td>NA</td>
<td>Parekh et al. 2009</td>
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<td>76</td>
<td>2</td>
<td>85% (dermis); 80% (dura mater)</td>
<td>Both completely recovered</td>
<td>Deng et al. 2009</td>
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<td>Surgisis</td>
<td>Chronic</td>
<td>10</td>
<td>1.5</td>
<td>100%</td>
<td>NA</td>
<td>Spiegel et al. 2005</td>
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<td>NA</td>
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<td>Rabbit</td>
<td>1995</td>
<td>Bovine type I/III collagen-elastin-fibrin matrix</td>
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<td>2</td>
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<td>Bonzon et al. 1995</td>
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<td>100%</td>
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Continued on next page
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<thead>
<tr>
<th>Animal model</th>
<th>Year</th>
<th>Scaffold materials</th>
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<th>Cases</th>
<th>Follow-up (months)</th>
<th>Healing rates (perforation closure)</th>
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<td>Water-insoluble 3D porous chitosan scaffold</td>
<td>Acute</td>
<td>10</td>
<td>14 days</td>
<td>60% (7 days); 80% (10 days); 100% (14 days)</td>
<td>Hearing completely recovered</td>
<td>Kim et al. 2011</td>
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<td>21 days</td>
<td>21.1% (7 days); 89.5% (14 days)</td>
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<td>HA</td>
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<td>EpiFilm</td>
<td>Acute</td>
<td>42</td>
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<td>No statistically significant difference between the pre- and postoperative ABR data</td>
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<td>1</td>
<td>100%</td>
<td>NA</td>
<td>Konakçılı et al. 2004</td>
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<td>1998</td>
<td>Cigarette paper</td>
<td>Acute</td>
<td>60</td>
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<td>94.4%</td>
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<td>Imamoğlu et al. 1998</td>
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<td>alginate</td>
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<td>Chinchilla</td>
<td>2006</td>
<td>Calcium alginate patch</td>
<td>Chronic</td>
<td>30</td>
<td>2.5</td>
<td>69.2%</td>
<td>NA</td>
<td>Weber et al. 2006</td>
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UBM: Urinary bladder matrix; SF: Silk fibroin; ABR: Auditory brainstem responses; NA: Data not available.
<table>
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<tr>
<th>Study type</th>
<th>Year</th>
<th>Scaffold materials</th>
<th>Perforation type</th>
<th>Cases (ears)</th>
<th>Follow-up (months)</th>
<th>Healing rates (perforation closure)</th>
<th>Hearing outcome</th>
<th>References</th>
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<td><strong>AlloDerm</strong></td>
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<td>Prospective</td>
<td>2011</td>
<td>AlloDerm</td>
<td>Chronic</td>
<td>42</td>
<td>3</td>
<td>95%</td>
<td>ABG gain 17dB</td>
<td>Raj et al. 2011</td>
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<td>Retrospective</td>
<td>2006</td>
<td>AlloDerm</td>
<td>Chronic</td>
<td>34</td>
<td>2-12</td>
<td>94%</td>
<td>Similar ABG, PTA and SRT results compared to the temporalis fascia group</td>
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<td>2005</td>
<td>AlloDerm</td>
<td>Chronic</td>
<td>114</td>
<td>31</td>
<td>88%</td>
<td>NA</td>
<td>Vos et al. 2005</td>
</tr>
<tr>
<td>Retrospective</td>
<td>2005</td>
<td>AlloDerm</td>
<td>Chronic</td>
<td>50</td>
<td>4-25</td>
<td>84%</td>
<td>53.8% ABG&lt;10dB</td>
<td>Fishman et al. 2005</td>
</tr>
<tr>
<td>Retrospective</td>
<td>2001</td>
<td>AlloDerm</td>
<td>Chronic</td>
<td>40</td>
<td>&gt; 6</td>
<td>100%</td>
<td>ABG 5.35dB</td>
<td>Benecke 2001</td>
</tr>
<tr>
<td>Case study</td>
<td>2001</td>
<td>AlloDerm</td>
<td>Chronic</td>
<td>24</td>
<td>6-36</td>
<td>87.5%</td>
<td>NA</td>
<td>Fayad et al. 2003</td>
</tr>
<tr>
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<td>1999</td>
<td>AlloDerm</td>
<td>Chronic</td>
<td>30</td>
<td>6</td>
<td>90%</td>
<td>80% ABG&lt;10dB</td>
<td>Saadat et al. 2001</td>
</tr>
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<td>Case study</td>
<td>2003</td>
<td>AlloDerm</td>
<td>Chronic</td>
<td>7</td>
<td>12</td>
<td>85.7%</td>
<td>NA</td>
<td>Saadat et al. 2001</td>
</tr>
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<td>Case study</td>
<td>1999</td>
<td>AlloDerm</td>
<td>Chronic</td>
<td>7</td>
<td>12</td>
<td>85.7%</td>
<td>NA</td>
<td>Saadat et al. 2001</td>
</tr>
<tr>
<td>Case study</td>
<td>2005</td>
<td>AlloDerm</td>
<td>Chronic</td>
<td>87</td>
<td>3</td>
<td>97%</td>
<td>NA</td>
<td>Lou et al. 2011</td>
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<tr>
<td>Case study</td>
<td>2011</td>
<td>Gelfoam/Gelfilm</td>
<td>VT removal</td>
<td>117 (155)</td>
<td>4.9</td>
<td>95.5%</td>
<td>NA</td>
<td>Hekkenberg et al 1995</td>
</tr>
<tr>
<td>Case study</td>
<td>1995</td>
<td>Gelfoam/Gelfilm</td>
<td>VT removal</td>
<td>115</td>
<td>6-24</td>
<td>94%</td>
<td>NA</td>
<td>Baldwin et al. 1992</td>
</tr>
<tr>
<td>Case study</td>
<td>1979</td>
<td>Gelfilm</td>
<td>Chronic</td>
<td>45</td>
<td>3-6</td>
<td>93.3%</td>
<td>44.1% ABG&lt;5dB; 70.6% ABG&lt;10dB</td>
<td>Karlan 1979</td>
</tr>
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</table>

Continued on next page
<table>
<thead>
<tr>
<th>Study type</th>
<th>Year</th>
<th>Scaffold materials</th>
<th>Perforation type</th>
<th>Cases (ears)</th>
<th>Follow-up (months)</th>
<th>Healing rates (perforation closure)</th>
<th>Hearing outcome</th>
<th>References</th>
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<tbody>
<tr>
<td>Gelfoam® or Gelfilm®</td>
<td></td>
<td></td>
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<td></td>
<td></td>
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<tr>
<td>Case study</td>
<td>1971</td>
<td>Gelfilm</td>
<td>Chronic</td>
<td>37</td>
<td>&gt; 12</td>
<td>43%</td>
<td>29.4% improved; 38.2% unchanged; 32.4% decreased</td>
<td>Harris et al. 1971</td>
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<td>HA</td>
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<tr>
<td>Prospective</td>
<td>2013</td>
<td>EpiFilm</td>
<td>Acute</td>
<td>155</td>
<td>6</td>
<td>94.8%</td>
<td>PTA and ABG significantly improved</td>
<td>Sayin et al. 2013</td>
</tr>
<tr>
<td>Prospective</td>
<td>2011</td>
<td>EpiDisc</td>
<td>Chronic</td>
<td>208 (213)</td>
<td>14.6-20.7</td>
<td>87%</td>
<td>ABG improved by 12.85dB</td>
<td>Saliba et al. 2011</td>
</tr>
<tr>
<td>Prospective</td>
<td>2011</td>
<td>EpiDisc</td>
<td>Chronic</td>
<td>234 (246)</td>
<td>15.5-20.6</td>
<td>92.7%</td>
<td>ABG improved by 12.8dB</td>
<td>Saliba et al. 2011</td>
</tr>
<tr>
<td>Case study</td>
<td>2008</td>
<td>EpiDisc</td>
<td>Chronic</td>
<td>21</td>
<td>4-16</td>
<td>91%</td>
<td>ABG improved by 17dB</td>
<td>Saliba 2008</td>
</tr>
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<td>Case study</td>
<td>2008</td>
<td>EpiFilm</td>
<td>Chronic</td>
<td>5</td>
<td>1.5</td>
<td>None TM healed and the study was aborted</td>
<td>NA</td>
<td>Prior et al. 2008</td>
</tr>
<tr>
<td>Paper</td>
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<tr>
<td>Prospective</td>
<td>2011</td>
<td>Steri-Strip tape</td>
<td>Acute</td>
<td>87</td>
<td>3</td>
<td>93.3%</td>
<td>PTA gain 13.5dB</td>
<td>Park et al. 2011</td>
</tr>
<tr>
<td>Prospective</td>
<td>2008</td>
<td>Carbon paper</td>
<td>Chronic</td>
<td>45</td>
<td>3</td>
<td>66.7%</td>
<td>ABG gain 5.4dB</td>
<td>Dursun et al. 2008</td>
</tr>
<tr>
<td>Prospective</td>
<td>1996</td>
<td>Steri-Strip tape</td>
<td>VT removal</td>
<td>88 (145)</td>
<td>12</td>
<td>96.7%</td>
<td>NA</td>
<td>Saito et al. 1996</td>
</tr>
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<td>Prospective</td>
<td>1995</td>
<td>Rice paper</td>
<td>Chronic</td>
<td>30 (64)</td>
<td>12</td>
<td>26.7%</td>
<td>PTA improved by 9.3dB</td>
<td>Spandow et al. 1995</td>
</tr>
<tr>
<td>Prospective</td>
<td>1991</td>
<td>Rice paper</td>
<td>Chronic</td>
<td>60</td>
<td>12</td>
<td>29%</td>
<td>No adverse effects</td>
<td>Laurent et al. 1991</td>
</tr>
<tr>
<td>Prospective</td>
<td>1987</td>
<td>Rice paper</td>
<td>Acute</td>
<td>60</td>
<td>2</td>
<td>90.6%</td>
<td>All patients recovered except one patient had ABG of 5dB</td>
<td>Linderman et al. 1987</td>
</tr>
<tr>
<td>Retrospective</td>
<td>1992</td>
<td>Cigarette paper</td>
<td>VT removal</td>
<td>131 (163)</td>
<td>18</td>
<td>87%</td>
<td>NA</td>
<td>Pribitkin et al. 1992</td>
</tr>
<tr>
<td>Retrospective</td>
<td>1990</td>
<td>Steri-Strip tape</td>
<td>Acute</td>
<td>108</td>
<td>0.5-26</td>
<td>99.1%</td>
<td>Patients had good hearing</td>
<td>Saito et al. 1990</td>
</tr>
<tr>
<td>Case study</td>
<td>2008</td>
<td>Paper patch</td>
<td>Chronic</td>
<td>90</td>
<td>3-18</td>
<td>52.2%</td>
<td>ABG&lt;10dB(TMP&lt;4mm); ABG&lt;15dB(TMP&gt;4mm)</td>
<td>Lee et al. 2008</td>
</tr>
<tr>
<td>Case study</td>
<td>2003</td>
<td>Cigarette paper</td>
<td>Chronic</td>
<td>77</td>
<td>3.4-7 years</td>
<td>55.7%</td>
<td>(TMP&lt;5mm); 12.5% (TMP&gt;5mm)</td>
<td>NA</td>
</tr>
<tr>
<td>Case study</td>
<td>1985</td>
<td>Micropore paper</td>
<td>Acute</td>
<td>47 (50)</td>
<td>5 weeks</td>
<td>92%</td>
<td>ABG gain 6.9dB</td>
<td>Camnitz et al. 1985</td>
</tr>
<tr>
<td>Case study</td>
<td>1980</td>
<td>Cigarette paper</td>
<td>Acute</td>
<td>4 (5)</td>
<td>1.5</td>
<td>100%</td>
<td>Immediate hearing improvement</td>
<td>Merwin et al. 1980</td>
</tr>
</tbody>
</table>

ABG: Air-bone gap; PTA: Pure tone average; SRT: Speech reception threshold; TMP: Tympanic membrane perforation; VT: Ventilation tube; NA: Data not available.
1.4.1. Decellularised matrices

Decellularised matrices are typically derived from mammalian tissues, including porcine and human. Tissues such as dermis (Haynes et al. 2005; Callanan et al. 1993; Deng et al. 2009), dura mater (Deng et al. 2009; Yetişer et al. 2001), temporalis fascia (Saraç & Gürsel 2002), small intestine submucosa (SIS) (Spiegel & Kessler 2005), and urinary bladder (Parekh et al. 2009) are harvested and treated to specifically remove the cellular components. These materials are then processed by stepwise disinfection, dehydration and terminal sterilisation, thereby leaving an intact ECM (Badylak et al. 2009). The ECM is composed of many kinds of structural and functional proteins, glycoproteins, proteoglycans and growth factors. For most tissues, collagen is the most abundant protein within the ECM, accounting for over 90% of its dry weight, with fibronectin being second to collagen in quantity (Badylak 2004). Of note, a variety of growth factors, such as keratinocyte growth factor (KGF), members of the fibroblast growth factor (FGF) family, and vascular endothelial growth factor (VEGF) may additionally be present within the ECM. These compounds serve various essential functions including maintenance of tissue architecture, regulation of cellular functions, and promotion of angiogenesis and tissue reconstruction (Badylak 2002). Consequently, decellularised matrices are appealing candidates for TM repair, as they contain native-like ECM composition, three-dimensional (3D) microstructure and biomechanical properties (Hoshiba et al. 2010). Some of the decellularised ECM scaffolds have already
been commercialised for clinical applications, with AlloDerm® being a representative example.

AlloDerm matrix is the most commonly used commercially available scaffold for TM applications in the clinical setting. It is an acellular dermal matrix sourced from human cadaver donor skin and consists of native collagens, elastin, vascular channels and proteins. Satisfactory outcomes have been well documented utilising AlloDerm for abdominal wall (Buinewicz & Rosen 2004) and breast reconstruction (Jansen & Macadam 2011) since the early 1990s. AlloDerm has also been applied for repair of the TM, and its efficacy has been shown in numerous animal studies (McFeely et al. 2000; Laidlaw et al. 2001; Downey et al. 2003; Johnson et al. 2007; Qin et al. 2012) and human clinical trials (Youssef 1999; Saadat et al. 2001; Benecke 2001; Fayad et al. 2003; Fishman et al. 2005; Vos et al. 2005; Lai et al. 2006; Raj et al. 2011). In 1999, Youssef conducted the first study in the literature using AlloDerm which found favourable results in the repair of chronic perforations in 30 patients, with a closure rate of 90% and a ABG less than 10dB in 80% cases (Youssef 1999). Later, AlloDerm was further investigated by other groups with reported success rates of 78% to 90% in chinchillas (McFeely et al. 2000; Laidlaw et al. 2001; Downey et al. 2003) and 84% to 100% in humans (Saadat et al. 2001; Benecke 2001; Fayad et al. 2003; Fishman et al. 2005; Vos et al. 2005; Lai et al. 2006; Raj et al. 2011), which is comparable to that of temporalis fascia at 88-95% (Sheehy & Anderson 1980; Vartiainen & Nuutinen 1993). Additionally, several clinical
trials have reported that the use of AlloDerm improved hearing outcomes to levels identical to those achieved by temporalis fascia (Benecke 2001; Fishman et al. 2005; Lai et al. 2006). In a prospective randomised clinical trial (RCT) with a short-term follow-up of 3 months, Raj et al. reported that the average hearing improvement was 17.0dB for the AlloDerm group and 14.5dB for the temporalis fascia group, without significant difference (Raj et al. 2011). During healing, AlloDerm is not replaced by native tissues but incorporated into the host TM with evident fibroblast infiltration and early revascularisation (Downey et al. 2003). Histologically, Laidlaw and colleagues found that AlloDerm-treated TMs healed with a normal trilaminar structure in chinchillas, while treatment with rice paper resulted in a bilaminar neomembrane with an absent fibrous layer (Laidlaw et al. 2001). Prior to use, a rehydration procedure is required to make AlloDerm soft and pliable. Postoperatively, it has been proven that different surgical methods such as underlay and overlay techniques exert little effect on the success rates (Qin et al. 2012; Lai et al. 2006). Of interest, AlloDerm has one smooth dermal surface and one coarse basement membrane surface. It has been shown that there is no difference noted in graft performance in terms of orientation (McFeely et al. 2000). Furthermore, Johnson et al. demonstrated that formaldehyde-treated AlloDerm was as safe and effective as normal AlloDerm for the repair of near-total perforation in a chinchilla model, which may overcome challenges associated with total perforation repair such as graft lateralisation and blunting (Johnson et al. 2007). Thus, the current
evidence suggests that AlloDerm is a valuable grafting material for the repair of a perforated TM, regardless of the surgical technique (Qin et al. 2012; Lai et al. 2006), graft orientation (McFeely et al. 2000), or formaldehyde fixatives usage (Johnson et al. 2007).

Zenoderm® is another acellular, collagen-based scaffold derived from porcine dermal tissue. It is treated with proteolytic enzymes to remove non-collagenous components and cross-linked with glutaraldehyde to prolong absorption and reduce antigenicity (Iosif 1987). Ironside (Ironside 1982) reported some early success using Zenoderm for tympanoplasty. A retrospective controlled trial, which matched the patient age, surgical method and perforation area, was performed by Callanan et al. to assess the efficacy of Zenoderm (Callanan et al. 1993). The results indicated that only four of ten Zenoderm recipients (40%) had perforations closure, whereas 41 of 43 patients (95%) achieved closure in the temporalis fascia group within a 10-month follow-up. This failure may be attributed to contraction or fragmentation of the Zenoderm implant during the healing process, suggesting that Zenoderm is not a suitable material for TM repair. However, a recent animal study in guinea pigs illustrated the feasibility of porcine acellular dermis as a scaffold to repair chronic TM perforations, with high success rates of 85% and complete hearing recovery 8 weeks postoperatively (Deng et al. 2009). This material was prepared by Triton X-100 and a nuclease solution for decellularisation. In addition, it could be seeded with
fibroblasts to facilitate the TM repair giving encouraging results of a higher healing rate (94.7%) and faster recovery in the early stage (Deng et al. 2009). Tutoplast® is an allograft material prepared from various human tissue sources. It is produced through a tissue cleaning and preservation process using solvent dehydration to reduce the possibility of disease transmission, while preserving its biological and mechanical properties (Oktem et al. 2007). Tutoplast has been commercially available for over 30 years and is utilised in numerous surgical areas including otology. Two clinical trials have reported positive results for its use in TM reconstruction. In a prospective RCT, of 43 patients who underwent tympanoplasty, Saraç and Gürsel (2002) showed that the use of Tutoplast temporalis fascia achieved similar success rates and hearing gains when compared to autologous temporalis fascia at 18 months post-surgery (Saraç & Gürsel 2002). By comparison, another retrospective case study by Yetişer et al. (2001) involving 45 patients who underwent revision myringoplasty using Tutoplast dura mater, with a mean follow-up of 4.7 years, reported excellent tissue compatibility and stability, in addition to a promising graft take rate of 86.7% (Yetişer et al. 2001). Tutoplast dura mater has been reported to have better dynamic characteristics compared with Tutoplast temporalis fascia under in vitro conditions, which may contribute to better audiological performance (Oktem et al. 2007). However, further studies are required to assess the actual in vivo behaviour of these materials.
Surgisis® is an ECM scaffold processed from porcine SIS that is acellular and predominately composed of non-cross-linked collagen (types I, III and V) (Rice et al. 2010). It functions as a surgical mesh for implantation to reinforce soft tissue, such as for hernia, abdominal wall, and rotator cuff repair. Studies have revealed tissue incorporation and neovascularisation of Surgisis postimplantation (Soiderer et al. 2004; Ayubi et al. 2008). In a chinchilla chronic perforation model, the Surgisis-repaired eardrums displayed a trilaminar structure with a success rate of 100% after 6 weeks, whereas treatment with autologous cartilage resulted in a lower graft take rate of 60% and a bilaminar repair with absence of the middle fibrous layer (Spiegel & Kessler 2005). These findings confirm previous studies, indicating that Surgisis is a viable alternative. However, Restore®, another SIS-derived biomaterial, has been shown to contain remnant xenocellular elements such as porcine DNA and serotonin, which may be associated with an inflammatory response postoperatively (Zheng et al. 2005). Therefore, future studies should be conducted on SIS-based materials to fully understand their safety and biocompatibility properties.

Urinary bladder matrix (UBM), another ECM scaffold, is derived from porcine urinary bladder and contains both stroma and a basement membrane. UBM has been previously employed for the repair of bladder, myocardium, oesophagus and other soft tissues (Badylak 2004). Recently, only one chinchilla model of chronic TM perforation repair has indicated promising results using the UBM scaffold, with accelerated tissue repair and a better
organised CT framework of the TM compared to spontaneous healing, but hearing function was not evaluated (Parekh et al. 2009).

1.4.2. Silk

Silk is a natural macromolecular protein polymer that is predominantly derived from the cocoons of the silk worm *Bombyx mori*. Silk is primarily composed of two proteins, fibroin (main structural protein) and sericin (glue-like coating protein) (Altman et al. 2003; Vepari & Kaplan 2007). Sericin has been identified as the antigenic agent of silk and therefore is generally removed to reduce immune responses (Meinel et al. 2005).

Traditionally, SF has been widely used as sutures for centuries. Subsequently, it has been investigated for the use in numerous regenerative medicine and tissue engineering applications. Promising results have demonstrated in bone, ligament, tendon, cornea, blood vessel and cartilage (Altman et al. 2003; Kasoju & Bora 2012), which may be due to its favourable properties such as biocompatibility, controllable biodegradability, appropriate mechanical properties, and ease of processing. In addition, SF can be manufactured into a variety of forms including film, foam, sponge, patch, membrane and mesh which is advantageous for diverse biomedical applications (Altman et al. 2003).

At present, SF has drawn much attention as an alternative grafting material for TM repair in otologic applications (Levin et al. 2009). Based on previous research, SF has been certified to be biocompatible and may cause very minor
local inflammatory reactions in vivo (Meinel et al. 2005; Vepari & Kaplan 2007). Moreover, it can be fabricated to match the rate of host tissue ingrowth and development through modifications of the processing methods and structural characteristics (Wang et al. 2008). In addition, SF allows direct observation of post surgical TM healing due to its transparency, which is favourable from an otologist’s point of view. Most importantly, SF can greatly contribute to cellular growth and wound healing (Altman et al. 2003).

Recently, our group has shown that human TM keratinocytes can adhere, proliferate and differentiate successfully on a formic acid-based SF membrane in vitro (Ghassemifar et al. 2010; Levin et al. 2010; Levin et al. 2012).

Currently, only one study has been performed to observe the effects of SF use for grafting TM perforations in vivo. An aqueous-based SF patch was compared with a paper patch and spontaneous healing in a rat model with large acute TM perforations (1.8mm in diameter). The SF patch was demonstrated to shorten the closure period (2 and 3 days, respectively) and accelerate the wound healing rate (92.5%, 67.5% and 65%, separately) (Kim et al. 2010a). However, this study neglected concerns regarding biocompatibility and safety when using this type of SF in vivo, especially within the ME cavity and with respect to hearing function, which are paramount for the application in TM tissue engineering.
1.4.3. Collagen

Collagen is a fibrous protein, and the most abundant within the human body. Collagen-based biomaterials have been utilised for various medical applications with several advantageous features including abundance, biodegradability, biocompatibility, weak immunogenicity, and the capability of promoting wound healing (Bunyaratavej & Wang 2001; Glowacki & Mizuno 2008; Ferreira et al. 2012). Thus far, 29 types of collagen have been characterised with collagen type I being the most commonly used (Parenteau-Bareil et al. 2010). These collagen-based biomaterials are typically represented as one of two forms, either as acellular tissue-derived ECM or as a reconstituted collagen matrix. The former is decellularised by physical, chemical, or enzymatic methods in order to preserve its original ECM structure. In contrast, the latter undergoes extraction, purification, and reconstitution of collagen molecules/fibres to produce a scaffold that mimics some of its native structure and function (Parenteau-Bareil et al. 2010). Furthermore, considering its rapid degradation rate, collagen material can be treated by various cross-linking techniques (e.g. physical, chemical, and enzymatic) to enhance the mechanical and enzymatic stability for specific tissue requirements. Among these, aldehyde (e.g. glutaraldehyde) treatment is the most common choice, but its clinical use is declining due to the reported nonspecific cytotoxicity associated with chemical residues remaining in the scaffold (Speer et al. 1980). Alternatively, tanning agents with lower
cytotoxicity profiles, such as hexamethylene diisocyanate, have also been employed (Glowacki & Mizuno 2008).

There has been a growing interest in collagen-based biomaterials for the closure of TM perforations. Collagen is the major component of the lamina propria of the TM, and the absence or deficiency of the collagen layers might result in an atelectatic TM with loss of stability and stiffness properties. Hence, exogenous collagen materials may be a logical choice for myringoplasty. Several animal studies have reported preliminary results indicating suitability of collagens from rat or bovine sources in experimental TM closure, including good host tolerance, enhanced eardrum regeneration, and ease in handling (Salen & Simbach 1965; Patterson 1967; Goycoolea et al. 1991; Bonzon et al. 1995). Furthermore, type IV collagen derived from human placenta has proven to be safe and effective in the closure of TM perforations in a dog model, in addition to a clinical trial involving 26 patients with chronic OM (Morgon et al. 1989; Truy et al. 1994). Likewise, analogous results were reported by Abbenhaus (Abbenhaus & Hemenway 1967; Abbenhaus 1978) utilising a reconstituted bovine collagen, and by Puls (Puls 1996), who used Parmatymp®, which is derived from bovine jugular vein.

However, some previous studies have shown unsatisfactory results, in part attributable to unfavourable purification or tanning techniques (Bonzon et al. 1995). For example, in a clinical study with patients treated with Parmatymp, hearing results were found to be comparable to those of temporalis fascia.
Nevertheless, the Parmatymp group possessed a higher percentage of granular myringitis with a lower closure rate, presumably due to the aldehydes used in the cross-linking procedure of the graft (Puls 1996). This result supports the idea of using alternate tanning agents. However, our group has recently developed a non-crosslinked bilayered ACS derived from porcine peritoneum that possesses suitable mechanical properties. In addition, ACS is acellular and contains no remaining reactive DNA that can invoke an inflammatory response, which suggests a potential use as a clinical treatment for TM perforations.

1.4.4. Gelatin

Gelatin is the product of thermal denaturation or disintegration of insoluble collagen derived primarily from animal sources (Gorgieva & Kokol 2011). Currently, gelatin-based biomaterials, such as Gelfoam® and Gelfilm® have been employed extensively in medical applications. Gelfoam is a porous absorbable gelatine sponge produced from denatured porcine skin. Due to its beneficial properties such as non-immunogenicity, non-toxicity, haemostasis and ease of handling, Gelfoam has been successfully utilised in otology since 1945, primarily as a packing agent for otologic surgeries (Shen et al. 2011). Although previous studies have revealed that the use of Gelfoam in the ME cavity may cause undesirable effects including adhesion, fibrosis and even osteoneogenesis (especially with denuded mucosa), it is noteworthy that Gelfoam today still remains a preferred option for otologic surgeons.
Gelfoam was initially used as an eardrum graft to seal perforations following surgical removal of ventilation tubes (VT) in paediatric patients, with success rates ranging from 95.5% to 100% (Hekkenberg & Smitheringale 1995; Puterman & Leiberman 2005). More recently, clinical trials have reported encouraging results of Gelfoam patching for both acute and chronic TM perforations. In a prospective RCT of 91 patients with acute traumatic perforation, Lou and He (Lou & He 2011) found that Gelfoam patching facilitated TM healing with a higher healing rate (97%) and significantly shorter healing times than spontaneous healing, though the actual underlying mechanisms are unclear (Lee 2011). A case study by Niklasson and Tano (2011) also reported the use of Gelfoam patching in persistent small TM perforations resulting in a high closure rate of 83%, similar to the routine fat plug technique (Niklasson & Tano 2011). Apart from a scaffold, Gelfoam has been widely used in TM tissue engineering therapies in addition to functioning as a vehicle for multiple growth factors (Kanemaru et al. 2011).

Gelfilm, homologous to Gelfoam, has found successful usage in otologic surgeries to prevent adhesion formation in the ME owing to its nonporous sheet-like structure (McGhee & Dornhoffer 1999). In addition, several retrospective studies have indicated that Gelfilm acts as an efficient material for eardrum grafting in clinical otology. Gelfilm patching presented the
similar success rates as the Gelfoam technique in paediatric cases after removal of VTs (Baldwin & Loftin 1992; Hekkenberg & Smitheringale 1995). For repair of chronic TM perforations, Gelfilm tympanoplasty also supports TM healing and induces neomembrane formation (Harris et al. 1971; Karlan 1979). In comparison with Gelfoam, Gelfilm allows easier manipulation intraoperatively and better visual inspection of the ME postoperatively due to its stiffness and transparency, respectively (Karlan 1979).

1.4.5. Chitosan

Chitosan is a polysaccharide derived from chitin, the main component of crustacean shells. This material has desirable haemostatic, biocompatibility, biodegradability and antibacterial properties. Furthermore, it accelerates wound healing and aids in scar prevention, and has been utilised for various biomedical applications. As for TM reconstruction, Kim et al. (2009) initially reported that the patch scaffolds fabricated by both a water-insoluble (Kim et al. 2009) and a water-soluble chitosan (Kim et al. 2010b) had acceptable mechanical properties and biocompatibility characteristics, and was more effective compared with spontaneous healing in the repair of acute TM perforations in rats. Histologically, both studies revealed that the healed TMs treated with chitosan patches had a better lamina propria layer structure with more collagen fibres than spontaneously healed TMs, but no hearing results were demonstrated. Subsequently, a 3D porous chitosan scaffold was investigated using the same animal model (Kim et al. 2011). Similar results
were reported, including accelerated TM healing and complete hearing recovery. However, in contrast to the two-dimensional chitosan membranes, there was cellular ingrowth within the pores of the 3D chitosan scaffold and integration of the scaffold into the regenerated TMs.

1.4.6. Hyaluronic acid derivatives

Hyaluronic acid (HA) (also known as hyaluronan, hyaluronate) is a naturally occurring high molecular weight glycosaminoglycan. It is distributed in the ECM of CTs (Laurent & Fraser 1992). HA has unique physico-chemical and biological properties, such as viscoelasticity and hygroscopicity. Furthermore, its safety and biocompatibility are well established in the literature (Becker et al. 2009; Friedman et al. 2002; Laurent et al. 1991a). Additionally, HA plays a critical role in cell biological functions, including cell proliferation and differentiation, and is beneficial for all phases of wound healing (Teh et al. 2012). To date, HA has been extensively investigated for biomedical use, with wide clinical applications in ophthalmologic and orthopaedic surgery (Polack 1986; Thomas et al. 1986). In the field of otology, HA has been explored for several applications including as a middle ear packing agent (MEPA) to reduce postoperative fibrosis (Shen et al. 2011), a novel sealing agent in cochlea implantation (Angeli 2006), and for promoting re-epithelialisation of the mastoid cavity following tympanomastoidectomy (Martini et al. 2000).
Of note, HA and its derivatives have been proven to be advantageous in TM wound healing (Teh et al. 2012). Initially, many studies in animals and humans indicated that topical application of HA in various concentrations resulted in an accelerated TM repair with shortened closure times and reduced TM scar formation compared with controls (Teh et al. 2012). In addition, HA is generally well tolerated without any documented medical complications such as ototoxicity or cholesteatoma. However, due to its rapid resorption and short residence time in the ME, topical HA normally requires repeated daily administration during the healing process. This possible inconvenience may limit its clinical use from both the patients’ and clinicians’ points of view.

Consequently, chemically modified HA by esterification coupling (e.g. MeroGel®, EpiFilm®, and EpiDisc® otologic lamina) and cross-linking (e.g. Seprafilm®) have been developed, which possess longer residence time in addition to enhancement in mechanical properties. In rats, a single administration of MeroGel, an esterified HA scaffold, presents similar efficacy as daily topical HA applications in the treatment of acute TM perforations (Ozturk et al. 2006). Furthermore, for patients with persistent perforations, the use of EpiDisc otologic lamina, a HA ester film scaffold, resulted in high healing success rates of 87-92.7% and hearing improvements of 12.8-17dB at frequencies of 500-4000Hz (Saliba 2008; Saliba & Woods 2011; Saliba & Froehlich 2011). These outcomes are comparable to those of the conventional temporalis fascia and perichondrium grafts using overlay or
underlay techniques. On the contrary, a previous study in a guinea pig model showed unsatisfactory results using EpiFilm, which is homologous to EpiDisc (Park et al. 2006). Furthermore, Prior et al. (2008) reported failure in the first five patients with a closure rate of zero at week 6 postoperatively, leading to subsequent abortion of the study (Prior et al. 2008). The reasons for failure were unclear, but it should be noted that the patient cohort were all over the age of 50 years with symptomatic perforations. Conversely, a recent clinical trial demonstrated that the use of EpiFilm yielded earlier closure times, but the results were not statistically significant when compared to spontaneous closure (Sayin et al. 2013). Future investigations will be valuable to clarify its benefit on TM perforations.

Seprafilm is a cross-linked HA that consists of HA and carboxymethylcellulose (CMC). This material is normally used as a barrier in abdominal and pelvic surgery to prevent adhesions. Currently, the use of Seprafilm in the repair of TM perforations is limited to one preclinical study, showing preliminary data of significantly faster healing compared to spontaneous healing (7.8 days vs. 14.9 days) (Konakçi et al. 2004). A recent study revealed that CMC has the potential for ototoxicity, causing sensorineural hearing impairment in rats (Antonelli et al. 2010). Therefore, its clinical application may need to be considered with caution.
1.4.7. Paper

Initially introduced by Blake in 1887, the technique of paper patching is commonly practiced in outpatient settings and has long been known to facilitate TM healing. Many types of paper have been utilised for this purpose in clinics, including cigarette, rice and carbon paper patches, with cigarette paper being the most common choice. In general, this technique is simple, safe and inexpensive with a good success rate, especially in small acute and traumatic perforations (Golz et al. 2003). In the literature, its documented recovery rates in clinical practice are 90.6-100% for acute perforations (Merwin & Boies 1980; Winerman et al. 1982; Lindeman et al. 1987), 87% for VT removal (Pribitkin et al. 1992), and 26.7-72.9% for chronic perforations (Laurent et al. 1991b; Spandow et al. 1995; Golz et al. 2003; Dursun et al. 2008; Lee et al. 2008). Moreover, the procedure is noninvasive and requires only local anaesthesia, with patients demonstrating an immediate improvement of hearing, as well as relief from other postoperative symptoms such as aural fullness (Merwin & Boies 1980; Winerman et al. 1982). Hence, paper patch has been widely accepted by most patients. In developing countries, the paper patch is still considered a first choice treatment to routine myringoplasty in patients with TM perforations.

However, many studies have reported that paper patches have inherent disadvantages such as nonbiocompatibility, nonflexibility, nontransparency, and easy detachment. Generally, this technique have been reported with unfavourable closure rates in large perforations (> 3mm) (Golz et al. 2003;
Dursun et al. 2008; Lee et al. 2008; İmamoğlu et al. 1998). In revision cases, there was no tendency for the perforations to heal with this treatment (Laurent et al. 1991b). Thus, paper patching is suggested to be more suitable for non-operated patients with dry and small TM perforations. Furthermore, paper tends to prematurely detach from the perforation margin during yawning or swallowing after surgery, thereby requiring repeated application over a prolonged period of time (Golz et al. 2003; Shen et al. 2013b). In addition, cigarette paper has been demonstrated to cause evident inflammatory responses with occasional purulent secretions postimplantation, which may result in delayed healing (Shen et al. 2013a; İmamoğlu et al. 1998). Animal studies have revealed that the paper-treated eardrums healed with an atrophic and retracted structure consisting of a minimal or absent lamina propria layer (Gold & Chaffoo 1991; İmamoğlu et al. 1998).

To avoid the limitation of easy detachment, the application of Steri-Strips™, an adhesive surgical tape has also been investigated. This material is made of synthetic paper coated on one side with latex-free, hypoallergenic adhesive, and is commonly used as a wound closure device. Clinical studies reported that traumatic perforations repaired with Steri-Strips had high success rates of 92-99.1%, similar to those of cigarette paper (Camnitz & Bost 1985; Saito et al. 1990; Park et al. 2011). Due to their adhesive strength, Steri-Strips are not easily detached from the eardrum, and thus decreases the need for repatching, which shortens healing times (Saito et al. 1996). However, Steri-Strips
patching was observed to be associated with an increased rate of otorrhea (23.3%) than cigarette paper patching (5.3%) (Park et al. 2011). Although otorrhea resolved following removal of the patch and was independent to closure rate and times, otologists must be cautious about this possibility when applying Steri-Strips.

1.4.8. Calcium alginate

Alginate, a natural organic polymer extracted from seaweed, is biocompatible, non-immunogenic and biodegradable. When chemically modified with calcium, its resilience and handling are improved. It is commonly utilised in various medical applications such as drug delivery devices, scaffold materials for tissue engineering and cutaneous wound dressings to promote healing. Moreover, a recent study demonstrates the potential of calcium alginate as an alternative to currently used commercial silicone based tympanostomy tubes owing to its high mechanical strength and tailored absorbability (Sherman et al. 2010). For TM repair, Hott et al. (2004) developed a preformed calcium alginate scaffold using computer-aided design and injection moulding techniques (Hott et al. 2004). These scaffolds possessed a precisely shaped structure and could successfully support chondrocytes in vitro. Subsequently, in a chinchilla chronic TM perforation model, this calcium alginate-based scaffold demonstrated significantly higher healing rates compared to the paper patch (69% vs. 22%) with less inflammatory responses 10 weeks postoperatively (Weber et al. 2006).
However, the theoretical ototoxicity potential of this biomaterial especially in
the ear has yet to be investigated.

1.5. Synthetic scaffolds

Synthetic scaffolds for TM repair consist of mainly synthetic polymers,
which are fabricated from chemical compounds and can be biodegradable or
nondegradable (Tables 1.4 and 1.5).

1.5.1. Nondegradable synthetic scaffolds

Nondegradable polymers used as TM scaffolds include polyvinyl (Johnson
1967), bisphenol-A polycarbonate (BAPC) (Feenstra et al. 1980; Feenstra et
al. 1984), polytetrafluoroethylene (PTFE) (Feenstra et al. 1980; Feenstra et
al. 1984), polydimethylsiloxane (PDMS) (Farhadi et al. 2012), cellophane
(Amadasun 2002), and silicone elastomers (Courteney-Harris et al. 1992;
Kartush 2000). These materials have superior mechanical properties and
physiological inertness, and may be incorporated into and remain within a
neomembrane to function as a permanent reinforcement. However, their
long-term biocompatibility is unfavourable and the nondegradable
characteristics may result in suboptimal hearing functions correlated with a
thickened structure of the TM.
Table 1.4  Animal experiments of synthetic scaffolds for TM regeneration

<table>
<thead>
<tr>
<th>Animal model</th>
<th>Year</th>
<th>Scaffold materials</th>
<th>Perforation type</th>
<th>Cases</th>
<th>Follow-up (months)</th>
<th>Healing rates (perforation closure)</th>
<th>Hearing outcomes</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chinchilla</td>
<td>2012</td>
<td>PGS spool-shaped plug</td>
<td>Chronic</td>
<td>11</td>
<td>4</td>
<td>91%</td>
<td>NA</td>
<td>Sundback et al. 2012</td>
</tr>
<tr>
<td>Chinchilla</td>
<td>2010</td>
<td>PGS plug</td>
<td>Chronic</td>
<td>16</td>
<td>1.5</td>
<td>91%</td>
<td>NA</td>
<td>Wieland et al. 2010</td>
</tr>
<tr>
<td>Rat</td>
<td>1980</td>
<td>Poly(D,L-lactic acid), PGA, poly(B-benzyl-L-aspartate-co-L-leucine) 50/50, poly(tetrafluoroethylene), BAPC</td>
<td>Acute</td>
<td>35</td>
<td>13</td>
<td>NA</td>
<td>NA</td>
<td>Feenstra et al. 1980</td>
</tr>
<tr>
<td>Cat</td>
<td>1967</td>
<td>Polyvinyl (Ivalon)</td>
<td>Acute</td>
<td>3</td>
<td>11</td>
<td>60%</td>
<td>NA</td>
<td>Johnson 1967</td>
</tr>
</tbody>
</table>

PGS: Poly(glycerol sebacate); PGA: Poly(glycolic acid); BAPC: Bisphenol-A polycarbonate; NA: Data not available.
<table>
<thead>
<tr>
<th>Study type</th>
<th>Year</th>
<th>Scaffold materials</th>
<th>Perforation type</th>
<th>Cases (ears)</th>
<th>Follow-up (months)</th>
<th>Healing rates (perforation closure)</th>
<th>Hearing outcomes</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Prospective</td>
<td>2002</td>
<td>Cellophane sheet</td>
<td>Acute</td>
<td>22</td>
<td>48 days</td>
<td>50%</td>
<td>50% improved</td>
<td>Amadasun 2002</td>
</tr>
<tr>
<td>Prospective</td>
<td>2000</td>
<td>Tympanic membrane patcher</td>
<td>Chronic</td>
<td>29 (30)</td>
<td>4-42</td>
<td>23.3% healed; 6.7% smaller; 70% unchanged</td>
<td>60% improved; others unchanged</td>
<td>Kartush 2000</td>
</tr>
<tr>
<td>Prospective</td>
<td>1995</td>
<td>Polyactive (poly(ethylene oxide-hydantoin)/poly(tetramethylene terephthalate) segmented polyether polyester copolymer)</td>
<td>Chronic</td>
<td>15</td>
<td>3-5 years</td>
<td>80% (1 year); 47% (3 years)</td>
<td>86.6% ABG&lt;30dB(1 year); 40% ABG&lt;30dB(3 years)</td>
<td>Grote 1995</td>
</tr>
<tr>
<td>Prospective</td>
<td>1992</td>
<td>Silastic sheeting</td>
<td>VT removal</td>
<td>108 (152)</td>
<td>9</td>
<td>93.2%</td>
<td>NA</td>
<td>Courteney-Harris et al. 1992</td>
</tr>
<tr>
<td>Case study</td>
<td>2011</td>
<td>Type I collagen-immobilised PDMS</td>
<td>Chronic</td>
<td>10</td>
<td>2</td>
<td>70%</td>
<td>ABG improved by 15.75dB</td>
<td>Farhadi et al. 2012</td>
</tr>
</tbody>
</table>

PDMS: Polydimethylsiloxane; VT: Ventilation tube; ABG: Air-bone gap; NA: Data not available.
Polyvinyl, a pliable plastic material, was proven to be unsatisfactory in the repair of tympanic perforations in a feline model. During the healing process, there was no observed TM replacement nor scaffold support (Johnson 1967). BAPC presented similar findings in rat and dog models. In addition to its high stiffness characteristics and a low tendency for tissue ingrowth, this material was not considered promising for TM grafting (Feenstra et al. 1980; Feenstra et al. 1984). PTFE, a nondegradable scaffold that is strong and smooth, is widely applied in plastic, gynaecological and urological surgeries. PTFE has been shown to support tissue ingrowth and becomes integrated within the TM, resulting in a thickened, reinforced neomembrane. However, it was found difficult to manipulate in ME surgery, and no further clinical data has been produced since (Feenstra et al. 1980; Feenstra et al. 1984). Cellophane was also found to be less effective compared to spontaneous healing (50% vs. 78%) in patients with traumatic perforations, and thus it was discouraged for use in TM repair (Amadasun 2002).

Several silicon-based products have also been investigated with promising results. PDMS is the most commonly used silicon-based organic polymer due to its beneficial properties such as inertness, nontoxicity and high mechanical properties. Following surface modification with type I collagen, this collagen-immobilised PDMS showed significantly increased cell adhesion and growth in in vitro conditions. A case series in 10 patients with small chronic perforations (≤5mm) later reported a 70% closure rate and an average ABG gain of 15.75dB, indicating its potential use in the clinical settings.
(Farhadi et al. 2012). Similarly, an early RCT of silastic sheeting reported a 93.2% success rate in 152 perforation closure procedures following VT removal (Courteney-Harris et al. 1992). By contrast, a prospective study of TM silicone patcher (Xomed, USA) reported only 23.3% chronic perforations healed with a long-term follow-up. Nevertheless, this material resulted in 69% hearing improvement postoperatively, and has advantages such as simplicity, cost-effectiveness and self-stabilising without adhesives. Consequently, this device is recommended as an alternative option for patients when surgery is contraindicated (Kartush 2000).

1.5.2. Biodegradable synthetic scaffolds

Subsequently, biodegradable polymers, such as poly(D,L-lactic acid) (D,L-PLA) (Feenstra et al. 1980; Feenstra et al. 1984), poly(glycolic acid) (PGA) (Feenstra et al. 1980; Feenstra et al. 1984), poly(β-benzyl-L-aspartate-co-L-leucine)50/50 (PBzAL) (Feenstra et al. 1980; Feenstra et al. 1984), polyactive® (Grote et al. 1991; Grote 1995), and poly(glycerol sebacate) (PGS) (Wieland et al. 2010; Sundback et al. 2012) have also been evaluated. Biodegradable materials serve primarily as a temporary scaffold for the migrating epithelium from the TM remnants during healing. In contrast to the nondegradable counterparts, these products will be degraded completely via hydrolysis and phagocytosis. Meanwhile, the structure of the scaffold may be modified to reduce the degradation rate to provide prolonged support following complete healing of the perforation.
PLA and PGA are the most commonly used biodegradable polymers in tissue engineering and other medical applications, owing to their ease of synthesis and high mechanical properties. However, their use in TM tissue engineering is limited. Animal studies have revealed that both polyesters were unsuitable candidates due to intense cellular reactions postimplantation associated with their rapid degradation (Feenstra et al. 1980; Feenstra et al. 1984). In comparison, PBzAL indicated potential for TM repair in animal models (Feenstra et al. 1980; Feenstra et al. 1984). However, no clinical data is available, which suggests this material has not been trialled in human patients yet. Polyactive®, a polyether polyester copolymer, is biocompatible and bioactive and has been proven to be an efficient temporary scaffold for the repair of TM perforations in rats (Grote et al. 1991). In a clinical study using polyactive, Grote (Grote 1995) reported complete healing in 12 of 15 patients at 1-year follow-up, with 7 possessing an ABG within 20dB. PGS, a highly biocompatible elastomer, has been explored in various applications such as vascular and cardiac scaffolds and drug delivery vehicles. Sundback (Sundback et al. 2012) described the fabrication of PGS spool-shaped plugs with elastic deformability, which are easy to manipulate during surgical procedures. In a chinchilla chronic perforation model, PGS plugs showed a healing rate of 91% with graft neovascularisation (Wieland et al. 2010).
1.6. Summary

1.6.1. Advantages and disadvantages of biological and synthetic scaffolds

Biological scaffolds have been extensively investigated for TM repair. The most commonly used bioscaffolds are decellularised matrices, which are protein-based ECMs that are usually derived from human or animal collagen-rich tissues. These materials are known to promote host cell ingrowth and neotissue formation owing to their natural 3D microstructure and native-like ECM composition. Among these, AlloDerm has been successfully used for TM applications in the clinic over several years. However, this cadaver tissue-derived product is limited by the shortage of suitable human donors and the risk of transmission of infectious diseases. Moreover, with evidence of remnant DNA components in some commercially available ‘decellularised’ scaffolds, particular attention is needed for allogeneic and xenogeneic scaffolds, and a full understanding of their safety is warranted prior to the clinical use. Animal based biomaterials have also been utilised for TM perforation closure. Although a majority of them have shown encouraging results, it is evident that high-quality controlled RCTs with long-term follow-up are limited. Following implantation, these materials undergo degradation and resorption at variable rates depending on material source. To provide longer support, there is a trend recently towards the use of modified bioscaffolds (e.g. cross-linking and coupling) with enhanced mechanical and enzymatic stability. However,
several chemical cross-linking agents such as aldehyde can cause nonspecific cytotoxicity and inflammatory reactions, and thus is not recommended for use. Plant based biomaterials are predominantly in the form of thin cellulosic sheets such as cigarette and rice paper. The application of paper is simple, safe and inexpensive, and is advantageous for dry, small- and medium-size perforations. However, these materials are unfavourable when used for large chronic perforations, particularly in revision cases. Other potential disadvantages include early detachment, severe inflammation and formation of atypical TM structure lacking a fibrous layer.

Synthetic scaffolds have not been commonly employed in TM perforation repair. However, these products possess superior mechanical properties and may be manufactured in large quantities. In addition, there is no risk of disease transmission. However, studies are currently limited with a majority using animal models. The initial utilisation of synthetic scaffolds consisted predominantly of nondegradable materials, which were aimed at providing permanent repair of the TM. However, their long-term biocompatibility was poor, and the thickened TM with remaining scaffold materials lead to suboptimal hearing. Biodegradable synthetic materials were later explored, and serve as a temporary scaffold guide for the migrating epithelium during healing. They have predictable degradation to match TM regeneration and can be absorbed by host tissue. This would be advantageous for optimal hearing results, and would possibly lead to the complete absence of foreign material and long-term foreign body associated complications. Though some
materials have associated preliminary results, no further clinical trials have been performed.

1.6.2. Safety and biocompatibility

One of the primary major concerns with biological and synthetic scaffolds is biocompatibility. Most importantly, for uses in the ear, the scaffold material should be non-ototoxic. Meanwhile, the material should be well-tolerated by the host and induce a minimal inflammatory response postimplantation into the ME cavity. As for a biodegradable scaffold, its degradation products should also be nontoxic without causing any local or systemic adverse effects. In contrast, a nondegradable scaffold should be sufficiently inert without inducing severe chronic inflammation and foreign body reactions. From the materials reviewed above, extra attention needs to be paid on Seprafilm, as its component CMC has been shown to cause sensorineural hearing impairment in rats. Even though Gelfoam is still widely utilised nowadays, cautious usage is necessary due to its potential complications such as adhesion, fibrosis and osteoneogenesis. In addition, chemical tanning agents such as aldehyde can induce nonspecific cytotoxicity and thus its usage is not advocated. It is known that the biocompatibility of a biomaterial is dependent of various intrinsic (e.g., chemical composition, physical characteristics) and extrinsic (e.g., implant site, surgical technique) factors. Comprehensive evaluation of the biocompatibility of a scaffold material is crucial.
Another major concern with biological scaffolds is the risk of infectious disease transmission. This is particularly applicable for decellularised matrices, although no such case of transmission has yet been reported. For scaffolds derived from human and animal tissue, the remaining allogeneic or xenogeneic cellular antigens may induce inflammatory and immunological reactions. This may potentially result in rejection of the implants leading to surgical failure. Therefore, the development and refinement of an appropriate decellularisation method is critical to completely remove cellular components. Theoretically, this method should be effective in completely decellularising the tissue without adverse effects on the composition, biological activity, and biomechanical integrity of the remaining ECM. Current methods include physical, chemical and enzymatic treatments. Given that all have their respective advantages and limitations, a combination of these methods is recommended to maximise the decellularisation process with minimal adverse effects. Moreover, terminal sterilisation processing should remove or inactivate microorganism, including virus, bacteria and prions to prevent infectious disease transmission (e.g., HIV, hepatitis, Creutzfeldt-Jakob disease). In addition, regulatory issues should be implemented to facilitate their clinical applications in a safe and controlled manner, such as donor screening, serological testing, and scaffold safety evaluation.
1.6.3. Ideal scaffolds for TM regeneration

For any medical treatment, it is important to reduce the possibility of adverse side effects. For TM regeneration, the most essential factors would include being biocompatible with a minimal localised inflammatory response and be free of ototoxicity. In addition, it should be biodegradable and possess the capability to induce host-cell growth (e.g. cellular adhesion, migration and proliferation) and TM reconstruction during the degradation process. Eventually this would lead to replacement by the TM neotissue with a native trilaminar structure. Meanwhile, the scaffold should process appropriate biomechanical properties such as elasticity and tensile strength in both dry and wet conditions. Hence, it can provide adequate stability to resist the negative pressure in the ME while allowing sufficient acoustic sensitivity during the healing procedure. Furthermore, the scaffold should be flexible and durable, for easy handling during surgery. Ideally, it should also be transparent to allow direct observation of the ME postoperatively. These characteristics are highly desirable from a surgeon’s point of view. In addition, the scaffold should be inexpensive and available in commercial quantities, and also be easy to fashion. Currently, no scaffold material satisfies all these aforementioned characteristics (Table 1.6), and thus an ideal scaffold is yet to be identified.
Table 1.6 Requirements for an ideal TM scaffold

<table>
<thead>
<tr>
<th>Requirement</th>
</tr>
</thead>
<tbody>
<tr>
<td>Safe</td>
</tr>
<tr>
<td>Biocompatible</td>
</tr>
<tr>
<td>Biodegradable</td>
</tr>
<tr>
<td>Appropriate biomechanical properties</td>
</tr>
<tr>
<td>Flexible yet durable</td>
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<tr>
<td>Easy handling during surgery</td>
</tr>
<tr>
<td>Low-cost</td>
</tr>
<tr>
<td>Easy to obtain and fashion</td>
</tr>
<tr>
<td>Available in commercial quantities</td>
</tr>
<tr>
<td>Transparent to allow observation of the ME</td>
</tr>
</tbody>
</table>

1.7. Hypothesis and aims

1.7.1. Hypothesis

This study was based on two hypotheses, namely that:

1. We hypothesise that SFS and ACS possess excellent biosafety and biocompatibility characteristics.

2. We hypothesise that when used as a graft material for TM perforations, SFS and ACS will provide a rapid improvement to hearing in addition to accelerating the wound healing process providing a superior neomembrane compared to spontaneous healing and other commonly used graft materials such as paper patch and Gelfoam.

1.7.2. Aims

The aims of this study were therefore:

1. To analyse the morphology, microstructure and mechanical properties of SFS and ACS.
2. To investigate the *in vivo* safety and biocompatibility of SFS and ACS compared to paper patch and Gelfoam in the SC tissues and ME cavity using a rat model.

3. To evaluate the efficacy (healing and hearing outcomes) of SFS and ACS as scaffolds for TM regeneration compared to more commonly used materials such as paper patch and Gelfoam in a rat acute TM perforation model.

4. To evaluate the efficacy of SFS and ACS for the repair of TM compared to paper patch in a guinea pig acute TM perforation model.
2. Materials and methods
2.1. Materials

2.1.1. Scaffolds

Figure 2.1 The gross morphology of the scaffolds
These include ACS (A), SFS (B), paper patch (C), and Gelfoam (D).

2.1.1.1. Production of SFS

The SFS was prepared at the Institute for Frontier Materials of Deakin University (Geelong, Australia) as previously described (Rajkhowa et al. 2011). Briefly, multivoltine *Bombyx mori* (*B. mori*) silk cocoons were degummed in 2 g/L sodium carbonate (Na$_2$CO$_3$) and 0.6 g/L sodium dodecyl sulphate (SDS) (Sigma-Aldrich, Australia) at 100°C for 20 min, washed in distilled water (dH$_2$O), and dissolved in 10M lithium thiocyanate (LiSCN) (Sigma-Aldrich) at room temperature (RT) for 2 h. Solutions were centrifuged at 4800rpm for 10 min then dialysed against milli-Q water for four days at 4°C. Solutions were again centrifuged and the concentration of silk solution was adjusted to 3% (w/v) with milli-Q water then lyophilised for dry SF foam production. The foams were dissolved in 98% formic acid (Sigma-Aldrich) to obtain 3% fibroin solutions (w/v). Silk membranes were cast in polyethylene discs (1ml fibroin solution in a 25mm diameter disc) and
dried in a fume hood (covered to allow slow drying). The films were then annealed in 75% ethanol for 6h to induce β-sheets. The treated films were washed thoroughly with milli-Q water to remove residual chemicals (ethanol/formic acid), dried at RT, and stored under desiccation. This formic acid-based silk film was approximately 30µm thick, transparent, and had high tensile strength (Figure 2.1 B).

2.1.1.2. Production of ACS

ACS (Cellgro™; Patent No 2008901451/PTO) was manufactured by Orthocell Ltd (Perth, Australia) as per a proprietary manufacturing process. The ACS membrane is sourced from porcine peritoneum and consists of type I and type III collagen without further cross-linking treatment. In brief, following mechanical removal of the fat, the peritoneum was incubated with 1% SDS and 0.2% lithium chloride (LiCl) to remove nucleic acids, and then washed with 100% acetone to remove the fat. The scaffold was processed into a bilayered membrane by incubation in 3N sodium hydroxide (NaOH) and 4% hydrochloric acid (HCl) overnight and then washed with 0.8% sodium chloride (NaCl). This collagen scaffold was approximately 40µm thick and opaque in a dry state (Figure 2.1 A). One surface was smooth with dense bundles of collagen and the other rough consisting of loose collagen fibres.
2.1.1.3. **Paper patch**

Paper patch was prepared from cigarette paper (Tally Ho, Imperial Tobacco Australia). It was approximately 20 µm thick, white, and opaque (Figure 2.1 C).

2.1.1.4. **Gelfoam**

Gelfoam® (absorbable gelatine sponge, Pharmacia & Upjohn Inc., New York, USA) is a highly absorbent, nonelastic sponge (Figure 2.1 D) that is approximately 4 mm thick with its pore size varying between 30 and 700 µm (Rohanizadeh et al. 2008). Gelfoam was supplied sterile in its original packaging, while the other three materials were packaged and sterilised with ethylene oxide gas.

2.1.2. **Animals**

All the animal experimental protocols were approved by both the Animal Ethics Committees at The University of Western Australia (Australia) (RA/3/100/965 and RA/3/100/903 for the rat project; RA/3/100/1078 for the guinea pig project) and Zhejiang University (People’s Republic of China) (#2010/51 for the rat project; #2011/17 for the guinea pig project). Animals were obtained from Shanghai Animal Centre (Chinese Academy of Science, Shanghai, China). All animals were housed in the experimental animal facility of the Zhejiang University and were provided with food and water *ad libitum* in a room with 12-h light/dark cycles. Experiments were performed in accordance with the Australian Code of Practice for the Care and Use of Animals for Scientific Purposes (2004). Prior to the study, all animals were
inspected using a S5 model otomicroscope (Carl Zeiss, Germany) to ensure
they were free of ME pathology.

2.1.3. Surgical instruments
All surgical instruments used for the animal experiments are listed in Table
2.1.

2.1.4. Animal drugs and reagents
All drugs and reagents used for the animal experiments are listed in Table 2.2.

2.1.5. Commercially purchased kits
All commercially available kits used are listed in Table 2.3.

2.1.6. Chemical reagents
All chemical reagents used during the course of this experiment were of
analytical grade and were obtained through the following manufacturers
listed in Table 2.4.

2.1.7. Buffers and solutions
The recipes of all buffers and solutions are provided in Table 2.5.

2.1.8. Laboratory instruments
All laboratory instruments used are listed in Table 2.6.

2.1.9. Software
All softwares used are listed in Table 2.7.
<table>
<thead>
<tr>
<th>Instruments</th>
<th>Manufacturer</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ear speculum (size 3, oval oblique)</td>
<td>Medtronic Xomed Inc. MN, USA</td>
</tr>
<tr>
<td>Haemostatic forceps (S)</td>
<td>Medical Instrument Inc. Shanghai, China</td>
</tr>
<tr>
<td>Metzenbaum scissors (curved)</td>
<td>Medtronic Xomed Inc. MN, USA</td>
</tr>
<tr>
<td>Needle (23-gauge)</td>
<td>Kindly Co., Ltd. Shanghai, China</td>
</tr>
<tr>
<td>Needle holder</td>
<td>Medical Instrument Inc. Shanghai, China</td>
</tr>
<tr>
<td>Operating scissors</td>
<td>Medical Instrument Inc. Shanghai, China</td>
</tr>
<tr>
<td>Otology Buck ear curette (sharp, size 00/0/1)</td>
<td>Medtronic Xomed Inc. MN, USA</td>
</tr>
<tr>
<td>Otology Buck ear curette (dull, size 00)</td>
<td>Medtronic Xomed Inc. MN, USA</td>
</tr>
<tr>
<td>Otology ear cup forceps (oblong cups)</td>
<td>Medtronic Xomed Inc. MN, USA</td>
</tr>
<tr>
<td>Otology Guilford-Wright suction tube (24-gauge)</td>
<td>Medtronic Xomed Inc. MN, USA</td>
</tr>
<tr>
<td>Otology House-Bellucci alligator scissors (curved)</td>
<td>Medtronic Xomed Inc. MN, USA</td>
</tr>
<tr>
<td>Otology House-Bellucci alligator scissors (straight)</td>
<td>Medtronic Xomed Inc. MN, USA</td>
</tr>
<tr>
<td>Otology House strut forceps (6.0mm smooth jaws)</td>
<td>Medtronic Xomed Inc. MN, USA</td>
</tr>
<tr>
<td>Otology House suction irrigator (90mm, 3.5&quot;)</td>
<td>Medtronic Xomed Inc. MN, USA</td>
</tr>
<tr>
<td>Otology House suction irrigator (70mm, 2.8&quot;)</td>
<td>Medtronic Xomed Inc. MN, USA</td>
</tr>
<tr>
<td>Otology House Wullstein miniature ear cup forceps</td>
<td>Medtronic Xomed Inc. MN, USA</td>
</tr>
<tr>
<td>Otology needle (dull, light curve)</td>
<td>Medtronic Xomed Inc. MN, USA</td>
</tr>
<tr>
<td>Otology pick (1.0mm point, angled 90°)</td>
<td>Medtronic Xomed Inc. MN, USA</td>
</tr>
<tr>
<td>Otology pick (bent right)</td>
<td>Medtronic Xomed Inc. MN, USA</td>
</tr>
<tr>
<td>Otology Royce ear knife (downcutting blade)</td>
<td>Medtronic Xomed Inc. MN, USA</td>
</tr>
<tr>
<td>Otology sickle knife (curved blade)</td>
<td>Medtronic Xomed Inc. MN, USA</td>
</tr>
<tr>
<td>Otology strut pick (0.50mm point, angled 90°)</td>
<td>Medtronic Xomed Inc. MN, USA</td>
</tr>
<tr>
<td>Scalpel handle (2&quot;)</td>
<td>Medical Instrument Inc. Shanghai, China</td>
</tr>
<tr>
<td>Suture (SA84G)</td>
<td>Ethicon, NJ, USA</td>
</tr>
<tr>
<td>Tissue forceps</td>
<td>Medical Instrument Inc. Shanghai, China</td>
</tr>
</tbody>
</table>
Table 2.2  Detail of drugs and reagents used for animal experiments

<table>
<thead>
<tr>
<th>Drugs</th>
<th>Manufactory</th>
</tr>
</thead>
<tbody>
<tr>
<td>Buprenorphine Hydrochloride (0.3mg/ml)</td>
<td>Qinghai Pharmaceutical, Co., Ltd., Xining, China</td>
</tr>
<tr>
<td>Diazepam (5mg/ml)</td>
<td>Nhwa Pharmaceutical, Co., Ltd., Jiangsu, China</td>
</tr>
<tr>
<td>Hypnorm® (fentanyl/fluanisone)</td>
<td>Janssen Pharmaceutical, Ltd., Beijing, China</td>
</tr>
<tr>
<td>Isofluorane (Forane®; 100ml)</td>
<td>Abbott Laboratories, Shanghai, China</td>
</tr>
<tr>
<td>Ketamine Hydrochloride (50mg/ml)</td>
<td>Hengrui Medicine Co., Ltd., Jiangsu, China</td>
</tr>
<tr>
<td>Medetomidine Hydrochloride (100µg/ml)</td>
<td>Hengrui Medicine Co., Ltd., Jiangsu, China</td>
</tr>
<tr>
<td>Ofloxacin Eye Ointment (Tarivid®; 3.5g)</td>
<td>Santen Pharmaceutical Co., Ltd., Osaka, Japan</td>
</tr>
<tr>
<td>Pentobarbitone Sodium</td>
<td>Sinopharm Chemical Reagent Co., Ltd., Shanghai, China</td>
</tr>
<tr>
<td>Povidone Iodine Solution (5%; 100ml)</td>
<td>Minsheng Pharmaceutical Co., Ltd., Hangzhou, China</td>
</tr>
</tbody>
</table>

Table 2.3  Detail of commercially purchased kits

<table>
<thead>
<tr>
<th>Kits</th>
<th>Manufactory</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dako Antibody Diluent Kit (S0809)</td>
<td>Dako Inc, California, USA</td>
</tr>
<tr>
<td>Dako DAB Chromogen Kit (K3468)</td>
<td>Dako Inc, California, USA</td>
</tr>
<tr>
<td>Masson’s Trichrome Stain Kit (MST-8003)</td>
<td>Maixin Bio Co., Ltd., Fuzhou, China</td>
</tr>
<tr>
<td>Rodent Block R (RBR962G)</td>
<td>Biocare Medical, California, USA</td>
</tr>
<tr>
<td>SPI-Pon™ 812 Epoxy Embedding Kit</td>
<td>SPI Supplies, USA</td>
</tr>
<tr>
<td>Reagent</td>
<td>Manufactory</td>
</tr>
<tr>
<td>----------------------------------------------</td>
<td>--------------------------------------------------</td>
</tr>
<tr>
<td>Acetone</td>
<td>Hangzhou Chemical Co., Ltd., Hangzhou, China</td>
</tr>
<tr>
<td>Boric Acid</td>
<td>Sigma, St Louis, MO, USA</td>
</tr>
<tr>
<td>DPX Mountant</td>
<td>Sigma-Aldrich, USA</td>
</tr>
<tr>
<td>Eosin</td>
<td>Sigma-Aldrich, USA</td>
</tr>
<tr>
<td>Ethanol (100%)</td>
<td>Rowe Scientific, Australia</td>
</tr>
<tr>
<td>Ethylenediaminetetraacetic Acid(EDTA)</td>
<td>Sangon Biotech Co., Ltd., Shanghai, China</td>
</tr>
<tr>
<td>Formaldehyde (37-40%)</td>
<td>Zhanyun Chemical Co., Ltd., Shanghai, China</td>
</tr>
<tr>
<td>Glutaraldehyde (25%)</td>
<td>Sigma, St Louis, MO, USA</td>
</tr>
<tr>
<td>Mayer’s Haematoxylin</td>
<td>Amber Scientific, Australia</td>
</tr>
<tr>
<td>Hydrochloric Acid</td>
<td>Sangon Biotech Co., Ltd., Shanghai, China</td>
</tr>
<tr>
<td>Hydrogen Peroxide (30%)</td>
<td>Sigma-Aldrich, USA</td>
</tr>
<tr>
<td>Isoamyl Acetate</td>
<td>Lingfeng Chem Co., Ltd., Shanghai, China</td>
</tr>
<tr>
<td>Lead Citrate</td>
<td>Sinopharm Chemical Reagent Co., Ltd., China</td>
</tr>
<tr>
<td>Methanol</td>
<td>BDH Laboratory Supplies, Poole, England</td>
</tr>
<tr>
<td>Milli-Q Water</td>
<td>Millipore Corporation, USA</td>
</tr>
<tr>
<td>Osmium Tetroxide</td>
<td>Sigma, St Louis, MO, USA</td>
</tr>
<tr>
<td>Paraffin Wax</td>
<td>Leica, Richmond, IL, USA</td>
</tr>
<tr>
<td>pH Calibration Solution</td>
<td>Merck KGaA, Darmstadt, Germany</td>
</tr>
<tr>
<td>1× Phosphate Buffered Saline (PBS)</td>
<td>Invitrogen, Shanghai, China</td>
</tr>
<tr>
<td>Scott’s Tap Water Substitute (STWS)</td>
<td>Amber Scientific, Australia</td>
</tr>
<tr>
<td>Sodium Chloride</td>
<td>Sangon Biotech Co., Ltd., Shanghai, China</td>
</tr>
<tr>
<td>Sodium Hydroxide</td>
<td>BDH Laboratory Supplies, Poole, England</td>
</tr>
<tr>
<td>Sodium Phosphate (dibasic) (Na₂HPO₄)</td>
<td>Sigma, St Louis, MO, USA</td>
</tr>
<tr>
<td>Sodium Phosphate (monobasic) (NaH₂PO₄)</td>
<td>Sigma, St Louis, MO, USA</td>
</tr>
<tr>
<td>Sucrose</td>
<td>Sangon Biotech Co., Ltd., Shanghai, China</td>
</tr>
<tr>
<td>Tween-20</td>
<td>AppliChem, Darmstadt, Germany</td>
</tr>
<tr>
<td>Uranyl Acetate</td>
<td>SPI Supplies, USA</td>
</tr>
<tr>
<td>Xylene</td>
<td>Rowe Scientific, Australia</td>
</tr>
</tbody>
</table>
Table 2.5  Detail of buffers and solutions

<table>
<thead>
<tr>
<th>Buffers and Solutions</th>
<th>Composition and Preparation</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>10% Aqueous EDTA (pH 7.0-7.4)</strong></td>
<td>Add 100g EDTA in 800ml dH₂O. pH solution and then make up to a final volume of 1000ml. Solution mixed and used fresh.</td>
</tr>
<tr>
<td><strong>0.2M Boric Acid Buffer (pH 7.0)</strong></td>
<td>Dissolve 12.366g boric acid in 800ml Milli-Q water. pH solution and make up to a final volume of 1000ml. Solution mixed and stored at RT.</td>
</tr>
<tr>
<td><strong>1% Eosin</strong></td>
<td>Dissolve 1g Eosin Y disodium salt in 20ml dH₂O and 80ml of 95% ethanol to make 1% stock solution. For working solution dilute 1:2 with 80% ethanol and add 0.5ml glacial acetic acid per 100ml before use.</td>
</tr>
<tr>
<td><strong>2.5% Glutaraldehyde</strong></td>
<td>Dissolve 36g sucrose in 600ml phosphate wash buffer, 200ml glutaraldehyde (25%), and 1200ml dH₂O. Solution mixed well and stored at 4°C.</td>
</tr>
<tr>
<td><strong>10% Neutral Buffered Formalin</strong></td>
<td>Dissolve 4.0g NaH₂PO₄ and 6.5g Na₂HPO₄ in 100ml formaldehyde (37-40%) and 900ml dH₂O. Mixed and stored at RT.</td>
</tr>
<tr>
<td><strong>1% Osmium Tetroxide</strong></td>
<td>Dissolve 1g osmium tetroxide in 50ml dH₂O. Shaken for 1 min then added to 50ml dH₂O. Solution mixed and stored at RT.</td>
</tr>
<tr>
<td><strong>1× PBS-Tween Buffer (0.1%)</strong></td>
<td>Add 0.5ml Tween-20 in 500ml 1× PBS. Mixed completely and stored at RT.</td>
</tr>
<tr>
<td><strong>0.4M Phosphate Buffer</strong></td>
<td>Dissolve 62.4g NaH₂PO₄ in 1000ml dH₂O. Mix and add to 170.4g Na₂HPO₄ and 3000ml dH₂O. Solution mixed and stored at 4°C.</td>
</tr>
<tr>
<td><strong>Phosphate Wash Buffer</strong></td>
<td>Add 100ml phosphate buffer (0.4M) in 200ml dH₂O. Mixed and stored at 4°C.</td>
</tr>
<tr>
<td>Reagent</td>
<td>Manufactory</td>
</tr>
<tr>
<td>--------------------------------------------------</td>
<td>--------------------------------------------------</td>
</tr>
<tr>
<td>Aperio ScanScope XT Automated Slide Scanner</td>
<td>Aperio Technologies Inc., Canada</td>
</tr>
<tr>
<td>Hitachi E1020 Scanning Electron Microscope</td>
<td>Hitachi Science Systems Ltd., Japan</td>
</tr>
<tr>
<td>Hitachi HCP-2 Critical Point Dryer</td>
<td>Hitachi Ltd., Japan</td>
</tr>
<tr>
<td>Hitachi S3000N Scanning Electron Microscope</td>
<td>Hitachi Ltd., Japan</td>
</tr>
<tr>
<td>IKA KMO-2 Magnetic Stirrer</td>
<td>IKA, Werke GmbH, Germany</td>
</tr>
<tr>
<td>Instron Tension/compression System (Model 5543)</td>
<td>Instron Inc., Canton, MA, USA</td>
</tr>
<tr>
<td>Kylin-Bell TS-2 Orbital Shaker</td>
<td>Kylin-Bell Co. Ltd., Haimen, China</td>
</tr>
<tr>
<td>Leica ASP200S Tissue Processor</td>
<td>Leica Microsystems, Germany</td>
</tr>
<tr>
<td>Leica DM3000 Light Microscope</td>
<td>Leica Microsystems, Germany</td>
</tr>
<tr>
<td>Leica EG1150H Heated Paraffin Embedding Module</td>
<td>Leica Microsystems, Germany</td>
</tr>
<tr>
<td>Leica EG1150C Cold Plate Modular Tissue Embedding System</td>
<td>Leica Microsystems, Germany</td>
</tr>
<tr>
<td>Leica HI1210 Water Bath for Paraffin Sections</td>
<td>Leica Microsystems, Germany</td>
</tr>
<tr>
<td>Leica RM2235 Manual Rotary Microtome</td>
<td>Leica Microsystems, Germany</td>
</tr>
<tr>
<td>Leica S260 Scanning Electron Microscope</td>
<td>Leica Cambridge Ltd., UK</td>
</tr>
<tr>
<td>Leica ST5010 Autostainer XL</td>
<td>Leica Microsystems, Germany</td>
</tr>
<tr>
<td>MEB-9100 Evoked Potential/EMG Measuring System</td>
<td>Nihon Kohden Co., Tokyo, Japan</td>
</tr>
<tr>
<td>MedRX Digital Video Otoscope</td>
<td>MedRX, Largo, FL, USA</td>
</tr>
<tr>
<td>Mettler Toledo Delta-320 pH Meter</td>
<td>Mettler Toledo Ltd., Shanghai, China</td>
</tr>
<tr>
<td>Mettler Toledo PL203 Balance</td>
<td>Mettler Toledo Ltd., Shanghai, China</td>
</tr>
<tr>
<td>Microwave Sharp Carousel(Model R-7280; 650 Watt)</td>
<td>Sharp, Australia</td>
</tr>
<tr>
<td>Minqiao JY6001 Balance</td>
<td>Minqiao Co. Ltd., Shanghai, China</td>
</tr>
<tr>
<td>Philips TECNAI-10 Transmission Electron Microscope</td>
<td>Philips Co., Netherlands</td>
</tr>
<tr>
<td>Tender Cooker (Microwave Pressure cooker)</td>
<td>Nordic Ware, Australia</td>
</tr>
<tr>
<td>Thermo PL6500 Lab Refrigerators</td>
<td>Thermo Fisher Scientific Inc., China</td>
</tr>
<tr>
<td>Vortex Genie II Model G560 Vortex Mixer</td>
<td>Scientific Industries Inc., NY, USA</td>
</tr>
<tr>
<td>Zeiss Model S5 Surgical Microscope</td>
<td>Carl Zeiss Surgical Inc., Germany</td>
</tr>
</tbody>
</table>
Table 2.7  Detail of software

<table>
<thead>
<tr>
<th>Description</th>
<th>Supplier</th>
</tr>
</thead>
<tbody>
<tr>
<td>Adobe® Acrobat® 9 Pro (Version 9.3.0)</td>
<td>Adobe Systems Inc., USA</td>
</tr>
<tr>
<td>Adobe® Photoshop® CS5 (Version 12.1)</td>
<td>Adobe Systems Inc., USA</td>
</tr>
<tr>
<td>Aperio ImageScope Viewer Software (Version 10.2.2.2319)</td>
<td>Aperio Technologies Inc., Vista, CA</td>
</tr>
<tr>
<td>Aurisview Software</td>
<td>Ear Science Institute Australia, Australia</td>
</tr>
<tr>
<td>EndNote® (Version X5)</td>
<td>Thomson Reuters Co., NY, USA</td>
</tr>
<tr>
<td>GraphPad Software (Version 5.03)</td>
<td>GraphPad Prism, La Jolla, USA</td>
</tr>
<tr>
<td>Instron® Tension/compression System</td>
<td>Instron, Canton, MA, USA</td>
</tr>
<tr>
<td>Fast-Track Software</td>
<td></td>
</tr>
<tr>
<td>Microsoft® Office Suite 2010</td>
<td>Microsoft Corporation, USA</td>
</tr>
<tr>
<td>Neuropack-µ Measuring Systems</td>
<td>Nihon Kohden, Tokyo, Japan</td>
</tr>
<tr>
<td>PASS Software (Version 11.0.7)</td>
<td>NCSS, Kaysville, UT, USA</td>
</tr>
<tr>
<td>SPSS Software (Version 16.0 for Windows)</td>
<td>SPSS Inc, Chicago, Illinois, USA</td>
</tr>
<tr>
<td>Statistical Software R (Version 2.11.1 for Windows)</td>
<td>The Comprehensive R Archive Network</td>
</tr>
</tbody>
</table>

2.2.  General methods

2.2.1.  Otoscopic observation

Before sacrifice, animals from each group were randomly chosen at each time point for otoscopic observation using a digital video otoscope (MedRX, Largo, FL) under general anaesthesia. Digital images were recorded using Aurisview software (Ear Science Institute Australia, Subiaco, Australia). For rats, the TMs were viewed with respect to perforation closure, infection, myringosclerosis, granulation tissue, and thickening. Each TM perforation was graded as either completely closed or unclosed. Only TMs that had completely closed were considered healed. For guinea pigs, the TMs were viewed with respect to perforation closure, signs of infection, and myringosclerosis. The perforation site of each TM was graded using Gnuechtel’s method (Gnuechtel et al. 2000) on a 0 to 7 scale: 0,
large perforation; 1, small perforation; 2, pinhole perforation; 3, moderate eschar; 4, minimal eschar; 5, moderate myringosclerosis; 6, small myringosclerosis; and 7, totally healed.

2.2.2. Tissue preparation for histology and IHC

Tissue specimens for histology and immunohistochemistry (IHC) were processed using an automated tissue processor (ASP200S, Leica, Germany) on an overnight cycle (Table 2.8), embedded in paraffin wax (Leica, USA), and transversely sectioned with a microtome (RM2235, Leica, Germany) at a thickness of 4µm.

Table 2.8 Automated processing procedures for rat and guinea pig tissues

<table>
<thead>
<tr>
<th>Sequence</th>
<th>Reagent</th>
<th>Time (hours)</th>
<th>Temperature (°C)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cylinder 1</td>
<td>70% Ethanol</td>
<td>1.0</td>
<td>30</td>
</tr>
<tr>
<td>Cylinder 2</td>
<td>70% Ethanol</td>
<td>1.0</td>
<td>30</td>
</tr>
<tr>
<td>Cylinder 3</td>
<td>80% Ethanol</td>
<td>1.0</td>
<td>30</td>
</tr>
<tr>
<td>Cylinder 4</td>
<td>95% Ethanol</td>
<td>1.0</td>
<td>30</td>
</tr>
<tr>
<td>Cylinder 5</td>
<td>100% Ethanol</td>
<td>1.0</td>
<td>30</td>
</tr>
<tr>
<td>Cylinder 6</td>
<td>100% Ethanol</td>
<td>1.0</td>
<td>30</td>
</tr>
<tr>
<td>Cylinder 7</td>
<td>Xylene</td>
<td>1.5</td>
<td>30</td>
</tr>
<tr>
<td>Cylinder 8</td>
<td>Xylene</td>
<td>1.5</td>
<td>30</td>
</tr>
<tr>
<td>Cylinder 9</td>
<td>Wax</td>
<td>1.0</td>
<td>60</td>
</tr>
<tr>
<td>Cylinder 10</td>
<td>Wax</td>
<td>2.0</td>
<td>60</td>
</tr>
<tr>
<td>Cylinder 11</td>
<td>Wax</td>
<td>2.5</td>
<td>60</td>
</tr>
</tbody>
</table>

2.2.3. H&E staining

Slides from each group were stained by haematoxylin and eosin (H&E) for general evaluation using the protocol described in Table 2.9.
Table 2.9  Detailed protocol for H&E staining

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Time (repeats)</th>
<th>Reaction</th>
</tr>
</thead>
<tbody>
<tr>
<td>100% Xylene</td>
<td>5 min (×3)</td>
<td>Dewax</td>
</tr>
<tr>
<td>100% Ethanol</td>
<td>5 min (×2)</td>
<td>Hydrate</td>
</tr>
<tr>
<td>95% Ethanol</td>
<td>5 min (×1)</td>
<td>Hydrate</td>
</tr>
<tr>
<td>70% Ethanol</td>
<td>5 min (×1)</td>
<td>Hydrate</td>
</tr>
<tr>
<td>Tap water</td>
<td>5 min</td>
<td>Wash</td>
</tr>
<tr>
<td>Mayer’s haematoxylin</td>
<td>5 min</td>
<td>Nuclear stain (purple/blue)</td>
</tr>
<tr>
<td>Tap water</td>
<td>5 min</td>
<td>Wash</td>
</tr>
<tr>
<td>STWS</td>
<td>2 min</td>
<td>Bluing agent</td>
</tr>
<tr>
<td>Tap water</td>
<td>2 min</td>
<td>Wash</td>
</tr>
<tr>
<td>1% Eosin</td>
<td>1 min</td>
<td>Cytoplasm stain (pink)</td>
</tr>
<tr>
<td>70% Ethanol</td>
<td>1 min (×1)</td>
<td>Dehydrate</td>
</tr>
<tr>
<td>80% Ethanol</td>
<td>1 min (×1)</td>
<td>Dehydrate</td>
</tr>
<tr>
<td>95% Ethanol</td>
<td>1 min (×1)</td>
<td>Dehydrate</td>
</tr>
<tr>
<td>100% Ethanol</td>
<td>2 min (×2)</td>
<td>Dehydrate</td>
</tr>
<tr>
<td>50:50 Xylene and ethanol</td>
<td>3 min</td>
<td>Clear</td>
</tr>
<tr>
<td>100% Xylene</td>
<td>3 min (×3)</td>
<td>Clear</td>
</tr>
</tbody>
</table>

2.2.4.  IHC staining

IHC was used to characterise inflammation and neovascularisation reactions after SC and ME implantation of the scaffolds in rats. The protocol described in Table 2.10 was used in conjunction with the primary antibodies listed in Table 2.11. For each antibody and tissue sample, the IHC protocol was optimised for primary antibody dilution and secondary antibody detection system. The primary antibody dilution was optimised by testing serial dilutions i.e. 1:50, 1:100, 1:200, 1:400 with the suggested dilution on the antibody specification sheet being the starting point. The secondary antibody detection system tested was Mouse-on-Rat Horseradish Peroxidase (HRP)-Polymer (MRT511G, Biocare Medical, California, USA). Optimised IHC conditions for the primary antibodies are shown in Table 2.11.
Table 2.10  Detailed protocol for IHC staining using diaminobenzidine (DAB)

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Time (repeats)</th>
<th>Reaction</th>
</tr>
</thead>
<tbody>
<tr>
<td>100% Xylene</td>
<td>5 min (×3)</td>
<td>Dewax</td>
</tr>
<tr>
<td>100% Ethanol</td>
<td>5 min (×2)</td>
<td>Hydrate</td>
</tr>
<tr>
<td>95% Ethanol</td>
<td>5 min (×1)</td>
<td>Hydrate</td>
</tr>
<tr>
<td>70% Ethanol</td>
<td>5 min (×1)</td>
<td>Hydrate</td>
</tr>
<tr>
<td>1×PBS</td>
<td>5 min</td>
<td>Wash</td>
</tr>
<tr>
<td>0.2M Boric acid (pH 7.0)</td>
<td>10 min</td>
<td>Microwave MED-HIGH</td>
</tr>
<tr>
<td>1×PBS</td>
<td>5 min (×2)</td>
<td>Wash</td>
</tr>
<tr>
<td>3% Hydrogen peroxide (H₂O₂)</td>
<td>5 min</td>
<td>RT; in methanol</td>
</tr>
<tr>
<td>1×PBS</td>
<td>5 min (×2)</td>
<td>Wash</td>
</tr>
<tr>
<td>Block R solution</td>
<td>20 min</td>
<td>RT</td>
</tr>
<tr>
<td>1×PBS-Tween</td>
<td>5 min (×2)</td>
<td>Wash</td>
</tr>
<tr>
<td>Primary antibody</td>
<td>Overnight</td>
<td>RT</td>
</tr>
<tr>
<td>1×PBS</td>
<td>5 min (×2)</td>
<td>Wash</td>
</tr>
<tr>
<td>Second antibody</td>
<td>30 min</td>
<td>RT</td>
</tr>
<tr>
<td>1×PBS-Tween</td>
<td>5 min (×2)</td>
<td>Wash</td>
</tr>
<tr>
<td>Chromagen DAB</td>
<td>3 min</td>
<td>RT; 1 drop DAB + 1ml buffer</td>
</tr>
<tr>
<td>1×PBS</td>
<td>5 min (×2)</td>
<td>Wash</td>
</tr>
<tr>
<td>Counter staining</td>
<td>1 min</td>
<td>Haematoxylin</td>
</tr>
<tr>
<td>Dehydrate, clear and mount</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Table 2.11  Detail of primary antibodies used in IHC staining

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Origin</th>
<th>Target species</th>
<th>Isotype</th>
<th>Dilution</th>
<th>Supplier (Cat #)</th>
<th>Antigen Retrieval</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD 68</td>
<td>Mouse</td>
<td>Rat</td>
<td>IgG1</td>
<td>1:100</td>
<td>AbD Serotec (MCA341R)</td>
<td>None</td>
</tr>
<tr>
<td>CD 31</td>
<td>Mouse</td>
<td>Rat</td>
<td>IgG1</td>
<td>1:20</td>
<td>Novus (TLD-3A12)</td>
<td>Boric acid</td>
</tr>
</tbody>
</table>

2.2.5. Masson’s trichrome staining

For rat TM sections, Masson’s trichrome staining was performed to examine the morphology of collagen fibres using the Masson’s Trichrome Stain Kit (MST-8003; Maixin Bio Co., Ltd., Fuzhou, China) as per the manufacturer’s instructions.
2.2.6. **ABR**

ABR was performed using the Nihon Kohden Neuropack-μ Measuring Systems (MEB-9100, Nihon Kohden, Japan) in a soundproof room. Animals were anesthetised prior to testing. Platinum subdermal needle electrodes were inserted at the scalp vertex (active electrode), both mastoids (reference electrode), and at the nose tip (ground electrode). The test stimuli (click) with 0.1ms duration were presented through an insert earphone. Animals were presented with a stimulus intensity series from 90- to 0-dB sound pressure level (SPL) in 10dB decrements for rats and in 5dB for guinea pigs. A total of 512 responses were averaged in each series of stimuli over a 10ms analysis period. Thresholds were defined as the lowest intensity to elicit a reproducible ABR waveform with typical wave III or wave IV morphology.

2.2.7. **Statistical analysis**

Average values are presented as mean±standard error of the mean (sem). Healing rates determined by otoscopic observation were compared using the chi-square test. Statistical analysis for mechanical testing results, otomicroscopic scores, ABR and TM thickness was evaluated using one-way analysis of variance (ANOVA), whereas hearing recovery in each group over time was performed using multiple linear regression analysis. Statistical significance for all tests was defined as $p<0.05$. 
3. The biocompatibility of SF and ACS for tissue engineering in the ear*

*Shen, Y et al. The biocompatibility of silk fibroin and acellular collagen scaffolds for tissue engineering in the ear. *Biomed Mater* 2014; 9(1): 015015 (Appendix 1)
3.1. Introduction

TM perforations are a common presentation in communities worldwide. If left untreated, large and chronic perforations can result in significant morbidity such as hearing impairment, recurrent infections, otorrhea, and acquired cholesteatoma (Lindeman et al. 1987). Although approximately 80% of acute TM perforations undergo spontaneous closure (Farrior 1983), chronic perforations are common, require surgical grafting (e.g. myringoplasty) as a means of repair. Currently, autologous grafts such as temporalis fascia (gold standard), cartilage, and perichondrium are the most favoured materials. However, they are associated with donor site morbidity, increased operative time, a shortage of supply in revision cases, and cosmetic defects (Levin et al. 2009). In addition, a variety of allografts and xenografts have been studied as promising materials for TM repair. Nevertheless, these graft types are limited by possible infection risks and different biomechanical properties compared to the normal TM (Levin et al. 2009). Thus far, no material has gained universal preference, and a better alternative is sought.

Recent research developments in the fields of biomaterials and tissue engineering may provide alternatives for TM reconstruction (Teh et al. 2013). Biomaterials such as silk, collagen, chitosan, and AlloDerm have been investigated extensively (Levin et al. 2009; Teh et al. 2013). SF, derived from silkworms, is a promising bioscaffold in regenerative medicine, due to its favourable properties such as high strength, low antigenicity, and
controllable biodegradability (Altman et al. 2003). Our previous *in vitro* studies have shown that SFS has suitable cytocompatibility (Levin et al. 2010; Levin et al. 2012; Ghassemifar et al. 2010) and mechanical properties (Rajkhowa et al. 2011), and can successfully support the growth and proliferation of human TM keratinocytes. Collagen based biomaterials have also proven useful, owing to their natural origin and wound healing properties (Bunyaratavej & Wang 2001). Previous studies report the application of collagen film for TM repair; however, no satisfactory outcome was achieved, in part due to unfavourable collagen purification (Bonzon et al. 1995). Our group has also developed a bilayered non-cross-linked ACS, which was derived from porcine peritoneum. A scaffold that simultaneously possesses biocompatibility and efficacy characteristics would be attractive for regenerative medicine applications in the ear. It has been shown that host reactions are tissue and organ dependent following material implantation (Shive & Anderson 1997). Moreover, a material that has proven to be safe for one application may not be safe for another (Anderson & Langone 1999). Therefore, *in vivo* studies are required to assess the ME host reactions following the applications of different materials. Indeed, a comprehensive understanding of a material’s biocompatibility properties is essential before clinical application.

The objective of this study was to evaluate the *in vivo* safety and biocompatibility of SFS and ACS in a rat model in comparison to Gelfoam and paper patch, which are commonly used for otological procedures in
outpatient settings. More specifically, this study aimed to evaluate the safety of these materials in the SC tissues in rats by histology, and in the ME cavity by otoscopy and histology. Specific emphasis was placed on the comparative host tissue reaction to the different scaffold materials, focusing on inflammation response, scaffold degradation, neovascularisation, and evidence of any adverse effects.

### 3.2. Materials and methods

#### 3.2.1. Scaffolds

The SFS, ACS, Gelfoam and paper were obtained and treated as per Section 2.1.1. Gelfoam was supplied sterile in its original packaging while the other three materials were packaged and sterilised with ethylene oxide gas.

#### 3.2.2. Animals

Eighty-one male Sprague-Dawley rats weighing 250-400g were obtained from Shanghai Animal Centre (Chinese Academy of Science, Shanghai, China). The experiments were approved by the Animal Ethics Committee of The University of Western Australia (Australia) and Zhejiang University (China). Experiments were performed in accordance with the Australian Code of Practice for the Care and Use of Animals for Scientific Purposes (2004). Animals were housed in the experimental animal facility of Zhejiang University under specific pathogen free conditions in a controlled environment (25°C, 12h light/dark cycle, 10-15 air exchange per hour, 30-70% relative humidity) and provided with food and water *ad libitum*. 
3.2.3. Experimental design

This experiment consisted of two separate studies: SC and ME implantation. In the SC implantation study, the rats (n=36) were randomly divided into four treatment groups according to the materials implanted: SFS, ACS, paper, and Gelfoam. Postoperatively, three rats from each group were sacrificed for histology on weeks 8, 12, and 26. The ME implantation study (n=45) consisted of five treatment groups with SFS, ACS, paper, Gelfoam ME implants through a myringotomy, and control (myringotomy without implant). Prior to experimentation, both ears of the rats in this study were inspected with an otomicroscope (S5, Carl Zeiss, Germany) to exclude ME disease. Three rats from each group were selected randomly for otoscopy and histology on weeks 2, 4, and 12 post-surgery.

3.2.4. Surgical procedures

3.2.4.1. SC implantation

All the surgical procedures were performed under general anaesthesia with intramuscular ketamine (80mg/kg) and medetomidine (0.5mg/kg). Prior to operation, the skin above the surgical area was shaved and disinfected with povidone iodine solution. Under sterile conditions, the various materials (10 × 10mm) were inserted into SC pockets on the mid-line dorsal region of each rat. The skin wounds were closed using 6-0 prolene sutures (Ethicon, NJ, USA). After the operation, all animals were given buprenorphine (0.02-0.08mg/kg) for postoperative analgesia. All procedures were performed by one surgeon to reduce variations in surgical technique.
3.2.4.2. **ME implantation**

Animals were anesthetised as described above. Following the removal of debris, the ear canals were sterilised with povidone iodine solution. Under aseptic conditions, a myringotomy incision was created in the posterior PT of the right TM using a sickle knife (Medtronic Xomed Inc. MN, USA). With the TM flap elevated, the materials, cut into small strips (1.5mm width × 20mm length), were gently inserted into the ME cavity, and the TM replaced. During surgical procedures, the mucosa of ME cavity was kept intact. The volume of implanted material was standardised for each animal except the control group.

3.2.5. **Otoscopic observation**

To investigate TM healing post-implantation, the animals in the ME implantation study were examined weekly using a digital video otoscope (MedRX, Largo, FL) under general anaesthesia. The TMs were observed with respect to TM perforation closure, signs of infection, myringosclerosis, and granulation tissue. Digital images were recorded using Aurisview software (Ear Science Institute Australia, Subiaco, Australia).

3.2.6. **Tissue harvest**

The animals were euthanised by intraperitoneal administration of pentobarbitone (160mg/kg) and death was confirmed by lack of heartbeat and pain response. In the SC implantation study, the implants were removed with surrounding SC tissues. Tissue specimens were rinsed with 0.1M PBS and fixed in 10% neutral buffered formalin for 24 h. Similarly, for the ME
implantation study, temporal bones were immediately harvested from the right ears of each animal following euthanasia. Specimens were fixed in 10% neutral buffered formalin for 24h and decalcified in 10% EDTA solution (pH 7.4). All tissues were then prepared for histology as per Section 2.2.2.

3.2.7. Histological evaluation

Three serial sections of each sample were stained with H&E for histological examination as per Section 2.2.3. Stained slides were digitally scanned using an Aperio ScanScope XT automated slide scanner (Aperio Technologies Inc., Vista, CA). The tissue response adjacent to the implants was evaluated for the presence of inflammatory cells (polymorphonuclear cells, lymphocytes, plasma cells, macrophages, and multinucleated giant cells), fibrosis, haemorrhage, necrosis, and neovascularisation using a semi-quantitative scoring system as described in International Organisation for Standardisation (ISO) 10993-6 (ISO10993-6). The intensity of inflammation was graded using a zero to four scale according to the number of cells per field of view (0=0 cells, 1=rare, 1-5 cells, 2=5-10 cells,3=heavy infiltrate, and 4=densely packed infiltrate). Other parameters such as fibrosis and neovascularisation were assessed using scoring scale ranging between zero and four (0=absent, 1=minor, 2=mild, 3=moderate, 4=extensive). The evaluation was performed by two independent examiners who were blinded to the experimental group. Three sections from each sample were randomly selected and evaluated. The average score value of each parameter was utilised for comparison.
3.2.8. IHC

Blood vessels and macrophages were immunohistochemically stained (Section 2.2.4) in two histological sections at the final time point of both studies. Following rehydration, the sections were treated by 3% H₂O₂ in methanol for 5 min to block endogenous peroxidase, and incubated in Rodent Block R (Biocare Medical, California, USA) for 20 min at RT. The sections were then incubated with an anti-rat monoclonal CD68 antibody (ED1, AbD Serotec, Oxford, UK; 1:100) or an anti-rat CD31 antibody (Novus Biologicals, Cambridge, UK; 1:20) at RT overnight to detect macrophages and vessel endothelial cells, respectively. Negative control sections received only diluents in place of the primary antibody. At the second day, secondary antibody, Mouse-On-Rodent HRP-polymer (Biocare Medical, California, USA) was applied to the sections for 30 min at RT. Diaminobenzidine was applied for 5 min and then the sections were counterstained with haematoxylin.

3.2.9. Statistical analysis

Statistical analysis was performed using the Prism software package (GraphPad Software 5.03, La Jolla, USA). All data were expressed as mean±sem and analysed by one-way analysis of variance followed by Tukey post hoc comparisons. Data were considered statistically significant at a level of p<0.05.
3.3. Results

3.3.1. Tissue response to SC implantation of the scaffolds

3.3.1.1. Histological evaluation

All animals survived the experiments with no infections or other postoperative complications (e.g. vestibular dysfunction) observed. The tissue response adjacent to the implants was evaluated. No signs of haemorrhage or necrosis at the implantation site were observed in any of the groups at any time point.

In the paper group, the SC tissue response was characterised by high cellularity and neoangiogenesis at 8 and 12 weeks following implantation. Large numbers of inflammatory cells, predominantly multinucleated giant cells and macrophages, were observed within and surrounding the paper remnants, reflecting a chronic granulomatous response (Figures 3.1 A and B). By 26 weeks, the granulomatous tissue was still present at the implantation bed, with increased lymphocytic infiltration and capillary formation around the unresorbed paper fragments (Figure 3.1 C).

Gelfoam was completely resorbed in all three samples at 8 weeks. In areas where the Gelfoam had been implanted, a vessel-rich granulation tissue was evident, containing numerous activated fibroblasts and occasional macrophages (Figure 3.1 D). Over a 26 week period, the number of capillaries and fibroblasts gradually diminished, returning to normal structure in all three samples (Figures 3.1 E and F).
ACS maintained its membrane integrity at 8 weeks post-surgery, with cellular ingrowth, mainly fibroblasts with a few macrophages, evident at both sides of the ACS membrane (Figure 3.1 G). At 12 weeks, more cells had penetrated to the central regions of the membrane, with obvious angiogenesis (Figure 3.1 H). At week 26, ACS was found to have integrated into the implantation bed, forming vascularised CT (Figure 3.1 I). Scant multinucleated giant cells and lymphocytes were observed within the ACS fibres throughout the study.

SFS implants demonstrated an indolent tissue response characterised by fibrous capsule formation and scant inflammatory cells. At 8 weeks, fibrous capsule surrounded the implants, with numerous well-oriented fibroblasts and occasional macrophages (Figure 3.1 J). The capsules became thinner but denser with time, and a few capillaries formed around the capsules (Figures 3.1 K and L). Silk degradation was not observed over this 26 week period.

3.3.1.2. Immunohistochemical evaluation

Using CD68 staining, we confirmed the presence of macrophages in the SC tissues 26 weeks postoperatively. The macrophages were predominantly infiltrated within and surrounding the paper residue (Figure 3.2 A). By contrast, there were scant macrophages observed in the other groups at this time point (Figures 3.2 B-D). We also evaluated the effect of scaffolds on neovascularisation by CD31 staining. At 26 weeks following paper
implantation, there were broad band of capillaries in the peri-implant space (Figure 3.3 A), and this appeared more than other material groups, which present only a few vessels in the peri-implant region (Figures 3.3 B-D).

3.3.1.3. Semi-quantitative evaluation of adjacent tissue histology

Implantation of paper resulted in some polymorphonuclear cell infiltration; however, this was not significant \((p>0.05)\) compared to the other groups at 8, 12, and 26 weeks (Figure 3.4 A). By contrast, there were significantly \((p<0.05)\) more lymphocytes, macrophages, and multinucleated giant cell infiltration in paper compared to Gelfoam, ACS, and SFS at each time point (Figures 3.4 B-D). Compared with Gelfoam, there were statistically \((p<0.05)\) more macrophages in ACS and SFS and more multinucleated giant cells in ACS at 12 weeks (Figures 3.4 C and D). Neovascularisation was similar \((p>0.05)\) among the four experimental groups at 8 and 12 weeks; however, paper resulted in significantly \((p<0.05)\) more neovessels than did Gelfoam at 26 weeks (Figure 3.4 E). Fibrous encapsulation produced significantly \((p<0.05)\) higher fibrosis values for SFS compared to Gelfoam and ACS at week 8, and all other groups at week 12 and 26 (Figure 3.4 F). Paper showed statistically \((p<0.05)\) higher fibrosis compared to Gelfoam at week 12 and 26, and ACS at week 26 (Figure 3.4 F).

3.3.2. Tissue response to ME implantation of the scaffolds

3.3.2.1. Otoscopic observation

Otoscopically, the myringotomy site in the Gelfoam, ACS, SFS, and control groups healed within two weeks after surgery. These healed TMs were
transparent at week 12, and no evidence of persistent perforation and infection such as hyperaemia or otorrhea was observed (Figures 3.5 G-J). In contrast, six of nine ears (66.7%) in the paper group presented with persistent perforation of the TM accompanied with congestion and serous otorrhea in two ears each at 2, 4, and 12 weeks, respectively (Figure 3.5 F).

### 3.3.2.2. Histological evaluation

The ME tissue response to the paper implants indicated a similar pattern to that observed after SC implants. This was characterised by infiltration of numerous inflammatory cells, primarily multinucleated giant cells, macrophages, and lymphocytes, within and around the paper residua throughout the study. In addition, there was moderate submucosal oedema in all samples during 12 weeks follow-up (Figures 3.6 A-C). At 12 weeks, non-healing TMs were characterised by the squamous epithelial layer migrating over the wound edge to meet the mucosal layer (Figure 3.7 B). There was no evidence for the keratin spur formation that was associated with normal healing.

ME cavities of the Gelfoam-treated group had evidence of a moderate inflammatory response infiltrating the Gelfoam remnant at 2 weeks, consisting of a few macrophages and scant multinucleated giant cells (Figure 3.6 D). At 4 weeks, the inflammation was replaced by moderate fibrosis with a great number of fibroblasts surrounding the Gelfoam remnants. Osteoneogenesis was seen in some Gelfoam remnants (Figure 3.6 E). By 12
weeks, Gelfoam was completely resorbed, but the mucosa was still thickened by prominent submucosal fibrosis and scattered macrophage infiltration in the ME cavities (Figure 3.6 F).

In the ACS group, an ingrowth of fibroblasts and macrophages was evident within the pores of ACS 2 weeks post-implantation (Figure 3.6 G). At week 4, the cellular ingrowth continued into the central region of the implanted ACS, with mild submucosal fibrosis also observed (Figure 3.6 H). At week 12, the ACS remnants were not readily visible and scarce fibroblasts and macrophages were observed in the ME cavities. The mucosal layer was normal with submucosal fibrosis reduced (Figure 3.6 I). There was no osteoneogenesis and only rare multinucleated giant cells and lymphocytes were observed throughout.

SFS resulted in an indolent tissue reaction in the ME cavity, similar to that observed in SC implantation. A fibrous capsule enveloped the implant at an early stage (week 2), with numerous well-oriented fibroblasts in the capsule and occasional macrophages in the interface between the host tissue and SFS. There was mild submucosal fibrosis at week 12, without visible inflammation. No signs of silk degradation or osteoneogenesis were observed during follow up examinations (Figures 3.6 J-L).

In the control group, no inflammation was found in the MEs. An increase in the thickness of the mucosal layer resembling submucosal oedema was noted
at 2 and 4 weeks postoperatively (Figures 3.6 M and N). However, this thickening resolved in all samples at 12 weeks (Figure 3.6 O).

### 3.3.2.3. Immunohistochemical evaluation

In the ME tissues, the extent of macrophages and neovascularisation were similar to that observed in the SC tissues. At 12 weeks, there were pronounced CD68 positive cells surrounding the paper remnant and also infiltrated within the thickened mucosal layer of the ME cavity (Figure 3.8 A). In the Gelfoam group, there were a few CD68 positive cells observed (Figure 3.8 B). However, only rare macrophages were present in the ACS and SFS groups, similar to the control group (Figures 3.8 C and D). For neovascularisation, 12 weeks after implantation, there were few vessels found in the peri-implant space and mucosal layer in all material groups, and the scaffolds were minimally vascularised (data not shown).

### 3.3.2.4. Semi-quantitative evaluation of adjacent tissue histology

There were significantly \((p<0.05)\) more macrophages and multinucleated giant cells infiltrating the paper compared with that of Gelfoam, ACS, and SFS at 2, 4, and 12 weeks (Figures 3.9 C and D). Similarly, significantly \((p<0.05)\) more lymphocytes were present in paper than all other groups at individual time points except at week 2 (Figure 3.9 B). However, no statistical differences \((p>0.05)\) were observed in polymorphonuclear cells, neovascularisation, and fibrosis among the four groups at each time point (Figures 3.9 A, E and F).
Figure 3.1 Representative histological sections of tissue surrounding (A-C) paper, (D-F) Gelfoam, (G-I) ACS, and (J-L) SFS at 8, 12, and 26 weeks after SC implantation (×200)

CT, connective tissue; V, vessels; FC, fibrous capsule. Asterisks indicate scaffold residua; arrowheads indicate multinucleated giant cells. H&E staining. Scale bars: 50µm.
Figure 3.2  Immunohistochemical staining for macrophages (CD68) in SC tissue surrounding (A) paper, (B) Gelfoam, (C) ACS, and (D) SFS 26 weeks after SC implantation (×200)

CT, connective tissue; FC, fibrous capsule. Scale bars: 40µm.

Figure 3.3  Immunohistochemical staining for blood vessels (CD31) in SC tissue surrounding (A) paper, (B) Gelfoam, (C) ACS, and (D) SFS 26 weeks after SC implantation (×400)

Arrows indicate blood vessels. Scale bars: 20µm.
Figure 3.4 Comparisons of tissue response of each group using semi-quantitative histological evaluation 8, 12, and 26 weeks after SC implantation

(*p<0.05, compared with paper group; #p<0.05, compared with Gelfoam group; +p<0.05, compared with ACS group). Data represent mean±sem.

Figure 3.5 Otoscopic images of the rat TMs
Upper row demonstrates (A) paper, (B) Gelfoam, (C) ACS and (D) SFS in the ME cavities following implantation and (E) control TM (without implantation). The arrows in (A-D) indicate the implanted materials, and the asterisk in (E) indicates the myringotomy incision. Lower row shows unhealed TM in the (F) paper group with otorrhea (arrowhead) and healed TMs in the (G) Gelfoam, (H) ACS, (I) SFS and (J) control groups at 12 weeks after implantation.
Figure 3.6 Representative histological sections of tissue surrounding (A-C) paper, (D-F) Gelfoam, (G-I) ACS, (J-L) SFS, and (M-O) control 2, 4, and 12 weeks after ME implantation (×200)

Bone, temporal bone; M, ME mucosa; FC, fibrous capsule; f, fibrous tissue. Asterisks indicate scaffold residua; arrowheads indicate multinucleated giant cells. H&E staining. Scale bars: 50µm.
Figure 3.7  Histology of the rat TM 12 weeks after ME paper packing

Numerous macrophages and multinucleated giant cells (asterisks) are seen in and around residual paper fragments (arrows) (A, ×50). The squamous epithelial layer (black arrowheads) abuts the mucosal layer (outlined arrowheads) at the wound edge (B, ×200). EAC, external auditory canal; ME, middle ear; m, handle of malleus. H&E staining. Scale bar: 200µm (A); and 50µm (B).

Figure 3.8  Immunohistochemical staining for macrophages (CD68) in ME tissue surrounding (A) paper, (B) Gelfoam, (C) ACS, (D) SFS, and (E) control 12 weeks after ME implantation (×200)

Bone, temporal bone; M, ME mucosa. Scale bars: 40µm.
Figure 3.9  Comparisons of tissue response to ME implantation for each group using semi-quantitative histological evaluation after 2, 4, and 12 weeks (*\(p<0.05\), compared with paper group; \(^{\#}p<0.05\), compared with Gelfoam group; \(^{\sim}p<0.05\), compared with ACS group). Data represent mean±sem.

3.4.  Discussion

This study investigated the \textit{in vivo} biocompatibility of SFS and ACS for ear applications compared with commonly used Gelfoam and paper, as controls, using SC and ME implantation models in rats. Our results revealed that SFS and ACS were well tolerated and compatible in rat SC and ME tissues throughout the study. Histological and otoscopic evaluation demonstrated that SFS and ACS invoked a milder tissue response with less inflammation compared to that of paper \textit{in vivo}. Gelfoam presented similar results to SFS and ACS after SC implantation; however, it was observed to be associated with pronounced fibrosis and osteoneogenesis following ME implantation. Therefore, this observation indicated that compared to Gelfoam and paper, both SFS and ACS were more biocompatible and may be preferred alternatives for tissue engineering in the ear.
Biocompatibility, an essential requirement for biomaterial applications in tissue engineering, is defined as ‘the ability of a material to perform with an appropriate host response in a specific situation’ (Williams 2008). The biocompatibility of a biomaterial is dependent upon multiple intrinsic and extrinsic factors (Katti et al. 2002; Říhová 1996). The intrinsic factors comprise the materials’ chemical composition, physical characteristics (e.g. form, size, surface, density), and degradation products (Katti et al. 2002; Říhová 1996; Fournier et al. 2003). Among these, surface characteristics, such as topography, chemical structure, hydrophilicity, or hydrophobicity, play a major role in biocompatibility (Salthouse & Matlaga 1983; Owen et al. 2005). Scaffolds with rough surfaces and porous structure have been reported to improve cell adhesion and tissue ingrowth (Wang et al. 2006; Kim et al. 2005). In Chapter 4, evident differences in these scaffolds’ surface morphology had been shown by SEM and may be related, in part, to the variable tissue responses observed. In addition, extrinsic factors such as implant site and surgical/insertion technique are also important factors to consider (Říhová 1996; Fournier et al. 2003; Mokrý et al. 2000). We evaluated scaffolds specifically in the ME cavities, since host reactions may be tissue- and organ-dependent. Moreover, the findings will then be relevant should any of these scaffolds be targeted for therapeutic use. The difference in scores we observed between SC and ME implantation confirms this expected tissue reaction. Surgical procedures may impact on the local tissue response at the implant site at an early stage; thus the test periods of our study
also included early time points at 2 and 4 weeks after ME and SC implantation, to minimise such influences on evaluation.

Inflammatory response is also a significant factor to consider in the application of biomaterials, as it may lead to surgical failure (Zheng et al. 2005; Malcarney et al. 2005). The ACS and Gelfoam used in this study presented a mild and localised inflammatory response after SC and ME implantation. This response was transient as it resolved with scaffold absorption by the host. Similarly, SFS showed minimal inflammatory response with a thin fibrous capsule observed around the implants, resembling a typical chronic inflammatory reaction process. In contrast, paper revealed significantly more inflammatory cell infiltration consisting of numerous multinucleated giant cells, macrophages, and lymphocytes, more than any of the other scaffolds, indicating a foreign body reaction caused by persistent and vigorous stimulation of the immune system by the paper residua. Foreign body reaction is the combined response of the inflammatory and wound healing reactions following biomaterial implantation (Anderson 2001), and can impact the biocompatibility of implanted materials and the short- and long-term tissue responses (Anderson et al. 2008). In light of these data, this study suggests that the application of SFS and ACS would be suitable for tissue engineering in the ear owing to the minimal inflammatory response in vivo. The latter is similar to that seen with Gelfoam but significantly less than with paper.
The SFS implant induced an indolent tissue response at the SC and ME implant sites, with early fibrous encapsulation around the SFS and a mild inflammatory response. The capsule thickness gradually decreased with time, but no signs of capsule breakdown, tissue ingrowth, or scaffold degradation were seen throughout the study. As a result, SFS was isolated from the host immune response by capsule formation at an early stage, seemingly developing inert-like properties in the subsequent post-implantation phase. By contrast, ACS evoked an early mononuclear cellular inflammatory response following implantation, but no encapsulation response was noted during the study. Early cell penetration into the scaffold, mainly macrophages and fibroblasts, meant that ACS eventually integrated into the SC implantation bed at 26 weeks and resorbed in the ME cavity at 12 weeks. Therefore, there were significantly different behaviours between the two scaffolds used. SFS underwent fibrous capsule formation, whereas ACS was incorporated into fibrous tissue. These differences may indicate a material’s suitability for specific applications, for example as a TM graft, SFS may remain in the reconstructed TM and function as a long-term reinforcement, especially in the presence of ongoing ME disease or absence of a fibrous TM layer. By contrast, the porous ACS may be incorporated into the neomembranes and allow host cellular and CT ingrowth and remodelling as is seen with some autografts. SFS and ACS had a similar extent of peri-implant neovascularisation in our study, and only scant multinucleated
giant cell infiltration was seen at the earliest stages of the study, indicating both scaffold types were compatible and devoid of a foreign body reaction.

The SFS used in this study was a formic acid-based SF processed from the domestic silkworm *B. mori*. The antigenic silk sericin protein had been removed following degumming (Rajkhowa et al. 2011), as it has been shown to elicit an adverse immune response after implantation (Soong & Kenyon 1984; Zaoming et al. 1996). Our previous *in vitro* studies have demonstrated that this sericin-deprived fibroin scaffold is cytocompatible and can successfully support the growth and proliferation of human TM keratinocytes (Levin et al. 2010; Levin et al. 2012; Ghassemifar et al. 2010). The current study has extended this previous research showing the *in vivo* biocompatibility of SFS at SC and ME tissues, two structurally and functionally different sites. Additionally, the experimental time frame of this study has been extended to address the potential for any long-term effects. Our results are consistent with previous studies, which showed only minimal inflammation following SC implantation of SF in rats, despite differences in silk processing conditions and observation period (Zhou et al. 2010; Lee et al. 2012; Meinel et al. 2005). The results of this study also demonstrated good biocompatibility of SFS with surrounding tissues in the ME of the rats, similar to that observed in the SC region. To our knowledge, this is the first report to show specific ME biocompatible properties of SFS designed for TM applications.
Similarly, we also observed that ACS presented a well-tolerated response in the SC and ME. The ACS used in this study consists of type I and type III collagen processed from porcine peritoneum and decellularised to remove xeno-DNA and xenocellular components which may cause antigenic reactions. The ACS is not cross-linked, which has been shown to inhibit the attachment and proliferation of human fibroblasts and osteoblasts in vitro (Rothamel et al. 2004), and has been associated with a severe foreign body reaction in vivo (Rothamel et al. 2005). We observed minimal inflammation after ACS implantation and the scaffold became well integrated into the SC implantation bed and supported complete neovascularisation during healing. Similar histological findings were reported recently by Ghanaati et al (Ghanaati et al. 2011; Ghanaati 2012). They showed favourable tissue reactions using a similar collagen product following implantation in a SC murine model and in human oral tissues. Furthermore, they found different tissue reactions due to differences in the surface morphology of this bilayered collagen scaffold. Although our study did not focus on such scaffold-tissue interface, similar studies are currently under way.

In this study, we chose Gelfoam as a control since this Food and Drug Administration (FDA) approved product is the most commonly used biomaterial in otological surgery worldwide (Shen et al. 2011). Our results indicate that Gelfoam evoked mild inflammation with increased vascularisation and fibroblast proliferation after SC and ME application. In the SC tissues, Gelfoam was relatively quickly resorbed and the adjacent
tissues returned to a normal appearance at an early stage. However, in the ME cavity, we noted evidence of osteoneogenesis in one rat at 8 weeks, and the ME mucosa was thickened by fibroblast infiltration and fibrous tissue formation at 12 weeks. These findings are similar to previous studies (Doyle-Kelly 1961; Hellstrom et al. 1983), and probably explained Gelfoam associated fixation of ME ossicles and TM which resulted in subsequent suboptimal hearing outcomes (Shen et al. 2011).

Furthermore, we used cigarette paper as an additional control. Paper is commonly used as a scaffold to repair traumatic TM perforations in outpatient settings. The histological results demonstrated that paper elicited a stronger inflammatory response than any of the other scaffolds in both SC and ME tissues, with a moderate foreign body reaction seen throughout. In the ME, we found persistent TM perforation with serous otorrhea in six of nine rats, likely due to the incorporation of paper fragments into the perforation edge and the subsequent inflammatory response resulting in delayed wound healing of the TM. Although paper patch has been utilised in clinical settings for decades (Merwin & Boies Jr 1980), no such in vivo study has been performed to assess its biocompatibility so far. Only Altuntas and Sümer (Altuntaş & Sümer 2013) reported relatively higher biocompatibility of cigarette paper than carbon paper under in vitro conditions. The present findings indicate that care needs to be taken when paper is used for TM applications. We further suggest that surgeons should avoid using paper in the ME.
Further studies in this area would benefit from including hearing function outcomes following ME implantation. Throughout the study, although cochlear toxicity remains unclear, there was no gross evidence of vestibular injury observed with any of these materials. Since alternative biomaterials for tissue engineering in the ear should be simultaneously safe and efficacious, further studies in other experimental models (e.g. guinea pigs) are warranted before clinical application. In addition, only Gelfoam was completely resorbed during the time frame of this study. Hence, studies with longer duration would be beneficial to fully evaluate the biological response of these materials. This preliminary information on biocompatibility of SFS and ACS will contribute to the design and optimisation of these scaffolds for specific otological applications.

In summary, this study demonstrated the biocompatibility characteristics of SFS and ACS in SC and ME tissues of rats. Our results showed that both SFS and ACS were well tolerated by the host and produced therapeutically accepted tissue reactions, including minimal inflammation, evident neovascularisation, and scaffold degradation. Therefore, these results suggest that SFS and ACS both are biocompatible and safe scaffolds and could serve as potential alternatives for tissue engineering in the ear.
4. Scaffolds for TM regeneration in rats*

4.1. Introduction

TM perforation is one of the most common problems in otolaryngology. If left untreated, it is associated with significant morbidity such as hearing loss, recurrent otorhhea, ME infection, and acquired cholesteatoma (Parekh et al. 2009). Although most acute TM perforations heal spontaneously, large or chronic TM perforations, especially from chronic suppurative OM, often fail to heal and may require grafting (Lindeman et al. 1987).

Currently, surgical methods such as myringoplasty are regarded as the most effective and reliable treatment for TM perforations (Sheehy & Anderson 1980; Karela et al. 2008). Various autologous grafts and allografts such as muscle fascia, cartilage, perichondrium, and AlloDerm have been used; however, all have their own limitations (Levin et al. 2009). For instance, temporalis fascia, which is regarded as the ‘gold standard’, is associated with donor site morbidity, additional incisions, long operation time, and a shortage of material in revision cases (Levin et al. 2009). AlloDerm, derived from human cadaver donor skin, is almost 10 times thicker than the normal TM, and its use is limited by the shortage of suitable human donors (Spiegel & Kessler 2005). To date, a range of xenografts and synthetic materials, including Gelfoam (Abbenhaus 1978), paper patch (Golz et al. 2003), and HA derivatives (Teh et al. 2012), have been investigated as suitable scaffolds that support the regeneration of TM. However, little evidence supports any of these as optimal materials for various types of perforations (Aggarwal et al. 91).
Moreover, several commercially available xenografts such as porcine SIS may evoke an inflammatory response due to the remnant xenocellular components, including serotonin (Zheng et al. 2005; Gilbert et al. 2009). In addition, many synthetic materials are nonbiodegradable, and their biomechanical and material properties are different compared with the normal TM, which may affect the long-term hearing function (Levin et al. 2009). Hence, there is a constant search for better materials to achieve improved healing and hearing.

Recently, with the advances in materials for tissue engineering, various alternative biomaterials have been developed as artificial eardrums for TM repair; these include silk (Kim et al. 2010a), collagen (Bonzon et al. 1995; Hakuba et al. 2010), chitosan (Kim et al. 2010b), and calcium alginate (Hott et al. 2004). SF, a protein polymer obtained from silkworm silk, possesses ideal properties such as biocompatibility, biodegradability, high tensile strength, and elasticity (Altman et al. 2003; Weber et al. 2006). More importantly, human TM keratinocytes have been shown to adhere and proliferate successfully on SF membranes in vitro (Levin et al. 2010; Ghassemifar et al. 2010). Collagen, a major ECM component, also has desirable physical characteristics such as high tensile strength, flexibility, nonreactivity, nontoxicity, and noncarcinogenicity (Patterson 1967; Abbenhaus 1978). As the main constituent of the lamina propria of the TM, collagen helps in maintaining the resilience and integrity of the TM and, hence, plays a key role in hearing (Knutsson et al. 2007; Knutsson et al.
The biocompatibility of both SFS and ACS in SC and ME tissues of rats was demonstrated in Chapter 3, indicating their potential use as a scaffold for TM perforation repair. However, only limited studies on silk and collagen as TM scaffolds have been performed, and the hearing outcomes for these scaffold materials have yet to be investigated. Hence, the aim of this study was to evaluate the efficacy (healing and hearing outcomes) of SFS and porcine-derived ACS as scaffolds for TM regeneration compared with more commonly used materials such as paper patch and Gelfoam in an acute TM perforation rat model.

4.2. Materials and methods

4.2.1. Description of scaffolds

Scaffolds used in this study include SFS, ACS, paper and Gelfoam. Full descriptions can be found in Section 2.1.1.

4.2.2. Animal models

The experimental protocols were approved by both the Animal Ethics Committees at The University of Western Australia (Australia) and Zhejiang University (China). One hundred and sixty male Sprague-Dawley rats, weighing 250-300 g, were obtained from Shanghai Animal Centre (Chinese Academy of Science, Shanghai, China). All rats were housed in the experimental animal facility of the Zhejiang University and were provided with food and water ad libitum in a room with 12-h light/dark cycles. Before the study, all animals were inspected using a S5 model otomicroscope (Zeiss)
to ensure they were free of ME pathology. A total of 150 rats were randomly divided into four scaffold repair groups, namely SFS (n=30), ACS (n=30), paper patch (n=30), Gelfoam (n=30), and control (spontaneous healing) (n=30). In addition, a group of 10 rats (n=10) were allocated as normal controls (without any perforation or scaffold).

All the surgical procedures were performed under general anaesthesia with intramuscular ketamine (80mg/kg) and medetomidine (0.5mg/kg). Debris from the external auditory canal (EAC) was removed using a 3.0mm aural speculum, and the EACs were prepped with povidone iodine solution. Bilateral TM perforations, measuring approximately 1.8mm in diameter, were created using a sterile 23-gauge needle in the posterior half of the PT via a transcanal approach. Four different materials were then trimmed into pieces (2.4mm in diameter), rinsed with 1× PBS solution (pH 7.4) (Invitrogen), and grafted onto the right TM perforation using onlay myringoplasty. Ofloxacin ointment (Tarivid® eye ointment; Santen Pharmaceutical Co., Ltd.) was applied to the margin of the scaffolds to maintain the scaffolds in place. For ACS, the rough surface was placed medially so that the smooth surface faced towards the EAC. The left ear served as an internal control where no graft material was placed on the perforated TM. All rats were given SC buprenorphine (0.02-0.08mg/kg) for postoperative analgesia.
Healing of the right ear TM in different treatment groups was evaluated by otoscopy, SEM, histology, and transmission electron microscopy (TEM); while the hearing function was analysed by ABR. Left ears were not evaluated. In each group, five rats (n=5) were selected randomly for both otoscopic and ABR assessment at 3, 5, 7, 9, 14, and 28 days postoperatively. In these subgroups of five rats, three were used for histological evaluation, and one each was used for SEM and TEM.

4.2.3. Otoscopic observation

To investigate TM healing, five rats from each group were randomly chosen at each time point for otoscopic observation using a digital video otoscope (MedRX, Largo, FL) under general anaesthesia. The TMs were viewed by two independent observers with regard to perforation closure, infection, myringosclerosis, granulation tissue, and thickening. Each TM perforation was graded as either completely closed or unclosed. Only TMs that had completely closed were considered healed. Digital images were recorded using Aurisview software (Ear Science Institute Australia, Subiaco, Australia).

4.2.4. SEM

SEM was performed to characterise the surface morphology of four individual scaffolds. Briefly, the scaffold samples were sputter coated with 5nm thick platinum (SEM coating unit, E 1020; Hitachi Science Systems Ltd.), and both sides were viewed under a scanning electron microscope (S260; Leica) at a low voltage (20kV).
In addition, SEM was performed to evaluate the healing process of TM after repair by scaffolds. Briefly, the rat TM specimens were fixed with 2.5% glutaraldehyde in 4°C overnight, dehydrated in ethanol solutions followed by critical point drying (HCP-2; Hitachi). Finally, the samples were coated with 5nm thick platinum, where the medial surface of the TMs was observed under SEM.

4.2.5. Histological evaluation

After sacrifice, right external ears were separated at the osteocartilaginous junctions, and the TMs along with the bony annulus were removed from the tympanic bulla. Harvested specimens were prepared for histology as per Section 2.2.2. All sections were evaluated using H&E staining as per Section 2.2.3. Masson’s trichrome staining (Section 2.2.5) was performed to examine the morphology of collagen fibres. All stained slides were digitally scanned using an Aperio ScanScope XT automated slide scanner (Aperio Technologies, Inc.; 40×/0.75 Plan Apo objective). Images were saved as SVS files for histological evaluation. TM thickness of healed TM sections on days 14 and 28 was measured using Aperio ImageScope Viewer software.

4.2.6. TEM

TEM was performed to investigate the microstructure of the healed TMs on day 28 postrepair. Briefly, after dissection, the perforation site of the harvested TM samples was fixed in 2.5% glutaraldehyde and stored overnight at 4°C. Tissue specimens were washed, postfixd (1% osmic acid for 1h at 4°C), dehydrated (graded alcohols), and embedded (epoxy resin) for
transmission observation. Thin transverse sections (120nm) were cut and examined with TEM (TECNAI 10, Philips Co.) at 80kV.

4.2.7. ABR

To assess the hearing of rats after grafting, ABR was performed using the Nihon Kohden Neuropack-μ Measuring Systems (MEB-9100; Nihon Kohden) in a soundproof room as per Section 2.2.6. Auditory thresholds of click stimuli were measured pre- and post TM perforation in the right ear of all rats, and at each time point after myringoplasty for the five animals from each group. The normal ears and the ears with TM perforation without materials served as controls.

4.2.8. Statistical analysis

Healing rates determined by otoscopic observation were compared using the chi-square test. Statistical analysis for ABR and TM thickness was evaluated using one-way analysis of variance, whereas hearing recovery in each group over time was performed using multiple linear regression analysis. All analyses were performed using the Statistical Software R (Version 2.11.1, package meta). Statistical significance was defined as $p<0.05$.

4.3. Results

4.3.1. Morphological characterisation of scaffolds

SEM of scaffolds before implantation showed that SFS was characterised by a smooth and nonporous surface (Figure 4.1 A). ACS had two distinct
surfaces: a smooth surface with compact collagen bundles (Figure 4.1 B) and a rough, porous surface with loose collagen fibres (Figure 4.1 C). Paper patches demonstrated an uneven surface topology with a few small pores (Figure 4.1 D). In contrast, Gelfoam showed substantial pores of varying sizes (Figure 4.1 E).

Figure 4.1 The surface morphology of scaffolds
SEM showed the surface morphology of four scaffolds (A-D; ×500, E; ×200). SFS was characterised by a smooth, dimpled, and nonporous surface (A). ACS possessed two distinct surfaces: a smooth surface featuring compact collagen bundles (B), and a rough, porous surface of loose collagen fibres (C). Paper patch surface was uneven with a few small pores (D). Gelfoam showed substantial pores of varying sizes (E). Scale bar: 500µm.

4.3.2. Promotion of TM regeneration induced by scaffolds

4.3.2.1. Otoscopic observation
An acute rat model of TM perforation was successfully established. All rats survived the surgical procedures with no complications postoperatively. The lateral aspect of TMs was observed via an otoscope to assess the effect of grafting at each time point. No signs of infection or abnormalities were observed in any of the rats.
In the control group, the TMs appeared thicker and opaque postperforation, with prominent microvessels visible close to the perforation margin. By 14 days, the TM became increasingly transparent, and majority of the perforations had fully closed. At 28 days, all the perforations were completely healed, although visible scars resembling an opalescent ring were observed at the perforation site (Figure 4.2). The transparency of SFS and semi-transparency of ACS allowed direct observation of the TM healing. Throughout the healing process, both SFS and ACS retained their structural stability and adhered well to the TM remnant. The opacification of TM and microvessels was less pronounced compared with those in the control group. The perforations had healed as early as 7 days after grafting where the healed TMs appeared normal (Figure 4.2). In contrast, paper patch and Gelfoam were opaque, making it difficult to examine the ME during healing. Moreover, these materials tended to detach easily from the healing TM. In particular, the bulk of Gelfoam shrunk, and its porous structure was lost over time. At 28 days, the TMs in the paper patch and Gelfoam groups appeared healed but with some scarring (Figure 4.2).
Figure 4.2  Otoscopic observation of TM healing after grafting

The lateral aspect of TMs was observed with otoscopy. The transparency of SFS (fully transparent) and ACS (semi-transparent) allowed direct observation of the TM healing, whereas the opacity of paper patch and Gelfoam resulted in difficulty with examination. SFS and ACS adhered well to the TM remnant throughout the healing process; however, paper patch and Gelfoam partially detached at 7 days. Microvessels were most prominent in the control group (3-14 days) compared with scaffold-treated TMs. Perforation closure of SFS and ACS was observed at 7 days compared with 9 days in the paper and Gelfoam groups and 14 days in the control group. Scar formation was visible at 28 days in the paper patch, Gelfoam, and control groups (white arrowheads). White arrows indicate scaffolds; asterisk indicates perforation.
Table 4.1  Healing of TM perforation at different time points after grafting

<table>
<thead>
<tr>
<th>Group</th>
<th>3 days (n=5)</th>
<th>5 days (n=5)</th>
<th>7 days (n=5)</th>
<th>9 days (n=5)</th>
<th>14 days (n=5)</th>
<th>28 days (n=5)</th>
</tr>
</thead>
<tbody>
<tr>
<td>SFS</td>
<td>0/5 (0.0%)</td>
<td>1/5 (20.0%)</td>
<td>4/5 (80.0%)*</td>
<td>5/5 (100%)*</td>
<td>5/5 (100%)*</td>
<td>5/5 (100%)</td>
</tr>
<tr>
<td>ACS</td>
<td>0/5 (0.0%)</td>
<td>0/5 (0.0%)</td>
<td>3/5 (60.0%)*</td>
<td>5/5 (100%)*</td>
<td>5/5 (100%)*</td>
<td>5/5 (100%)</td>
</tr>
<tr>
<td>Paper patch</td>
<td>0/5 (0.0%)</td>
<td>0/5 (0.0%)</td>
<td>2/5 (40.0%)</td>
<td>5/5 (100%)*</td>
<td>5/5 (100%)*</td>
<td>5/5 (100%)</td>
</tr>
<tr>
<td>Gelfoam</td>
<td>0/5 (0.0%)</td>
<td>0/5 (0.0%)</td>
<td>0/5 (0.0%)</td>
<td>3/5 (60.0%)*</td>
<td>5/5 (100%)*</td>
<td>5/5 (100%)</td>
</tr>
<tr>
<td>Control</td>
<td>0/5 (0.0%)</td>
<td>0/5 (0.0%)</td>
<td>0/5 (0.0%)</td>
<td>2/5 (40.0%)</td>
<td>4/5 (80.0%)</td>
<td>5/5 (100%)</td>
</tr>
</tbody>
</table>

*p<0.05, statistically significant difference between control (spontaneous healing) and the other scaffold.

After sacrifice at individual time points, closure of the perforation was confirmed by observing the internal surface of the harvested TMs using an otomicroscope. TM healing in the SFS and ACS groups was markedly quicker compared with the other groups (Table 4.1). In fact, one TM in the SFS group had healed as early as day 5. At 7 days, TMs had healed in 80% (4/5) of SFS-treated ears and in 60% (3/5) of ACS-treated ears; while none had healed in the control group (0/5) (*p<0.05). After 9 days, the TM was completely healed in all five rats in the SFS, ACS, and paper patch groups, which was significantly different compared with the control group (2/5) (*p<0.05). At 14 days, all ears were completely healed except one TM in the control group (4/5). By 28 days postsurgery, all the TMs had completely healed.
4.3.2.2. **SEM**

The medial aspect of TMs was observed with SEM to assess scaffold attachment, cellular integration with scaffold, and perforation closure (Figure 4.3 A). The SFS and ACS showed steady attachment to the perforation margin throughout the healing process, thereby preserving their scaffold function. TM epithelial cells migrated across the wound margin and adhered to the internal surface of SFS on day 3 (Figure 4.3 B-a) and to ACS on day 5 (Figure 4.3 B-b). By 9 days, the TMs of both groups had healed, and the internal surface of neo-membranes was smooth. In contrast, paper patch demonstrated early partial detachment from the TM surface, and its scaffold function was partially lost. Exudate formation and inflammatory cell infiltration were evident at the perforation site in the paper group (Figure 4.3 B-c). Gelfoam showed early disintegration of its sponge structure (Figure 4.3 B-d). As shrinkage and absorption progressed, most of the Gelfoam dissolved, resulting in loss of its support function. The healed TMs in paper and Gelfoam groups showed some scarring at 14 days. In the control group with no scaffold implantation, a rolled perforation edge of the unhealed TM was visible at 9 days (Figure 4.3 B-e). The TM eventually healed by 14 days, but with an obvious scar.

4.3.2.3. **Histological evaluation**

The histology of the TM healing and effects of the four scaffolds were examined over 28 days (Figure 4.4). Compared with other groups, TM healing in the control group was relatively slower. In the first week, the
perforation remained patent, although hyperplasia was observed in the epithelial and CT layers of the TM. On day 5, a keratin spur was seen, and the perforations started closing at 9 days with significant thickening throughout the three TM layers. By 28 days, the healed TM became thinner but with residual thickening at the previous perforation site. The CT layer was found to be disorganised with loosely packed collagen fibres (Figures 4.5 I and L).

In the SFS group, TM healing was characterised by prominent hyperplasia of the epithelial and CT layers as well as at the perforation edge. The perforation was seen to close early at 7 days. By 28 days, the structure of healed TM appeared normal, with three obvious layers consisting of dense, well-organised collagen fibres in the middle layer (Figures 4.5 B and E). Throughout the healing process, only a minor inflammatory response was observed. In the ACS group, epithelial hyperplasia and vascular proliferation were evident in the early stages (day 7). Infiltrating cells resembling fibroblasts were abundant in the CT layer with occasional lymphocytes surrounding the graft. At 28 days, the healed TM appeared normal with a trilaminar structure (Figures 4.5 C and F).

In contrast, numerous inflammatory cells (predominantly lymphocytes) and prominent exudates were observed surrounding the paper patch. Although the TM perforation eventually healed, the TM remained thickened with disorganisation of the newly synthesised fibres (Figures 4.5 G and J). Likewise, Gelfoam induced the infiltration of inflammatory cells at
implanted site. Unlike other materials, prominent fibroblast proliferation and erythrocyte-filled blood vessels were found in the CT layer. After 28 days, the healed TM remained thickened with atypical disorganised collagen fibres in the CT layer (Figures 4.5 H and K).

The TM cross-sections were used to quantify changes in the TM thickness after treatment (Figures 4.5 M and N). At 14 days, TMs in all groups were substantially thickened compared with normal TM ($p<0.05$) except SFS-treated TM, which had a similar thickness (14.13±4.04µm) to the normal TM ($p>0.05$). By 28 days, statistically significant differences in TM thicknesses were found in the control, paper patch, and Gelfoam groups ($p<0.05$). However, no statistically significant difference in TM thicknesses was seen in SFS (4.01±0.63µm) and ACS groups (8.55±4.25µm) compared with the normal TM ($p>0.05$).

4.3.2.4. **Ultrastructural morphology**

TEM was performed to investigate the ultrastructure of healed TMs 28 days post-surgery. In scaffold-treated and spontaneously healed TMs, the CT layer was moderately thickened, and fibroblast proliferation was apparent compared with the normal TM (Figures 4.6 B-F). In SFS and ACS groups, the three layers of the TM were readily identified, and the CT layer was compact with well-orientated collagen bundles (Figures 4.6 C and D). However, in paper patch and Gelfoam groups, collagen fibres were loosely
and irregularly arranged in the fibrous layer, with obvious oedema seen (Figures 4.6 E and F).

4.3.3. Improvement of hearing induced by scaffolds

Hearing thresholds were similar in all treatment groups measured preperforation ($p>0.05$) and postperforation ($p>0.05$). The average auditory threshold of the normal rat was $15.0\text{dB}$, and this significantly increased to $29.5\text{dB}$ after perforation, indicating that TM perforation caused significant hearing loss ($p<0.01$). Audiometric assessment using ABR demonstrated hearing recovery for all groups after treatment (Figure 4.7). The hearing recovery was defined as the difference between auditory threshold immediately after perforation (prerepair) and at specific time points after grafting (postrepair). Auditory threshold of all rats recovered over time, and significant differences were observed when comparing between different treatments ($p<0.01$). Most obviously, hearing in the animals treated with the SFS or ACS recovered significantly faster compared with those treated with paper patch ($p<0.05$ for SFS; $p<0.01$ for ACS), Gelfoam ($p<0.01$ for SFS and ACS), and spontaneous healing ($p<0.01$ for SFS and ACS). Hearing recovery between SFS and ACS groups was similar ($p>0.05$).
The medial aspect of TMs was observed with SEM (A). SEM confirmed the attachment of SFS and ACS to the perforation margin throughout the healing process. TM epithelial cells were seen to have migrated to the medial surface of SFS on day 3 (B-a; ×500) and ACS on day 5 (B-b; ×500). TMs grafted with ACS and SFS had healed on day 9, where the medial surface of neo-membranes was smooth. Paper patch showed partial detachment from the TM surface on day 3. Exudate and inflammatory cell infiltration was evident at the perforation site on day 5 (B-c; ×150). Gelfoam showed early disintegration with a majority of Gelfoam dissolving by day 3 (B-d; ×100). At 14 days, the healed TMs in paper patch and Gelfoam groups showed some scarring (white arrows). In the control group, a rolled perforation edge in the unhealed TM was evident at 9 days (B-e; ×150), and the TM healed by 14 days with an obvious scar (white arrow). Tears in the TMs were artefacts during the drying process. Asterisks indicate scaffolds; white arrowheads indicate perforation margin. M, handle of malleus; E, epithelial cells. Scale bar: 500µm.
Figure 4.4  Histological observation of the TM healing after grafting

In the SFS group, prominent hyperplasia was evident in the epithelial and CT layers and at the perforation edge on day 3 and 5, with minor inflammatory responses seen throughout the healing process. In the ACS group, epithelial hyperplasia and vascular proliferation were evident in the early stages (day 3-7). Infiltrating cells resembling fibroblasts were abundant in the CT layer with a few lymphocytes surrounding the implant. The perforations in SFS and ACS groups closed early at 7 days, and the structure of healed TMs appeared normal at 28 days. Paper patch elicited lymphocytic cell infiltration with prominent exudates at 9-28 days. Gelfoam also induced the infiltration of inflammatory cells, and fibroblast proliferation was prominent in the CT layer. The perforations in paper and Gelfoam groups closed on day 7 and 9, respectively, with atypical and thickened healed TMs at 28 days (white arrows). In the control group, TM healing was relatively slower compared with scaffold-treated groups. The perforation remained patent in the first week, and a keratin spur was seen on day 5. The perforation had closed at 9 days with significant thickening throughout the three TM layers. By 28 days, the healed TM became thinner with residual thickening at the previous perforation site (white arrow). Black arrowheads indicate TMs; black arrows indicate scaffolds. EAC, external auditory canal; ME, middle ear; m, handle of malleus; spur, keratin spur. H&E staining. Scale bars: 200µm.
Figure 4.5 Photomicrographs of healed TMs at 28 days after grafting

A normal TM shows a thin uniform structure (A, D). At 28 days, TMs treated with SFS (B, E) and ACS (C, F) had a normal trilaminar structure, consisting of dense and well-organised collagen bundles in the CT layer. TMs treated with paper patch (G, J) and Gelfoam (H, K) remained thickened in the healed area with loose and disorganised collagen fibres in the middle layer. TMs in the control group (I, L) remained thick with an atypical structure and regions of irregular collagen fibres. At 14 days, all TMs except the SFS group were significantly thickened compared with the normal TM (M). By 28 days, TM thickness in the SFS and ACS groups showed no significant differences compared with the normal TM (N). (*p<0.05, **p<0.01). Arrowheads indicate the residual scaffolds. H&E and Masson trichrome staining. Scale bars: 50µm.
Figure 4.6  Transmission electron microscopic observation of the TMs

TEM images demonstrated the ultrastructure of a normal TM (A), spontaneously healed TM (B), and TMs repaired with SFS (C), ACS (D), paper patch (E), and Gelfoam (F) at 28 days. A normal TM (A) consists of an outer epidermal (arrows), middle CT, (containing radial fibres (Rf) and circular fibres (Cf)), and an inner mucosal layer (arrowheads). The CT layer was moderately thickened, and fibroblast proliferation was apparent in spontaneously healed (B) and scaffold-treated TMs (C-F). TMs repaired with SFS (C) and ACS (D) showed more compact tissues with dense, well-orientated collagen bundles compared with spontaneously healed TM (B) or TMs treated with paper patch (E) and Gelfoam (F). Oedema (*) in the CT layer was apparent in paper patch (E) and Gelfoam (F) groups. Scale bars: A, 1µm; B, C, and E, 2µm; D and F, 5µm.
The hearing recovery was defined as the difference between auditory threshold immediately after perforation (prerepair) and at specific time points after grafting (postrepair). The values represent mean±sem (n=5). Hearing recovery after grafting in each group was performed using multiple linear regression analysis. Auditory threshold of all rats recovered over time, and significant differences were observed when comparing between different treatments (p<0.01). Hearing in the rats treated by the SFS or ACS recovered significantly faster compared with those treated with paper patch, Gelfoam, and spontaneous healing (control); however, no significant difference was found between SFS and ACS. Statistical significance between groups was as follows: aSFS and spontaneous healing (p<0.01); SFS and Gelfoam (p<0.01); bACS and spontaneous healing (p<0.01); ACS and paper (p<0.01); ACS and Gelfoam (p<0.01); cSFS and ACS (p>0.05); and dSFS and paper (p<0.05).

4.4. Discussion

This study demonstrated that two bioscaffolds (SFS and ACS) significantly shortened the perforation closure time and promoted TM wound healing compared with two commonly used scaffolds (paper patch and Gelfoam) and spontaneous healing in a rat model. The healed TMs in SFS and ACS groups showed improved morphology with the regeneration of compact collagen fibres, a rapid return to a normal TM thickness, and complete hearing recovery at an earlier stage compared with the other groups. Since the goals of surgical treatment for TM perforation are to achieve complete closure of the perforation and restoration of the hearing (Albera et al. 2006), these
results suggest that both SFS and ACS are efficient and may serve as alternative scaffolds for improving both TM healing and hearing.

In this study, it was evident that the use of scaffolds enhanced TM wound regeneration when compared with spontaneous healing. Scaffolds provided structural support in guiding the regenerating tissue across the perforation as seen with SEM. Moreover, scaffolds improved structural organisation of the healed TMs and resulted in prominent hyperplasia of the epithelial and CT as observed via histology. Cell in-growth into the pores of scaffolds and integration of the scaffolds into healed TM were not observed, suggesting that scaffolds used with this onlay technique serve to provide structural support rather than form a part of the regenerated TM. This finding is different in studies in which 3D scaffolds are placed with an inlay technique and found to integrate into the regenerated TM (Kim et al. 2011). Among the four scaffolds used, SFS and ACS were found to attach closely to the TM perforation margin. In contrast, paper patch was found to attach weakly and was often lost from the TM surface whereas Gelfoam rapidly lost its sponge-like structure with evident swelling and shrinking. Paper and Gelfoam were unstable and detached early from the TM remnant, thereby losing their scaffold function and resulting in a delay in healing and resultant hearing. Where no scaffold was used in the spontaneously healed TMs, a keratin spur was observed on day 5. This keratin spur was thought to serve as a natural scaffold for the epithelium to bridge the perforation (Spandow et al. 1996), suggesting that scaffolds are indeed essential in promoting TM wound
healing by providing support for early migration of keratinocytes. This is particularly important for TM repair, as the wound margin is suspended in air without an underlying tissue matrix that supports the regenerating epithelium and migrating neovasculature (Santa Maria et al. 2010).

Our study showed SFS achieved earlier structural repair of TM compared with paper patch and spontaneous healing. This is similar to a recently published study by Kim et al. (Kim et al. 2010a). They reported a significantly shorter healing time (1.9 days) between aqueous-based silk and paper patch; however, they did not report the hearing outcome. ACS also achieved similar outcomes as SFS in this study. Although previous studies have shown promising results for collagen membranes for TM reconstruction (Bonzon et al. 1995; Morgon et al. 1989; Truy et al. 1994), no commonly used scaffolds were used as control materials in these studies, and different animal models were used. AlloDerm, a decellularised ECM in which the main component is collagen, has recently reported high perforation closure rates (87.5% to 100%) (Haynes et al. 2005). However, its therapeutic capacity is restricted by its cadaveric origin, with a limited supply by tissue donations. Compared with AlloDerm, the porcine-derived ACS has been fashioned to a similar thickness to TM and, thus, provides a unique alternative for tympanic reconstruction. In terms of paper patch, our study showed similar results to previous research in terms of biocompatibility, fragility, and poor adhesion (Golz et al. 2003; Kim et al. 2010b; Chun et al. 1999). Recent clinical trials showed encouraging results of Gelfoam patching for acute or small TM
perforations (Lou & He 2011; Niklasson & Tano 2011). However, Gelfoam should be carefully applied, as it has been shown to cause adhesion, fibrosis, and even osteoneogenesis in the ME (Hellstrom et al. 1983; Doyle-Kelly 1961; Bahadir et al. 2003; Shen et al. 2011).

In addition to a more rapid healing rate, our study also demonstrated that SFS and ACS achieved significantly faster hearing recovery compared with the other groups. A possible explanation may relate to improved organisation of collagen fibres of healed TMs and early remodelling to achieve comparable thickness to a normal TM. On the other hand, the healed TMs treated with paper patch, Gelfoam, and especially the spontaneous healing groups remained thickened with loosely disorganised collagen fibres accompanied by oedema in the lamina propria layer. Since this lamina propria layer is critical in maintaining the mechanical and vibroacoustic properties of the TM, spontaneously healed TMs tend to result in higher reperforation rates and suboptimal hearing outcomes, particularly where a dimeric structure lacking the middle fibrous layer is formed. In addition, collagen fibres are essential in the preservation of the anatomical integrity of a TM by avoiding a retraction pocket caused by negative ME pressure (Knutsson et al. 2009). In clinical practice, retraction pockets in weakened TMs result in complications such as cholesteatoma, which can be destructive and require further surgeries as well as adhesive fibrous OM, which can result in suboptimal hearing (Forsen 2000; Cassano & Cassano 2010). Moreover, radial collagen fibres in the TM have been proved to be essential for sound conduction, especially in
frequencies above 4kHz (O'Connor et al. 2008). In clinical cases with unsatisfactory hearing, it was found that the collagen fibres had not been restored, despite the re-establishment of an intact TM (O'Connor et al. 2008).

The requirements for an ideal TM scaffold include biocompatibility, biodegradability, and appropriate physical properties (Kim et al. 2010b). More importantly, a bioscaffold should be safe and non-ototoxic. All materials selected in this study have previously been found to be non-ototoxic when packed into the ME cavity over 12 weeks in rats (see Chapter 3). Recent studies have shown SF to be cytocompatible, free of toxicity and genotoxicity (Liu et al. 2010). Collagen has also been known to be nonreactive, nontoxic, and noncarcinogenic (Patterson 1967; Abbenhaus 1978); hence, many collagen-based materials have been approved by the FDA. Since the introduction of Gelfoam in 1945, it has been extensively used in otology mainly as a MEPA due to its nonantigenicity and nontoxicity (Hellstrom et al. 1983). Paper patch has also been used in outpatients for decades, as it is well tolerated with no severe complications reported to date.

Biocompatibility of a scaffold is an important element to consider, as an inflammatory response after the application of biomaterials may lead to failure in surgery (Zheng et al. 2005; Malcarney et al. 2005). In this study, the SFS and ACS accelerated and improved TM healing, partly attributed to minimal inflammatory response at the implantation sites, which is consistent with previous reports (Kim et al. 2010a; Truy et al. 1994).
biocompatibility of SFS has been established after the removal of sericin, an antigenic glue-like coating protein (Altman et al. 2003). We recently showed its biocompatibility and ability to support human TM keratinocyte growth *in vitro* (Levin et al. 2010; Ghassemifar et al. 2010). Collagen is also known to elicit minimal inflammatory and antigenic responses (Pachence 1996); however, previous research showed unsatisfactory results with collagen films in repairing TM perforation due to unfavourable collagen purification (Bonzon et al. 1995). The ACS used in this study is acellular and contained no remaining reactive DNA that can invoke an inflammatory response. In contrast, we found that the paper patch was associated with severe inflammation, which may contribute to the delayed healing seen. With Gelfoam, only moderate inflammation was observed; however, complications such as adhesion and fibrosis may occur when it is used in the ME cavity (Shen et al. 2011).

Finally, physical properties of a scaffold should be considered. SFS and ACS were found to be easy to handle during surgery, as they are not as fragile as paper or bulky and spongy as Gelfoam. Moreover, the transparency of SFS and ACS allowed direct observation of the TM, whereas the opacity of paper and Gelfoam obstruct the direct visibility of TM healing. From a clinical point of view, these characteristics make SFS and ACS more favorable compared with paper and Gelfoam. Although Gelfoam was significantly thicker compared with other materials, the thickness of the scaffold was not found to be a significant factor in the delay of TM wound healing.
With the advances in tissue engineering and biomaterials, recent research has focused on using tissue engineering techniques for TM reconstruction. With encouraging results, this regenerative method has been described as potentially the ‘greatest advance in otology since the cochlea implant’ (Jackler 2012). Compared with conventional surgery, it is clear that tissue-engineered constructs could be applied in an outpatient setting without needing a surgery, with obvious cost and accessibility benefits. Although many biomaterials have been investigated, an ideal scaffold does not exist. Based on the findings of our studies, SFS and ACS are promising scaffolds for a bio-engineered TM. More importantly, improved TM healing with these materials may reduce complications such as recurrent otorrhea, ME infection, and acquired cholesteatoma.

A limitation of this study was the use of an acute rat TM perforation model, due to the lack of a robust and reliable animal model for chronic TM perforation (Santa Maria et al. 2007). TM perforations in rodents tend to heal naturally and rapidly (Krupala et al. 1998) as was confirmed in this study; hence, further study in a larger animal model (e.g., rabbit or monkey) may be needed. In addition, another limitation of the rat model is that the more commonly used scaffolds such as temporalis fascia were not used as controls due to the lack of this material in rats (Schiller & Wormald 1992). Despite these limitations of our model, we have identified a residual scarring and thickening within the time frame for our study and also demonstrated that the TM regeneration significantly improved when scaffolds were used, with less
scarring and a rapid improvement to hearing. Hence, while these results may not extrapolate directly to chronic perforations in the clinical setting, they show encouraging improvements and indicate that further investigation in a suitable chronic perforation model may prove worthwhile.

In summary, this study showed that both SFS and ACS significantly accelerated acute TM wound healing and achieved hearing recovery from an early stage. The healed TMs in SFS and ACS groups demonstrated better morphology, comparable thickness to a normal TM, and improved organisation of collagen fibres in the CT layer. Improved morphology was associated with significantly faster hearing recovery compared with the paper patch, Gelfoam, and spontaneous healing. In contrast, paper patch and Gelfoam lost their scaffold function in the early stages and showed an inflammatory response, which may have contributed to delayed healing. SFS and ACS also satisfy the requirements of an optimal scaffold for TM tissue engineering, including safety and biocompatibility, and have appropriate physical properties such as transparency and ease of handling, making them more favourable in the clinical setting. Taken together, these data show that both SFS and ACS are comparatively better than Gelfoam, paper, and spontaneous healing and are potential clinical substitutes in the repair of TM perforations.
5. TM repair using SF and ACS in guinea pigs*

5.1. Introduction

Chronic perforations of the TM represent a significant source of morbidity worldwide (Levin et al. 2009). These complications include conductive hearing loss, ME infections, acquired cholesteatoma, or even intracranial complications if left untreated (Lindeman et al. 1987). Acute perforations of the TM usually heal without treatment, with up to 80% undergoing spontaneous closure (Farrior 1983). Those that persist and become chronic usually result from infection (e.g. OM) or traumatic injury.

Failure of a TM perforation to heal is treated surgically by myringoplasty, where a graft is applied to restore the integrity of the TM, to improve hearing, and to decrease the incidence of further ME infection (Aggarwal et al. 2006). Typical graft materials used are temporalis fascia (gold standard), cartilage, perichondrium, and fat. Success rates for perforation repair are high. However, when anatomical, functional, and audiometric factors are taken into account, the initial graft take rates are substantially reduced (e.g., to 63-67%) (Kumar et al. 2010; Yung et al. 2007). Graft complications may include reperforation, cholesteatoma, lateralisation, blunting, epithelial pearl, and inclusion cysts (Albera et al. 2006). Reperforation and retraction formation are generally due to lack of regeneration of the fibrous middle layer, which makes the TM acoustically and mechanically less efficient (O'Connor et al. 2008). Furthermore, myringoplasty is a costly procedure due to the requirement of anesthesia, surgery, and an overnight hospital admission (Laidlaw et al. 2001; Kim et al. 2010b).
Recent advances in biomaterials research and tissue engineering have provided alternative materials for TM regeneration (Teh et al. 2013). Biomaterials such as silk, collagen, AlloDerm, chitosan, and calcium alginate have been investigated as potential TM grafting materials, and have shown promising results in animal models and clinical studies (Levin et al. 2009; Teh et al. 2013). In Chapter 4, we showed in a rat TM perforation model that silk and ACS grafts improved acute healing and recovery of hearing (Shen et al. 2013b). SF, a structural protein processed from silkworms, is a natural biomaterial, with beneficial properties including biocompatibility and biodegradability. Previous studies have shown its efficacy to promote wound healing of skin, ligament, nerves, and bone (Altman et al. 2003). Our group has demonstrated that SF can successfully support human TM cells in vitro (Levin et al. 2010; Levin et al. 2012). Collagen has been widely used as a bioscaffold due to its biocompatibility and ability to promote the growth of many types of cells and tissues (Yarlagadda et al. 2005). As a major substance of the TM lamina propria, collagen fibres play an essential role in maintaining a resilient TM and in sound conduction (O'Connor et al. 2008). Previous studies have shown unsatisfactory results using collagen film for TM repair, partly due to unfavourable collagen purification (Bonzon et al. 1995). However, we have developed an ACS derived from porcine peritoneum that may be suitable for TM repair (Shen et al. 2013b). The cellular components are removed in the processing procedure, without
retaining reactive DNA or other biological components that may provoke inflammatory responses.

We hypothesised that SFS and ACS may act as effective TM graft materials to improve TM regeneration and provide better acoustic function. In Chapter 4, a rat model was used to show that the scaffolds were effective for an acute TM perforation (Shen et al. 2013b). However, restoration of hearing was not measured due to high baseline variability in this model. The purpose of the present study was to confirm the efficacy of SFS and ACS as graft materials for TM repair in a guinea pig acute TM perforation model, and to gain further insight into restoration of hearing. We compared TM healing and hearing against untreated controls that healed spontaneously, and against paper patch, which has been used clinically in outpatient settings.

5.2. Materials and methods

5.2.1. Materials

The SFS, ACS and paper were obtained and treated as per Section 2.1.1. All three materials were packaged and sterilised with ethylene oxide gas prior to use.

5.2.2. Mechanical tests

Mechanical properties of the scaffolds were measured using an Instron testing system with Fast-Track software (Model 5543, Instron Inc., MA) at a displacement rate of 15mm/min. Scaffolds (6mm width × 70mm length) were
prepared to measure the tensile strength and percentage elongation to failure. Tests were repeated 10 times, and the averages of the properties were calculated from stress/strain plots. The thickness of each scaffold type was measured by a scanning electron microscope (S3000N, Hitachi, Japan) at a voltage of 20kV.

### 5.2.3. Animals

The animal experiments were approved by the Animal Ethics Committee of Zhejiang University (China) and The University of Western Australia (Australia). Treatment and care of the animals was in accordance with the Australian Code of Practice for the Care and Use of Animals for Scientific Purposes. Eighty-two male albino guinea pigs (*Cavia porcellus*), weighing 350 to 400 g, were obtained from Shanghai Animal Centre (Chinese Academy of Science, Shanghai, China). Before experimentation, both ears of all animals were inspected with an otomicroscope (Zeiss S5, Germany) to exclude ME disease. Animals were housed in the experimental animal facility of the Zhejiang University and provided with food and water *ad libitum* in a room with 12-h light/dark cycles.

### 5.2.4. Experimental design

The animals were allocated serially into four treatment groups according to the scaffolds implanted: SFS (n=18), ACS (n=18), paper patch (n=18), and control (spontaneous healing) (n=18). An additional group of guinea pigs (n=10) were allocated as a normal TM group (without perforation or scaffold implantation) used for acoustic analysis. Postoperatively, six guinea pigs
from each treatment group were randomly chosen and evaluated at 7, 14, and 28 days. TM healing was examined by otomicroscopic and histologic evaluation, whereas the hearing function was analysed by ABR. Guinea pigs were sacrificed by intraperitoneal injection of pentobarbital sodium (80mg/kg).

5.2.5. Surgical procedure

Surgical procedures were performed under general anaesthesia using intramuscular hypnorm (1ml/kg) and intraperitoneal diazepam (5mg/kg). Following the removal of debris, ear canals were sterilised with povidone iodine solution. TM perforations consisting of approximately 40% of the PT were created in the right ear using a sterile 23-gauge needle. Three different scaffolds were rinsed with sterile PBS solution (Invitrogen, Shanghai, China), and grafted onto the perforations using onlay myringoplasty. Ofloxacin ointment (Tarivid® eye ointment, Santen Pharmaceutical Co., Ltd., Japan) was applied to the margin of each scaffold to adhere the scaffolds in situ. For ACS, the rough surface was placed medially and the smooth surface faced laterally, towards the ear canal. After the operation, all guinea pigs were given buprenorphine (0.02-0.08mg/kg) for postoperative analgesia.

5.2.6. Otomicroscopic evaluation

Before sacrifice, six guinea pigs in each treatment group were examined using an otomicroscope at each time point. Under general anaesthesia, the TMs were viewed by three independent observers who were blinded to the
study groups with respect to perforation closure, signs of infection, and myringosclerosis. The perforation site of each TM was graded using Gnuechtel’s method (Gnuechtel et al. 2000) on a 0 to 7 scale: 0, large perforation; 1, small perforation; 2, pinhole perforation; 3, moderate eschar; 4, minimal eschar; 5, moderate myringosclerosis; 6, small myringosclerosis; and 7, totally healed. Images were recorded with a video otoscope (MedRX, Largo, FL) and converted to digital images using Aurisview software (Ear Science Institute Australia, Subiaco, Australia).

5.2.7. ABR

Prior to sacrifice, ABR tests were performed to assess the hearing of guinea pigs using a Nihon Kohden Neuropack-µ Measuring Systems (MEB 9100, Nihon Kohden, Japan) as per Section 2.2.6. Auditory thresholds of the click stimuli were measured for the right ears of each animal before and after TM perforation. Following grafting, ABR thresholds were again measured for six randomly selected animals from each treatment group at each time point. The normal ears and the ears with TM perforations, but without treatment, served as controls.

5.2.8. Histological examination

Following sacrifice, right TMs along with the bony annulus were extracted from the tympanic bulla. Specimens were prepared for histology as per Section 2.2.2. A total of 4µm sections were cut and stained with H&E (Section 2.2.3). Stained sections were digitally scanned using an Aperio
ScanScope XT automated slide scanner (Aperio Technologies Inc., Vista, CA) with a 2× doubler inserted.

5.2.9. Statistical analysis

Statistics were analysed using the Prism software package (GraphPad Software 5.03, La Jolla, USA). Data are presented as mean±sem. Healing rates determined by otologic observation were compared using Fisher’s exact test. Mechanical testing results, otomicroscopic scores, and hearing threshold data were analysed by one-way analysis of variance, followed by Tukey post hoc comparisons. Statistical significance was defined as $p<0.05$.

5.3. Results

5.3.1. Characteristics of SFS and ACS

SFS was transparent while ACS and paper were white and opaque in the dry state (Figures 5.1 A-C). Mechanical properties of SFS and ACS were measured as shown in Figure 5.1. The tensile strength of SFS (42.0±2.2MPa) was significantly higher than paper (34.4±1.7MPa; $p<0.05$) and both were stronger than ACS (21.4±1.6MPa). ACS had significantly higher elongation properties (19.6±1.4 %) in comparison with that of paper (2.2±0.1 %) or SFS (2.3±0.1 %) ($p<0.05$) (Figures 5.1 D and E).
Figure 5.1 Characteristics of grafting materials

Gross morphology of (A) paper, (B) ACS, and (C) SFS. Comparison of mechanical properties of the materials: (D) tensile strength; (E) elongation at the rupture point (*p<0.05, compared with paper; **p<0.001, compared with paper). Data represent mean±sem. n=10 for each group.

5.3.2. SFS and ACS improved the structural healing of repaired TM

All animals survived the surgery, with no infections or other postoperative complications observed. The structural healing of TM was significantly improved in the SFS and ACS groups compared to the control group. Perforation closure according to otomicroscopic examination was faster in the SFS and ACS groups than in the control group (Table 5.1). At 7 days, perforations were closed in all six animals (100%) in both SFS and ACS groups, compared with four of six animals (66.7%) in the paper group and no animals (0%) in the control group. Perforation closure was achieved in all six animals (100%) in the SFS, ACS, and paper groups at 14 days, compared to
three of six animals (50%) in the control group. Perforations in all animals were healed at 28 days.

Otomicroscopic evaluation was performed to observe gross morphology of the repaired TM. The healed TMs in the SFS and ACS groups appeared transparent and smooth on day 28 (Figures 5.2 G and H). In contrast, control and paper-treated TMs showed increased opacity, resembling scar formation at the perforation site (Figures 5.2 E and F). The semi-quantitative otomicroscopic score in the SFS and ACS groups were significantly higher than in the paper or control group ($p<0.05$) at each of the time points, but with no significant differences when compared to each other (Table 5.1). In terms of paper, although it showed higher otomicroscopic scores compared to control group at 7 days, there were no significant differences between two groups at 14 and 28 days postoperatively.

Histological examination demonstrated obvious differences in the morphology of the neomembranes (Figure 5.3). At 28 days, the TMs in the SFS and ACS groups showed a well-organised trilaminar membrane consisting of an outer epidermal layer, a middle fibrous layer, and an inner mucosal layer, which was similar to the native TM. The TMs in both groups appeared normal with uniform thickness throughout (Figures 5.3 D and E). In contrast, the paper and control groups had thickened TMs at the perforation site, mainly due to a disorganised fibrous layer (Figures 5.3 B and C). It is noteworthy that part of the paper patch was found to incorporate into the
regenerated TM in two of the animals at 28 days postoperatively. Numerous inflammatory cells, predominantly lymphocytes, were observed around the remaining graft, with scant giant cells, macrophages, and eosinophils visible (Figure 5.3 F).

5.3.3. SFS and ACS facilitated the hearing recovery of treated TM

ABR testing demonstrated that hearing recovery in the SFS and ACS groups was significantly improved compared to the control group (Figure 5.4). Auditory thresholds were similar in all guinea pigs measured preperforation ($p>0.05$) and postperforation ($p>0.05$). The average hearing threshold of all guinea pigs was $18.2\pm0.3$dB; this significantly increased to $29.9\pm0.2$dB after perforation, indicating that TM perforation caused significant hearing loss ($p<0.001$). Hearing thresholds in the SFS and ACS groups were not significantly different compared to the normal TM group from the earliest time point measured at day 7 ($p>0.05$), suggesting that the animals underwent rapid hearing recovery. By contrast, hearing thresholds in the control group were still significantly worse than in normal TM, ACS, and SFS groups on day 28 ($p<0.05$). In the paper group, the hearing significantly improved when compared to the control group on day 7 ($p<0.01$). Although hearing thresholds were significantly worse when compared to the other groups on day 14 (for normal and SFS, $p<0.05$; for ACS, $p<0.01$), the hearing thresholds in the paper group were similar to the normal TM group on day 28.
Table 5.1  Perforation closure and otomicroscopic scores of three materials at different time points following grafting

<table>
<thead>
<tr>
<th>Group</th>
<th>Perforation Closure</th>
<th>Otomicroscopic Score&lt;sup&gt;1&lt;/sup&gt;</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>7 days</td>
<td>14 days</td>
</tr>
<tr>
<td>Control</td>
<td>0/6</td>
<td>3/6</td>
</tr>
<tr>
<td>Paper</td>
<td>4/6</td>
<td>6/6&lt;sup&gt;*&lt;/sup&gt;</td>
</tr>
<tr>
<td>ACS</td>
<td>6/6&lt;sup&gt;†&lt;/sup&gt;</td>
<td>6/6&lt;sup&gt;*&lt;/sup&gt;</td>
</tr>
<tr>
<td>SFS</td>
<td>6/6&lt;sup&gt;†&lt;/sup&gt;</td>
<td>6/6&lt;sup&gt;*&lt;/sup&gt;</td>
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</table>

<sup>1</sup>Otomicroscopic scores (n=6) are presented as mean±sem.

<sup>*</sup><sub>p<0.05</sub>, control (spontaneous healing) vs. other groups (Fisher's exact test).

<sup>†</sup><sub>p<0.01</sub>, control vs. other groups (Fisher's exact test).

<sup>a</sup><sub>p<0.05</sub>, control vs. other groups (one-way ANOVA).

<sup>b</sup><sub>p<0.05</sub>, paper vs. ACS, or paper vs. SFS (one-way ANOVA).

Figure 5.2  Otoscope images of guinea pig TM

Upper row shows (A) a TM perforation (asterisk) prior to grafting and following repair with (B) paper, (C) ACS, and (D) SFS. The arrows in (B-D) indicate the grafts after surgery. Lower row shows healed TMs 28 days postoperatively. The arrowheads indicate scar formation at the perforation site in (E) control and (F) paper groups. The healed TMs treated with (G) ACS and (H) SFS appeared transparent and smooth.
Figure 5.3  Photomicrographs of histological sections

(A) Normal TM, (B) control (spontaneous healing) TM, and TMs repaired with (C) paper, (D) ACS, and (E) SFS at 28 days postoperatively. (F) Shows paper patch incorporating into the regenerated TM at 28 days, and inflammatory cell (yellow arrows) infiltrates were observed around the remaining paper (blue arrowheads). The black arrowheads in (E) indicate the residual graft materials. EAC, external auditory canal; ME, middle ear; m, handle of malleus. H&E staining. Scale bar: 20µm.

Figure 5.4  Comparison of hearing thresholds in each group assessed by ABR

(A) Shows preoperative baseline hearing thresholds for all animals. (B-D) Shows average hearing thresholds for TMs treated with three materials at 7, 14, and 28
days postoperatively ($p<0.05$, **$p<0.01$, ***$p<0.001$, ANOVA). The values represent mean±sem. n=6 guinea pigs per group.

5.4. Discussion

The primary goal of TM repair is to achieve restoration of both structural integrity and hearing function of the TM (Albera et al. 2006). The results of this study confirm that SFS and ACS may serve as suitable graft materials for TM regeneration. Otomicroscopic and histological analysis demonstrated that TMs treated by SFS or ACS possessed superior outcomes compared to paper patch or control, with complete closure of the perforation at an earlier stage and more native structure formation by 28 days. ABR assessment demonstrated that SFS and ACS facilitated a faster recovery of hearing function compared to paper patch and control probably related to faster healing. This observation suggests that SFS and ACS are effective graft materials and may be utilised as alternatives to current grafts for TM repair.

Grafting is an essential element for successful repair and healing of TM wounds. As the TM is suspended in air without an underlying tissue matrix (Santa Maria et al. 2010), an ideal graft material should be maintained at the perforation margin, provide structural support, and function to guide epithelial migration and neovascularisation. The eventual healing would ideally create a trilaminar, transparent membrane with appropriate vibro-mechanical properties. In this study, we compared the efficacy of two materials (SFS and ACS) to spontaneous healing and a clinically available material (paper patch). The SFS and ACS were effective TM graft materials
in that they attached closely to the TM remnant and provided stable support over the study period. They accelerated the healing process, possibly through regrowth of epithelium and fibroblasts, as seen in vitro (Altman et al. 2003). Precisely how silk and ACS are able to promote the healing mechanisms is not known and is the subject of ongoing research.

Mechanical properties such as elasticity and strength are also thought to be important for the TM grafts (Kim et al. 2010b). Graft materials need to provide sufficient stability to resist constant negative pressure (e.g. eustachian tube dysfunction) while allowing adequate acoustic sensitivity during regeneration of the TM (Huttenbrink 2004). A strength of a graft material range from 1 to 400MPa was considered as sufficient based on previous studies (Kim et al. 2010b). This study showed SFS and ACS to be in this range, with significantly higher elasticity for ACS compared to paper. Moreover, we have shown previously that SFS was highly flexible (136.6%) in a wet state (Rajkhowa et al. 2011). Thus, our study indicated that SFS and ACS had adequate strength and flexibility, which are important for the vibroacoustics of TMs.

By contrast, our study revealed that the paper patch tended to detach from the TM contour post-surgery, losing its scaffold function at an early stage. This finding coincides with previous findings in rats (Kim et al. 2010a; Shen et al. 2013b). Since first introduced by Blake in 1887, paper patching has been commonly used in outpatient settings to treat acute and traumatic TM
perforations (Merwin & Boies Jr 1980). In this method, paper has been thought to serve as a scaffold to guide the migrating epithelium to close the defect (Golz et al. 2003). However, we found evident paper fragments embedded in the fibrous layer of the TM in two of the six guinea pigs on day 28, with accompanying inflammatory infiltration. To our knowledge, this is the first report showing that paper may be incorporated into the TM and through the inflammation response may delay healing. Moreover, paper has been shown to produce bilaminar membranes when used to repair chronic perforations in a chinchilla model (Laidlaw et al. 2001). However, this was not observed in our study.

Guinea pigs were chosen for this study as their TMs are similar to those of humans and have a well-documented history in auditory research (Albuquerque et al. 2009). In this study, we demonstrated that ears treated with SFS or ACS underwent faster hearing recovery than the paper and control treatments, which correlated with a higher otomicroscopic score and well-organised histological structure. Hearing thresholds in the SFS and ACS group were already normalised at day 7. However, hearing thresholds in the paper or control group were significantly worse than the normal TM at 14 and 28 days. This may relate to the formation of scar tissue and pronounced thickening at the perforation site, which may alter the sound impedance of the TM and result in rigid and less elastic TM (Rahman et al. 2007). This finding was consistent with that of Bigelow et al (Bigelow et al. 1996), who reported that hearing loss up to 12dB was still present even when the experimental
perforated TM healed due to its abnormal structure. Thus, the results of ABR demonstrated the efficacy of SFS and ACS in promoting hearing restoration.

The formic acid-based SF used in this study was processed from the domestic silkworm *Bombyx mori*. Following the removal of sericin, an antigenic glue-like coating protein, the core SF fibres are biocompatible with little immune response postimplantation (Meinel et al. 2005). In Chapter 4, we demonstrated hearing and healing benefits of SF membranes using onlay myringoplasty in rats, with improved structural outcomes. Kim et al. (Kim et al. 2010a) also found rapid healing in rat TM with a similar but aqueous-based silk film. However, they did not report any associated hearing outcomes. In the current study, we not only confirmed healing benefits from silk scaffold using a guinea pig model, but also have shown full restoration of hearing at the earliest time point evaluated. Similarly, we also observed that ACS enhances healing of TM repair. The ACS used in this study was processed from porcine peritoneum and decellularised without any xeno-DNA and xenocellular components to invoke an inflammatory response. Furthermore, unlike SFS and paper, ACS has two distinct surfaces. During placement, its rough side was placed medially to allow for cellular in-growth, while its smooth side faced towards the external ear canal, which we speculate may reduce bacteria and foreign body seeding.

A limitation of this study was the use of an acute TM perforation model to evaluate the materials, mainly due to the lack of a reliable chronic model
(Santa Maria et al. 2007). Acute TM perforations often heal spontaneously, and may have different responses to the graft materials compared to chronic TM perforations. Nevertheless, the results of our acute perforation model have provided us with preliminary information on SFS and ACS for TM grafts for future studies. In addition, this is a short-term study of 4 weeks duration, and long-term studies would be necessary before the application of SFS and ACS in clinical settings.

In conclusion, this study has demonstrated that SFS and ACS are effective graft materials for TM repair in a guinea pig acute perforation model. SFS and ACS possess advantages, including optimal mechanical properties, transparency, and ease of handling. Treatment with SFS and ACS improved the structure and function of TM repair compared to paper patch and control, with complete closure of the perforation and restoration of hearing at an earlier stage and more native structure formation. The results suggest that SFS and ACS are promising substitutes for TM repair and may be utilised as alternatives to the materials currently in use.
6. General discussion and future directions
6.1. General summary

TM perforations are a significant source of morbidities such as conductive hearing loss and infection of the ME (Parekh et al. 2009). Myringoplasty is commonly performed to repair chronic perforations (Karela et al. 2008). The principles of myringoplasty are to reinstate the integrity of the TM and restore audiologic function for an ongoing period of time (Aggarwal et al. 2006). Autologous TM grafts have been utilised for decades, such as temporalis fascia (gold standard), perichondrium and cartilage. However, various limitations and complications are associated with autografts, including donor site morbidity, additional incisions, long operation time and a shortage of material in revision cases. Meanwhile, a range of allografts, xenografts and synthetic materials have been investigated for TM regeneration, but all have associated disadvantages (Levin et al. 2009). Recently, with the advances in materials for tissue engineering, various alternative biomaterials have been developed as artificial eardrums. Therefore, significant research now focuses on the development of tissue engineering for TM repair to promote success rates and address limitations (Teh et al. 2013; Hong et al. 2013).

In myringoplasty, a suitable scaffold material is considered essential, to provide structural support for epithelial migration and supply a suitable environment for cell infiltration, angiogenesis and neotissue synthesis (Gladstone et al. 1995). Given that the TM is suspended in air without underlying tissue, the use of scaffolds is particularly important for the repair
of TM perforations (Santa Maria et al. 2010). In addition, MEPAs are also commonly utilised in myringoplasty to provide support to the TM grafts, aerate the ME cavity, and for haemostasis (Krupala et al. 1998). However, MEPAs may potentially cause detrimental effects such as adhesion, fibrosis, osteoneogenesis and ossicular fixation, thereby resulting in suboptimal hearing outcomes postoperatively (Hellstrom et al. 1983). Thus, the decision to use MEPA remains controversial, and there is an ongoing search for innovations in surgical techniques and devices to obviate the need for ME packing (Shen et al. 2011) (see Appendix 4).

This thesis has investigated the use of SFS and ACS as scaffolds for the promotion of TM regeneration in vivo. Both scaffolds have proven to be biocompatible and effective and may offer alternatives to the conventional autografts. In addition, they would not require the use of MEPA.

To provide insight into the safety of SFS and ACS for tissue engineering in the ear, the in vivo biocompatibility of SFS and ACS were evaluated and compared to two commonly used graft materials, paper patch and Gelfoam in a rat model (Chapter 3). The scaffolds were implanted in SC tissue and the ME cavity followed by histological and otoscopic evaluation for up to 26 weeks. Our results revealed that SFS and ACS were well tolerated and compatible in rat SC and ME tissues throughout the study. The tissue response adjacent to the implants evaluated by histology and otoscopy showed that both SFS and ACS have a milder tissue response with minimal
inflammation compared to that of paper. Gelfoam gave similar results to SFS and ACS after SC implantation, but it was found to be associated with pronounced fibrosis and osteoneogenesis after ME implantation. It is concluded that SFS and ACS both were biocompatible and could serve as potential alternative scaffolds for tissue engineering in the ear.

Furthermore, we evaluated the efficacy of SFS and ACS compared with Gelfoam and paper for TM reconstruction. These scaffolds were implanted via transcanal onlay myringoplasty on acute TM perforations of both rat (Chapter 4) and guinea pig (Chapter 5) models. During myringoplasty, antibacterial ointment was applied to the margin of the scaffolds as an adhesive to maintain the scaffolds in place. Therefore, no MEPA was applied in this study so that undesirable side effects were avoided. Prior to the study, surface morphology of the scaffolds was observed with SEM, while their mechanical properties were investigated by tensile testing.

Postoperatively, the morphology of the TMs was assessed at various time points with a 4-week follow-up using otoscopy, light and electron microscopy, and functional hearing outcomes by ABR. The results demonstrated that mechanical properties, such as tensile strength and elasticity of SFS and ACS were within the known range for human TM, which are important for the vibroacoustics of TMs (Kim et al. 2010b). An ideal TM scaffold should contain adequate strength to resist ME negative pressure especially with eustachian tube dysfunction while allowing adequate
acoustic sensitivity during regeneration of the TM (Huttenbrink 2004; Kim et al. 2009). Moreover, the healing patterns post-surgery were very similar between the rats and guinea pigs. We found that SFS and ACS significantly accelerated TM perforation closure, obtained optimal TM thickness, and resulted in better trilaminar morphology with well-organised collagen fibres and early restoration of hearing. By contrast, paper patch and Gelfoam lost their scaffold function in the early stages and showed an inflammatory response, which may have contributed to delayed healing.

In summary, the results of this thesis suggest that SFS and ACS are safe, biocompatible and effective TM scaffolds that, when in comparison with paper patch and Gelfoam, can be used as potential substitutes for the TM repair in myringoplasty procedures.

6.2. Clinical significance

The application of SFS and ACS has various clinical advantages based on this study. First, both materials can be applied in an office setting without packing (Shen et al. 2011), obviating the need for general anaesthesia and tissue harvesting. Thus, it can not only reduce the operative time and costs, but also alleviate donor site morbidity such as pain, scar, and discomfort associated with conventional surgical procedures (Shen et al. 2013b; Shen et al. 2013a). This makes the treatment simplified, less invasive and more acceptable by most patients (Teh et al. 2013). Second, both biological materials are readily available, which is highly desirable for revision cases, given that temporalis
fascia may not be available and a second incision is needed for harvesting.

Third, SFS and ACS can be processed into a variety of structures such as fibres, membranes, and sponges, to meet specific clinical requirements (Yarlagadda et al. 2005). Finally, we found both materials were easy to handle during surgery. Unlike the paper and Gelfoam, which are opaque, the transparency of SFS (fully transparent) and ACS (semi-transparent) allowed direct observation of the TM healing postsurgery. From a surgical point of view, these features make SFS and ACS favourable alternatives to current available materials. Although SFS and ACS may be more expensive than autologous materials, the added cost of the scaffold products may be more than offset by diminished operating times and its related benefits, including more rapid healing, less pain, and reduced surgical risks.

6.3. Limitations of this study

This study investigated alternative tissue engineering scaffolds for TM regeneration and demonstrated significantly improved TM healing and recovery of hearing under *in vivo* conditions. However, several limitations should be recognised and remain to be resolved in the future.

6.3.1. Animal models

A limitation of this study was the utilisation of acute TM perforation animal models, due to the lack of a robust and reliable animal model for chronic TM perforations treated clinically (Shen et al. 2013b; Shen et al. 2013a; Santa Maria et al. 2007). Today, the most commonly used animal models for ME
research are mainly rodents, including rat, mouse, guinea pig and chinchilla (Deng et al. 2009). However, spontaneous TM healing in rodents is rapid (7 to 10 days) and occurs regardless of any intervention (Santa Maria et al. 2007). Furthermore, rodents have no commonly used autologous grafts such as temporalis or periauricular fascia for comparison (Downey et al. 2003; Hanna et al. 1993). In addition, responses to treatment are likely to be different between acute and chronic animal models, suggesting a complex wound healing scenario and the importance of using a chronic animal model when developing clinical treatments.

Currently, the challenge in evaluating any novel therapy for TM repair is the lack of an appropriate chronic TM perforation model. In man, a chronic perforation is a stable non-healing wound after 3 months; and in animal experiments it is termed chronic at 8 weeks after perforation. Histologically, chronic perforations have squamous epithelium growing over the perforation edge to meet the inner mucosal layer (Gladstone et al. 1995). To date, various attempts have been made to create a chronic TM perforation model in animals, albeit with limited success (Santa Maria et al. 2007). Specifically, a chinchilla model has been the most successful. However, the availability of this animal is limited worldwide, and the chinchilla has no temporalis fascia (Downey et al. 2003; Hanna et al. 1993). Additionally, the infolding technique used in this model, in combination with thermal myringotomy, results in an alteration to the histology of the perforation edges that is different to that found in clinical cases. The application of hydrocortisone,
mitomycin C, or 5-fluorouracil causes a delay in the healing process, but not a chronic perforation. Similarly, partial excision of the handle of malleus also significantly alters the TM wound healing process (Santa Maria et al. 2007).

6.3.2. Other limitations
There are several other limitations in this project. Firstly, the most commonly used autografts such as temporalis fascia were not utilised as control materials due to the lack of this material in rodents (Schiller & Wormald 1992). Moreover, the efficacy studies of SFS and ACS for TM repair were short-term observations of only 4 weeks in durations. Long-term investigations of these scaffolds would be necessary before their clinical use. In addition, in the biocompatibility study, the potential cochlear toxicity of SFS and ACS remains unknown. We found that the rat is a difficult model to perform ABR evaluation due to the high baseline variability in hearing threshold (Shen et al. 2013a), and further studies using other animal models (e.g. guinea pigs) are warranted before clinical application.

6.4. Future directions
Despite the essential achievements from this dissertation that SFS and ACS both are effective and biocompatible TM scaffolds with potential use in the clinical settings, several obstacles remain on the road to their clinical applications for TM repair. Further research is required to investigate the clinical potential of SFS and ACS. This includes the development of a robust
and clinically relevant animal model for chronic TM perforation, investigating the efficacy of SFS and ACS compared to autografts (e.g. temporalis fascia), determining the suitability of SFS and ACS using alternative surgical techniques, and biomechanical testing of the repaired TMs.

6.4.1. Development of an animal model for chronic TM perforation

Recent research has seen a number of novel tissue engineering therapies developed for TM regeneration, with appropriate bio-scaffolds and bioactive molecules (Teh et al. 2013; Hong et al. 2013). Notable successes have been seen to reduce the need for microsurgery and thus increasing the availability to a greater population (Jackler 2012). Experimental studies to develop these methods may provide insight into mechanisms, but have been hindered by the lack of a suitable chronic TM perforation animal model. Various attempts have been made to create such models, but with limited success (Santa Maria et al. 2007). The ideal chronic animal model is yet to be identified and would fulfil requirements such as availability of animals, surgical accessibility of the TM, low costs, duration of perforation patency over 8 weeks, and most importantly, an appropriate histological morphology.

In this study, we found that implantation of paper into the ME cavity of rats prevented TM perforation healing (Chapter 3). During 2-12 weeks follow-up, 6 of 9 (66.7%) TM perforations failed to heal as indicated by otoscopy (Figure 3.5). This was characterised histologically by
re-epithelialisation of the wound edge margin and an absence of fibrous layer healing. Residual paper fragments were observed in the ME cavity throughout the study. Numerous macrophages and multinucleated giant cells were found in and around these fragments, with a few scattered lymphocytes (Figure 3.7). Serous otorrhea was noted in some of the persistent TM perforations, without any signs of infection. Hence, we hypothesised that ME paper packing induces chronic disruption of wound healing in rats, which potentially can be a similar method for creating a chronic TM perforation animal model for future studies. The non-healing TM is likely due to a foreign body response as evidenced by the presence of macrophages and multinucleated giant cells, causing an inflammation in the ME and at the edge of the perforation. However, future studies with larger sample sizes are required to investigate this method as a chronic animal model. The chronic TM perforation in a clinical setting is often associated with otorrhea, mucosal change and Eustachian tube dysfunction, which the paper implantation method may be imitating. Thus, further development is likely required to replicate all of these pathological features of chronic suppurative OM, including mucosal disease and discharge.

Recently, several new methods also have been developed to create chronicity of the TM perforation in animals. Choi and colleagues (2011) reported an animal model using two different methods in Sprague-Dawley rats (Choi et al. 2011). In brief, following thermal myringotomy, the perforations were treated with local application of Gelfoam soaked in mitomycin C.
(0.5mg/ml) for 1 week. Alternatively, they were treated with Gelfoam soaked in mitomycin C (0.5mg/ml) for 10 min and then in dexamethasone (5mg/ml) for 1 week. Perforation maintenance rates were found to be 65% and 75%, respectively. Both methods have also been proven to have consistent reproducibility, and later both techniques were used successfully to assess the efficiency of chitosan patch scaffolds (Choi et al. 2011; Seonwoo et al. 2013b). The mechanism of chronicity in this animal model is thought to be the synergistic effect. Thermal injury, plus the chemical effects of mitomycin C (inhibition of DNA synthesis and function via cross-linking of DNA) in addition to dexamethasone (reduced cellular proliferation, delay macrophage chemotaxis and decrease hyaluronan synthesis), and mechanical inhibition of Gelfoam after placement result in an additive effect preventing healing (Seonwoo et al. 2013a; Choi et al. 2011). Although this model remains controversial (Santa Maria 2013), a recent study reports the success of a similar method in guinea pigs, utilising thermal myringotomy, topical application of Gelfoam with mitomycin C (0.2mg/ml) for 5 min, and then hydrocortisone (100µl) with ciprofloxacin for 7 days (Jang et al. 2013). Currently, in the literature, there is a lack of evidence on these potential methods in creating a chronic animal model, which warrant future investigations in order to compare and confirm their efficacy.
6.4.2. Efficacy of SFS and ACS compared to the autografts

Currently, autografts such as temporalis fascia and cartilage remain the most commonly used grafting materials. However, this study has indicated SFS and ACS to be optimal synthetic TM scaffolds for potential use. Therefore, further studies to evaluate their efficacy compared to autografts as controls are warranted prior to clinical trials. This would be technically challenging with rodents, due to the lack of fascia in the temporal or periauricular region in these animal models (Hanna et al. 1993; Schiller & Wormald 1992). To overcome this issue, large animal models (e.g. rabbit, dog, pig or monkey) may be required as they possess the required tissues. If rodents are to be used, then autologous fascia harvested from alternative regions, such as lumbar fascia or fascia lata may be used (Downey et al. 2003; Indorewala 2002). However, this may be associated with disadvantages such as an additional incision and longer operation times.

6.4.3. Suitability of SFS and ACS using different myringoplasty techniques

There are several surgical techniques for placing the TM graft for myringoplasty: onlay, overlay and underlay. In brief, the graft material can be placed lateral to the TM perforation (onlay or overlay) or medial to it (underlay). In general, the choice of operative technique depends on the site of the perforation and the surgeons’ preference (Aggarwal et al. 2006; Levin et al. 2012). The underlay technique is ideal for posterior perforations, while the overlay technique is typically reserved for total perforations anterior perforations, or failed underlay surgery (Kartush et al. 2002). The surgical
outcomes from this study were based on the onlay technique for experimental convenience. Further investigation of the efficacy of SFS and ACS using other operative techniques may be beneficial, especially the more commonly used underlay technique (Singh et al. 2003). This may provide valuable information for different clinical situations and aid to design an appropriate scaffold. From an otologist’s point of view, a TM graft/scaffold suitable for all types of perforation and technique will be attractive in myringoplasty surgery.

6.4.4. Biomechanical testing of the repaired TMs

A major goal of myringoplasty is the restoration of sound conduction towards the inner ear to recover hearing. Following an operation, graft materials need to provide sufficient stability to resist ME negative pressure, especially in chronic tubal dysfunction, while allowing adequate acoustic sensitivity during the TM healing (Huttenbrink 2004). Therefore, specific mechanical properties (e.g. elasticity and tensile strength) are thought to be essential for the TM graft materials (Kim et al. 2009). Our study indicated that SFS and ACS had adequate strength and flexibility, suggesting that both may be suitable scaffolds for TM reconstruction.

Although the histological results in this study showed the therapeutic potential of SFS and ACS, further detailed acoustic and biomechanical testing of the repaired TMs is required. Indeed, studies have shown that when a TM perforation heals, its fibrous structure and elasticity features may not be
restored (Huttenbrink 2004). This may cause loss of acoustic transfer properties of the TM and its stability in response to atmospheric pressure changes. From an acoustic point of view, elasticity is of primary importance for the healed eardrum (Kim et al. 2010b). As a consequence, this may result in a weakened and flaccid TM, which is associated with complications such as suboptimal hearing, retraction pockets, adhesive fibrous OM, and even reperforation (Forsen 2000; Cassano & Cassano 2010). This may in part explain the significant decrease of long-term healing results postoperatively. Meanwhile, normal hearing (ABG<10dB) is reported in only 37-80% of cases in the literature (Packer et al. 1982; Levin et al. 2009). Hence, we believe biomechanical testing to be a crucial parameter in evaluating the success of TM repair. To do this, current modern laser-assisted measuring techniques, such as laser doppler vibrometry (LDV) can be utilised. In addition, the correlation among histology, biomechanical and acoustic properties needs to be further investigated as a whole in the future for assessing therapeutic outcomes.

6.5. Conclusion

In summary, based on the preclinical outcomes, this thesis has demonstrated a collection of evidence that suggests SFS and ACS as potential substitutes for TM perforation reconstruction. We have evidenced that tensile strength and elasticity of SF and AC were in the range for human TM. SF and AC were compatible in SC and ME implant assays for up to 26 weeks. In rats and
guinea pigs, the healing of perforated TM was similar. SF and AC significantly accelerated TM perforation closure, obtained optimal TM thickness, and resulted in better trilaminar morphology with well-organised collagen fibres and early restoration of hearing. By contrast, paper patch and Gelfoam lost their scaffold function in the early stages and showed an inflammatory response, which may have contributed to delayed healing. In conclusion, the findings presented in this thesis suggest that SFS and ACS both are biocompatible and clinically efficacious grafting materials for repairing TM perforations. Future research will be aimed at further characterising the SFS and ACS. This would involve fully investigating the mechanism through which these scaffolds promote TM regeneration. Additionally, further assessment and development of SFS and ACS would bring the work in this thesis from a lab setting towards future clinical applications. In the near future, we are likely to see major advancements in this area of research, which will be beneficial not only to patients but also to otolaryngologists.
7. Bibliography


8. Appendices
Appendix 1


doi:10.1088/1748-6041/9/1/015015

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Appendix 2


doi:10.1089/ten.tea.2012.0053

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DOI: 10.1002/lary.23940

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DOI: 10.1002/lary.21470

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doi:10.1089/ten.teb.2012.0389

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DOI:10.1517/14712598.2012.634792

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