PHYSICO-MECHANICAL AND BIOLOGICAL EVALUATION OF THREE-DIMENSIONAL PRINTED THERMOPLASTIC POLYURETHANE AND POLYLACTIC ACID SCAFFOLD FOR TRACHEAL TISSUE ENGINEERING

ASMAK BINTI ABDUL SAMAT

UNIVERSITI SAINS MALAYSIA

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by

ASMAK BINTI ABDUL SAMAT

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LIST OF SYMBOLS

α	Alpha
μ	micro
T_g	Glass transition temperature
T_m	Melting temperature

LIST OF ABBREVIATIONS

2D	Two dimensional
3D	Three dimensional
AA	Antibiotic-Antimycotic
ABS	Acrylonitrile butadiene styrene
AM	Additive manufacturing
ANOVA	Analysis of variance
ARASC	Animal Research and Service Centre
ASTM	American Society for Testing and Materials
ATCC	American Type Culture Collection
CAD	Computer-aided design
CHAPS	3-[(3-cholamidopropyl) dimethylammonio]-1-propanesulfonate
CO_2	Carbon dioxide
CT	Computed tomography
DMSO	Dimethyl sulfoxide
DPX	Dibutylphthalate Polystyrene Xylene
DSC	Differential scanning calorimetry
ECM	Extracellular matrix
EO	Ethylene oxide
ELISA	Enzyme-linked immunosorbent assay
FBS	Fetal bovine serum
FDM	Fused deposition modelling
FESEM	Field Emission Scanning Electron Microscope
FTIR	Fourier Transform Infra-red
H&E	Hematoxylin and eosin
HDPE	High-density polyethylene
HMDS	Hexamethyldisilazane
IPS	Institut Pengajian Siswazah
ISO	International Organization for Standardization
LA	Lactic acid
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide
PBS	Phosphate buffered solution

PCL	Poly(Ɛ-caprolactone)
PGA	Polyglycolic acid
PLA	Polylactic acid
PLGA	Polylactide-co-glycolide acid
POSS-PCU	Polyhedral oligomeric silsesquioxane poly(carbonate-urea) urethane
PTFE	Polytetrafluoroethylene
RP	Rapid prototyping
SD	Standard deviation
SEM	Scanning electron microscopy
STL	Standard triangle/tessellation language
T_g	Glass transition temperature
T_m	Melting temperature
TPU	Thermoplastic polyurethane
USM	Universiti Sains Malaysia
YM	Young's Modulus
α-MEM	Alpha Minimum Essential Medium

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PENILAIAN FIZIKO-MEKANIKAL DAN BIOLOGI PERANCAH BERCETAK TIGA DIMENSI TERMOPLASTIK POLIURETANA DAN ASID POLILAKTIK BAGI KEJURUTERAAN TISU TRAKEA

ABSTRAK

Pembedahan penggantian trakea merupakan proses yang rumit dan memerlukan penggunaan bahan yang serasi, stabil secara mekanikal, terdegradasi secara perlahan-lahan dan tidak toksik, sebagai satu kaedah untuk mengatasi kekurangan dan kebergantungan ke atas penggunaan trakea alograf. Perancah trakea bercetak tiga dimensi (3D) yang dihasilkan daripada polimer merupakan satu pilihan untuk menangani beberapa cabaran dalam pemindahan trakea. Pengadunan polimer merupakan satu pendekatan untuk mencipta bahan dengan sifat khusus untuk membina perancah trakea. Penyelidikan ini bertujuan untuk menilai sifat fizikal, mekanikal serta biologi campuran dua bahan polimer iaitu termoplastik poliuretana (TPU) dan asid polilaktik (PLA) sebagai bahan yang berpotensi untuk penggantian trakea. Kedua-dua bahan terkenal dengan ciri-ciri yang menarik apabila digunakan secara berasingan, oleh itu digunakan secara meluas dalam pelbagai aplikasi, terutamanya dalam bidang bioperubatan. Oleh yang demikian, gabungan kedua-dua bahan berpotensi menghasilkan komposit dengan kualiti yang sesuai untuk penggantian trakea. Komposisi TPU dan PLA yang berbeza dicampur, diikuti dengan penilaian pencirian sifat fizikal dan mekanikal, ciri terdegradasi secara *in vitro,* serta sifat toksik kedua-dua bahan tersebut. Selepas itu, perancah berbentuk cakera telah dibina daripada TPU/PLA dengan nisbah filamen 90:10 menggunakan kaedah cetakan 3D dan dinilai untuk morfologi, keliangan, dan tindak balas sel terhadap perancah. Selain itu, ekstrak biodegradasi telah diuji pada sel BEAS2B untuk menilai

keupayaan percambahan dan penyembuhan luka secara *in vitro*. Akhirnya, implantasi subkutaneus perancah bercetak 3D telah dilakukan untuk menilai kesesuaian biologi perancah dalam model tikus pada titik masa yang berbeza. Penilaian histopatologi telah dijalankan untuk mengkaji kesan tempatan perancah pada percambahan selular dan tisu, tindak balas keradangan, angiogenesis, dan ketoksikan sistemik. Keputusan menunjukkan bahawa, walaupun tidak bercampur, TPU dan PLA adalah serasi secara fizikal sebagai komposit, dan sifat mekanikalnya adalah hampir sama dengan trakea manusia. Kompaun dan ekstrak yang diadun adalah biokompatibel dan tidak toksik kepada sel, dan kadar biodegradasinya yang perlahan sesuai untuk menjana semula tisu seperti trakea. Selain itu, perancah berliang TPU/PLA menunjukkan keserasian biologi dan menggalakkan perlekatan selular, penghijrahan dan percambahan secara *in vitro*. Penemuan ini disokong oleh tindak balas tisu tempatan yang menggalakkan dan *angiogenesis* berikutan implantasi subkutan dalam model arnab. Kesan perancah ini pada tisu sekeliling dan organ distal mencadangkan keselamatan dan sifat tidak toksiknya pada organ penting dan juga seluruh badan. Kajian ini mencadangkan bahawa teknologi percetakan 3D membolehkan pengeluaran rekabentuk yang sesuai berdasarkan parameter tertentu, dan TPU dan PLA sebagai bahan boleh digunakan untuk membina perancah dengan ciri-ciri yang diingini, menawarkan alternatif kepada cabaran pemindahan trakea.

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ABSTRACT

Surgical restoration of extensive tracheal lesions is complicated, and it necessitates the use of a biocompatible, mechanically stable, and non-toxic material that degrades gradually to overcome the limitation of allografts. A three-dimensional (3D) printed tracheal scaffold produced from polymers is one option for addressing some of the challenges in tracheal transplantation. Polymer blending is one approach for creating material with specific properties for a trachea scaffold. The goal of this study was to evaluate the physical, mechanical, and biological properties of thermoplastic polyurethane (TPU) and polylactic acid (PLA) blend as prospective tracheal replacement materials. Both materials are well-known for their promising properties when utilised independently, thus broadly employed in various applications, especially in the biomedical field. It is hypothesised that combining the two materials and the subsequent 3D printing method produces a composite with suitable qualities for tracheal replacement. Different TPU and PLA compositions were melt-blended and characterised for their physical and mechanical properties, including *in vitro* degradability and toxicity. Subsequently, disc-shaped scaffolds were constructed from TPU/PLA with a ratio of 90:10 filament using the 3D printed method and evaluated for their morphology, porosity, and cell response towards the scaffold. In addition, the biodegradation extract was tested on BEAS2B cells to assess its proliferation and wound-healing ability *in vitro*. Finally, subcutaneous

implantation of the 3D printed scaffold was performed to assess the biocompatibility of the scaffolds in a rat model at different time points. Histopathological assessment was conducted to examine the local effects on cellular and tissue proliferation, inflammatory response, angiogenesis, and systemic toxicity. TPU and PLA are physically compatible as a composite, despite being immiscible, and their promising mechanical qualities are almost equivalent to the human trachea. The blended compound and extract were biocompatible and non-toxic to the cells, and its slow biodegradation rate was suitable for regenerating tissues such as the trachea. In addition, TPU/PLA porous scaffolds showed good biocompatibility and promoted cellular attachment, migration and proliferation *in vitro*. These findings were corroborated by favourable local tissue response and angiogenesis following subcutaneous implantation in a rabbit model. The effects of these scaffolds on surrounding tissue and distal organs suggest their safety and nontoxicity on vital organs and, consequently, the entire body. This study suggested that 3D printing technology allows for the creation of personalised designs based on specific parameters, and TPU and PLA as materials could be employed to construct scaffolds with desirable characteristics, offering an alternative to tracheal transplant challenges.

CHAPTER 1

INTRODUCTION

1.1 Introduction

The trachea is one of the vital organs in the respiratory system. Disorders of the trachea can lead to serious health complications and potentially fatal conditions in both paediatric and adult populations (Best *et al.*, 2018; Etienne *et al.*, 2018; Damiano *et al.*, 2021). Airway disorders can be caused by multiple etiologies, including cancer, infection, congenital abnormalities, trauma, or stenosis (Greaney and Niklason, 2021). Airway diseases requiring surgical intervention have increased in recent years due to the higher incidence of respiratory tract cancer. Based on the data published in the Global Burden of Disease Study 2019, cancer of the trachea, bronchus, and lung became the largest cause of cancer fatalities and the second major cause of new cancer cases worldwide (Abbafati *et al.*, 2020). Similarly, in Malaysia, according to the Department of Statistics Malaysia, the cases of malignant neoplasm of the trachea, bronchus and lung showed an increment of 0.1% from 2019 to 2020, ranked the fifth most common cause of death (Department of Statistics Malaysia Official Portal, 2020).

The treatment for extensive trachea injury is complex. Since the 1950s, the "gold standard" clinical strategy for the management of most airway disorders, such as tracheal stenosis, tracheomalacia, and tumours, was tracheal resection and end-to-end anastomosis (Kalathur, Baiguera and Macchiarini, 2010; Bogan, Teoh and Birchall, 2016). However, in extensive lesions, resection retains insufficient length of the original airway for primary reconstruction, making safe reconstruction results unattainable (Grillo, 2002). By rule of thumb, severe lesions of greater than half of

the tracheal length in adults or one-third in small children indicate the need for a tracheal reconstruction or replacement (Grillo, 2002; Kucera *et al.*, 2007; Haykal *et al.*, 2014; Etienne *et al.*, 2018). Severe injuries pose a significant challenge to clinicians because of the inherent difficulties associated with any surgical intervention on the airway (Bogan, Teoh and Birchall, 2016; Greaney and Niklason, 2021). It may cause a delay in diagnosis, resulting in early fatal outcomes or late sequelae such as airway stenosis and recurrent pulmonary infections (Prokakis *et al.*, 2014; Damiano *et al.*, 2021).

For almost a century, clinicians and researchers have attempted to develop a graft to replace long-segment tracheal defects. The spectrum of tracheal replacements used clinically ranges from autologous tissue flaps and patches, allograft transplants, synthetic stents and prostheses to tissue-engineered scaffolds (Kucera *et al.*, 2007; Delaere and Van Raemdonck, 2016; Etienne *et al.*, 2018; Greaney and Niklason, 2021). Tracheal transplantation was once considered one of the best options (Grillo, 2002). However, finding a suitable donor is a great obstacle due to global shortages of tissues and organs, which has been a significant public health problem for many years (HRSA, 2020). According to the Organ Procurement & Transplantation Network, United States Department of Health and Services, the number of patients on the national transplant waiting lists for all types of organ in the United States of America (USA) until July 2019 has increased to more than one hundred thousand. Out of the number, two-thirds are above 50 years old, while almost 2,000 are below eighteen years old, and only one-third of the total number received organ transplants (Jones and Bes, 2012; Giwa *et al.*, 2017; US Health Resources and Services Administration, 2019). Even then, if a transplant is possible, there is a high chance of immune rejection requiring long-term immunosuppressants, which increases the

patient's morbidity and mortality risks (Grillo, 2002; Haykal *et al.*, 2014; Hancox *et al.*, 2019). In addition, the transplant is compromised by the lack of mechanical strength and unmatched size, which requires stenting and invasive repeated surgeries due to various complications (Grillo, 2002; Delaere and Van Raemdonck, 2016).

Even though experimental and clinical tracheal repair dates back to the late 1800s, no clinically convincing tracheal replacement procedure has been established (Tan *et al.*, 2006; Bogan, Teoh and Birchall, 2016; Greaney and Niklason, 2021). Before 1950, nondegradable synthetic materials were used as replacement airways to minimize the narrowing and collapse of soft fascial-derived grafts. Success was variable, beginning with rigid tubes made from polymers (Longmire, 1948) and metal or glass tubes to provide patency to the conduit. Still, all grafts eventually migrated, dislodged, blocked, and encouraged infection at the graft interface. Erosion of the arteries was inconsistent and frequently lethal (Chiang *et al.*, 2016; Greaney and Niklason, 2021). Regardless of these complications, a stiff prosthesis can briefly maintain an open airway without a healing response.

Additional attempts were made using porous materials to encourage tissue ingrowth and reepithelization, and avoid dislodgement. These were employed by porous metallic or polymeric wire, mesh (Beall *et al.*, 1962), or gauze (Koontz and Kimberly, 1953) as an intermediate ground between soft fascia or skin and hard polymeric grafts to resist collapse of the luminal area while allowing some flexibility which is crucial to normal physiologic head and neck movements. Host cell infiltration and deposition of connective tissue were expected to support epithelial migration. Fabric meshes were often reinforced with wire, plastic rings, or coils and covered with biopolymers such as fibrin or collagen to ensure an airtight seal at the

implant. However, scar tissue deposition in these grafts frequently resulted in blockage and stenosis. Large areas of mesh frequently remained uncovered, leading to bacterial growth. Only sufficiently short grafts were fully recellularized (Greaney and Niklason, 2021).

Following the introduction of immunosuppressive therapies in the 1980s and 1990s, fresh, frozen, or decellularized allograft transplants were utilised, resulting in considerable increases in survival in patients with solid organ and composite tissue transplants (Cooper *et al.*, 1989; Strome *et al.*, 2001). However, preserved allograft transplants in patients ended with necrosis and collapse of the conduit rendering support from the stents (Jacobs *et al.*, 1996; Delaere *et al.*, 2010). Failure to promote revascularization in the nonviable grafts at the transplant site led to attempts of heterotopic transplantation. In the heterotopic transplant method, the allografts were transplanted in a highly vascularized region of the recipient before tracheal implantation (Delaere & Hermans, 2003). Yet, many complications were reported, and the most common are stricture, forearm fistula, and infection at both the forearm incision site and after orthotopic transplantation (Delaere et al., 2012). There were also reports of the procedure failing when the graft in the forearm was necrosed (Loos *et al.*, 2016).

Another approach of allotransplantation employs contemporary decellularization methods to remove all cellular components from the cadaveric trachea, leaving primarily extracellular matrix proteins (Gilbert, Sellaro and Badylak, 2006). The decellularization procedure aims to produce immunologically inert tissue. Thus, provides a trachea scaffold with superior biomechanical qualities and a functional extracellular matrix (ECM) and reduces the chance of immunological

response (Gilbert, Sellaro and Badylak, 2006; Haykal *et al.*, 2012). Despite providing temporary functional airways to patients for several years, these procedures were also associated with severe morbidity. Survived patients required multiple post-operative surgeries to repair the complications related to the grafts, such as stenosis, graft collapse or adverse host response (Haykal *et al.*, 2012; Partington *et al.*, 2013; Hamilton *et al.*, 2015). In particular, when applied to paediatric patients, the technical feasibility is obscured by a high incidence of major complications and long-term uncertainty (Jungebluth *et al.*, 2012).

Advances in tissue engineering provide promising alternative approaches to assembling functional constructs that repair, preserve, or enhance defective tissues or organs (Lysaght and Reyes, 2001; Law *et al.*, 2016; Han *et al.*, 2020). Cells expanded in the three-dimensional scaffolds serve as an adhesive substrate for the implanted scaffold and provide a mechanical framework leading to the formation of new organs (Viola, Lal and Grad, 2003). The approach was initially founded to deal with the critically unmet demand of the markedly growing number of organ transplant patients on waiting lists. Langer and Vacanti were the first to demonstrate that scaffold composition is a crucial element that can speed tissue regeneration even without cells or growth agents (Vacanti and Langer, 1999). The scaffolds can be biological, such as a decellularised allotransplant or non-biodegradable or biodegradable synthetic materials.

Biodegradable polymers are gaining popularity in tracheal tissue engineering, particularly in pediatric populations, due to the limited treatment options available to children compared to adults (Gao *et al.*, 2017). Apart from providing mechanical support for the injured trachea, the scaffold should facilitate cellular migration and

proliferation, tissue modification and degradation at an appropriate rate during growth to eliminate recurrent surgeries (Bogan, Teoh and Birchall, 2016; Park *et al.*, 2019). Although synthetic scaffolds demonstrate promise for future applications, biocompatibility, graft mobility, and poor integration with the host tissue are concerns that must be addressed. Additionally, biodegradable scaffolds face particular challenges, such as the release of toxic degradation products and the loss of mechanical characteristics over time (Bogan, Teoh and Birchall, 2016).

Polylactic acid (PLA) (Wu *et al.*, 2017; Wang *et al.*, 2019), polyglycolic acid (PGA), and poly(Ɛ-caprolactone) (PCL) (Tsao *et al.*, 2014; Chan *et al.*, 2020), polyurethane (Hsieh *et al.*, 2018), and polyethylene terephthalate (PET) (Gustafsson *et al.*, 2012) are the most extensive biodegradable polymers utilised in tracheal tissue engineering. PGA, PLA, and their copolymers, PLGA, were reported to have good mechanical properties and are biocompatible with adjustable degradation rates (Kim *et al.*, 1994; Dean *et al.*, 2003). However, each of these elements poses several other issues. For instance, the biodegradable PGA products evoke some local acid environment which is not conducive to cell growth and development (Luo *et al.*, 2009). As an ECM, the scaffold plays an important role; however, it is often unable to determine the exact microenvironment during tissue growth to facilitate *in vitro* or *in vivo* tissue development. In addition, these artificial trachea scaffolds are yet to be clinically successful, and most of the research and experiments are up to preclinical stages only. Thermoplastic polyurethane (TPU) is often employed for blood vessel scaffolds due to its high flexibility. TPU has been utilised to construct a tracheal replacement in an effort to increase the rigidity and elasticity of the trachea scaffold (Ahn *et al.*, 2019).

Different procedures are used to fabricate tracheal scaffolds with high precision. Electrospinning (Townsend *et al.*, 2018), solvent casting (Naito *et al.*, 2011) or thermally induced phase separation (Jing *et al.*, 2014), three-dimensional (3D) printed technology or additive manufacturing (Gao *et al.*, 2017; Xia *et al.*, 2019), and the most recent technology of 3D bioprinting using bio-ink (Bae *et al.*, 2018), are some of the methods used to produce tracheal scaffold that has been evaluated in animal models.

The safety of implanted biodegradable materials is critically important because they are implanted into the body and remain close to human tissues for an extended time (Fournier *et al.*, 2003; Vaisman *et al.*, 2010; Nyska *et al.*, 2014). Once implanted, the local response around grafted biomaterials becomes a major indicator of the safety of the biomaterial. The aspects of tissue response to biomaterials are critical in research and development for performance, safety, and regulatory purposes, thus requiring proper evaluation. Robust protocols of widely respected standards are available for evaluating the nature of such tissue reactions (Muhamed *et al.*, 2015; Khorramirouz *et al.*, 2018). According to the International Organization for Standardization (ISO) and the Food and Drug Administration (FDA), not only *in vitro* assessment but *in vivo* testing must be performed to assess the local effects after implantation before new biomaterials are used as medical devices (ISO/EN10993-6, 2007)(US FDA, 2020). The primary objective of the test method is to characterise the history and evolution of tissue response after implantation of a medical device or biomaterial, including final integration and absorption or degradation of the material. The degradation characteristics of the material, as well as the resulting tissue response, should be determined, particularly for degradable materials. The tests provide researchers with information on the proposed manufacturing processes and

design of tissue-engineered scaffolds during the early phases of development. Subsequently, these tests should be performed again using the final manufacturing and sterilisation conditions as in the final product (Khorramirouz *et al.*, 2018).

1.2 Problem statement

Surgical replacement of extensive tracheal defects was first undertaken in the late 1800s, with clinical advances in materials and techniques. Since then, various materials, such as autografts, allografts, tissue flaps, prosthetic materials, stents, or a combination of these approaches, have been described in experimental and clinical settings (Damiano *et al.*, 2021; Greaney and Niklason, 2021). However, the outcomes vary greatly due to the presence of several complications. There have been reports of failure at the site of implantation, such as migration and dislodgement of the scaffold; as well as infection, granulation tissue, necrosis, and erosion of major blood arteries that lead to stenosis. In addition, the requirement for lifelong immunosuppression and lack of eligible donor sources are the limiting factors, particularly in allograft transplants. (Haykal *et al.*, 2014; Law *et al.*, 2016; Boazak and Auguste, 2018).

Due to the limitation of organ transplants for allografts, and with the advanced technology in the tissue engineering field, the use of biomimetic material as a scaffold in tissue engineering is gaining worldwide attention (Kojima and Vacanti, 2014; Bogan, Teoh and Birchall, 2016). Tissue-engineered polymeric implants are preferable as they do not elicit a significant immune response, often seen in allografts (Bogan, Teoh and Birchall, 2016). Tracheal transplantation requires a biological and mechanically stable scaffold with some flexibility. An ideal synthetic scaffold that is biocompatible, timely degraded and eliminated by the body

system, with appropriate and physical-mechanical qualities that can be easily replicated when needed, and individually custom-made to prevent prosthesis failure is required (Grillo, 2002; Etienne *et al.*, 2018). 3D printing is one of the widely employed methods for medical research in recent years because it allows rapid production of complicated multi-layered structures tailored to an individual trachea for tissue replacement (Soriano *et al.*, 2021). Thus, the physical and mechanical properties of the materials can be customised according to the specific application through modification of the techniques (Ligon *et al.*, 2017). Among the materials widely investigated are PLA and its copolymers, PGA and PLGA; PCL and PET; however, each possesses problems related to either mechanical instability, inflammatory response or degradation rate or its by-products.

Thus, we aimed to develop a material from biodegradable polymers, namely TPU and PLA. Both materials are considered biocompatible and biodegradable, which have been used widely in biomedical fields. The TPU and PLA blends were produced through the melt blending technique to produce filament feedstock. Subsequently, the scaffold was fabricated using a 3D printing technique and characterized to evaluate its physical, mechanical, and biological properties. The TPU/PLA blended matrix is expected to provide good mechanical properties by possessing suitable mechanical strength and flexibility between the TPU and PLA and biocompatible with the cells and tissues as potential material for tracheal tissue engineering. This study involves the development of the material, characterization of the blended materials, and evaluation of the 3D printed scaffold *in vitro* and in the animal model of a rat.

1.3 Objectives of the study

1.3.1 General objective

To evaluate the physicomechanical and biological properties of thermoplastic polyurethane (TPU) and polylactic acid (PLA) blends as a potential 3D scaffold for tracheal tissue engineering.

1.3.2 Specific objectives

1. To fabricate and characterise the physical and mechanical properties of different compositions of TPU and PLA blends for the tracheal scaffold.
2. To evaluate the physical and biological properties of the 3D-printed TPU/PLA scaffolds in a cell culture study.
3. To evaluate the inflammatory response, angiogenesis, and tissue integration of the 3D tracheal scaffolds following subcutaneous implantation in the rat; and to assess the toxicity of the 3D printed TPU/PLA in the kidney and liver by histopathological analyses.

1.4 The hypothesis of the study

The 3D printed TPU/PLA blend scaffold has suitable physical and mechanical properties, as well as biocompatibility for tracheal tissue engineering.

CHAPTER 2

LITERATURE REVIEW

2.1 Anatomy of the trachea

The trachea is a fibrocartilaginous hollow conduit that connects the larynx to the bronchi of the lungs which is responsible for air transmission. The trachea starts from the lower border of the cricoid cartilage extending to the carina level (Minnich and Mathisen, 2007; Mehran, 2018). It comprises semicircular cartilaginous rings, dorsally flattened and connected by annular ligaments of fibroconnective tissue (Furlow and Mathisen, 2018). The tracheal wall is cartilage-free in the posterior and composed of longitudinally oriented smooth muscle, called trachealis muscle and fibrous connective tissue. The length and diameter of the trachea vary with age, sex, race, and stature of an individual. Taller-statured individuals tend to have longer trachea and vice versa (Allen, 2003; Furlow and Mathisen, 2018). Upon deep inspiration, the length increases by ten per cent and may decrease up to thirty per cent while coughing. During expiration, the trachealis muscles of the posterior walls are pulled towards each other while the configuration of cartilaginous walls remains unchanged (Mehran, 2018). The luminal shape varies, being almost circular in children, gradually changing to an ovoid shape with growth and age, and in the presence or absence of disease (Breatnach, Abbott and Fraser, 1984; Holbert and Strollo, 1995; Allen, 2003; Minnich and Mathisen, 2007; Drevet, Conti and Deslauriers, 2016).

The cartilage is one of the stiffest anatomical components essential in providing the trachea's mechanical stability (Partington *et al.*, 2013; Safshekan *et al.*, 2016). Despite variations in intrathoracic pressure, the airways are prevented from collapsing and air passage narrowing, directly affecting the trachea's physiological

respiratory function (Roberts *et al.*, 1998). The free dorsal end of the rings at the posterior is connected by predominantly transverse-oriented, with some longitudinal or oblique muscle fibres known as the trachealis muscle (Aung *et al.*, 2019). These muscle fibres contract on physiological reflexes or actions such as coughing, generating bending and tensile stresses that modulate the luminal diameter of the trachea and increase the airflow velocity, aiding the dislodging of mucus and foreign particles (Roberts *et al.*, 1998; Allen, 2003). The lengthening and shortening of longitudinal collagenous and elastic connective tissue fibres are related to the individual cartilages during swallowing or movements of the neck. Airway cartilage undergoes several stresses over relatively long and brief periods. In normal respiration, which occurs for minutes or hours, cartilage provides a reasonably robust load to the trachealis muscle. It maintains tracheal wall rigidity, produced by changes in trachealis muscle tones (Murray, 1993). In contrast, transmural pressure change occurs during forced expirations or cough in less than a second; viscoelastic characteristics of the airway cartilage play a vital role in preserving the airway calibre (Roberts *et al.*, 1998).

Physiologically, the trachea serves not solely as the conduit to pass air from the nostril to the lungs. It warms, humidifies and cleans the air and withstands the intrathoracic stresses or forces from normal respiration, swallowing and coughing reflexes (Weinberger, Cockrill and Mandel, 2018). Its histological structures can be divided into four distinct parts, representing their functions: the mucosa, submucosa, cartilaginous layer, and the outermost part of the trachea, adventitia. The luminal surface is lined by pseudo-stratified ciliated columnar epithelium composed of ciliated cells, goblet cells, basal cells, and neuroendocrine cells (Salassa, Pearson and Payne, 1977; Rock, Randell and Hogan, 2010; Brand-Saberi and Schäfer, 2014). Mucus and

water secreted by goblet cells humidify and moisten the luminal parts and eliminate foreign particles from the air as it flows through the trachea. Motile cilia clusters on the apical surface that beats in coordinated waves are crucial in aiding mucociliary clearance (Brand-Saberi and Schäfer, 2014; Tilley *et al.*, 2015). Basal cells and brush cells are other primary cell types of the tracheal epithelium. Basal cells are centred on the basal lamina and exhibit stem cell-like properties that allow the homeostatic event of the normal epithelium following an injury or during tissue renewal (Rock, Randell and Hogan, 2010). The deeper layer of the submucosa is mainly made up of elastin, submucosal glands, and smooth muscle supplied by blood vessels. Externally, the submucosa layer comprises C-shaped cartilage that provides support for the airway, anteriorly and laterally. The adventitia is the outermost layer encompassing a loose connective tissue band that links the trachea to the oesophagus and other adjacent structures (Brand-Saberi and Schäfer, 2014).

Blood flow is segmented over its whole length throughout the lateral walls of the trachea. Its vessels made their way from the lower thyroid, subclavian, superior intercostal, inner thoracic, native, and upper and mid-bronchial arterials and emerge from their lateral pedicles. These vessels attach along the lateral surface and form significant vascular anastomoses in the longitudinal direction. The lateral and anterior tracheal walls are supplied by the transverse segmental arteries that pass between the cartilage rings from these two lateral longitudinal networks. The transverse vessels provide endotracheal mucosa with capillary beds, which nurture the cartilage through diffusion. The posterior membranous part of the cartilage is supplied by the esophagal arteries and subdivisions (Salassa, Pearson and Payne, 1977; Haykal *et al.*, 2014). The anatomy of the trachea is illustrated in Fig. 2.1.

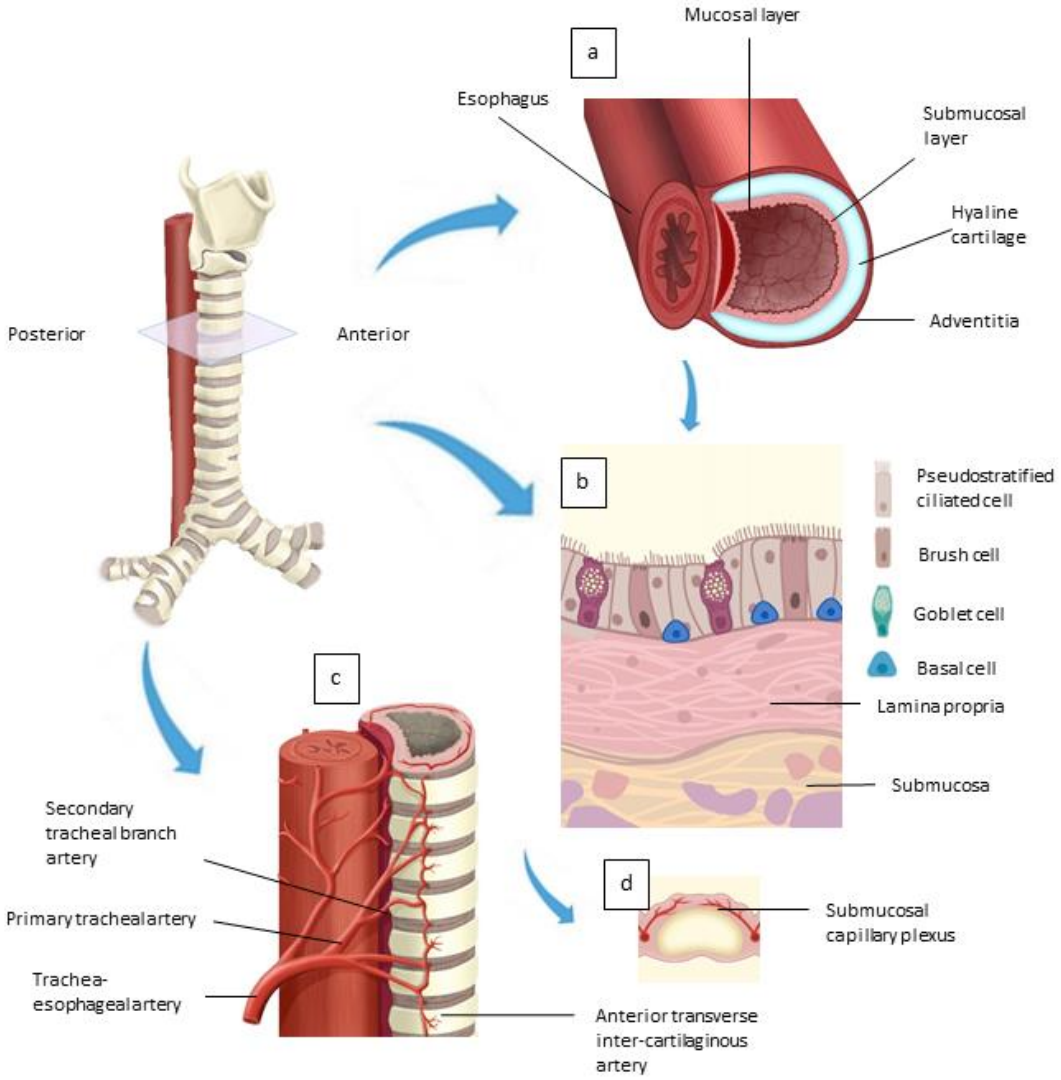

Figure 2.1 Illustrations of the trachea and oesophagus, and multiple layers of the trachea. a) Cross-sectional anatomy showing layers of the trachea, b) Mucosa and submucosal layers indicating types of cells, c) Blood supply to the trachea, and d) submucosal capillary plexus supplying the cartilage ring.

2.2 Types of tracheal disorders indicated for replacement

Since the trachea is a vital organ in the respiratory system, any disorders can lead to serious health complications and potentially fatal conditions in both paediatric and adult populations (Best *et al.*, 2018; Etienne *et al.*, 2018). Current tracheal reconstruction developments include tracheal dilatation with a rigid bronchoscope, laser surgery with endoluminal stent implantation, and surgical excision. Unfortunately, the first two techniques have a high rate of stenosis recurrence (Damiano *et al.*, 2021). Thus, tracheal resection with direct end-to-end anastomosis is the gold standard for treating tracheal stenoses, particularly when the stenotic tract is less than 4 cm (Bogan, Teoh and Birchall, 2016; Damiano *et al.*, 2021). However, in more extensive lesions, the general limits for safe resection are about one-half of the tracheal length in adults and one-third in small children (Haykal *et al.*, 2014; Abouarab *et al.*, 2017; Etienne *et al.*, 2018; Greaney and Niklason, 2021). If the limits are exceeded, surgical excision within safe margins of the affected airway is impossible, leaving only palliative therapies such as stenting, tumour debulking in cases of tumours, or radiotherapy to reduce the disease progression (Haag, Jungebluth and Macchiarini, 2013; Madariaga and Gaissert, 2018).

Several conditions most commonly indicated for tracheal reconstruction or replacement include malignant tumours (squamous cell carcinoma, adenoid cystic carcinoma), tracheoesophageal fistula, tracheomalacia and stenosis.

2.2.1 Tracheal cancer

Tracheal cancer can develop from epithelial cells, mesenchymal cells, or salivary glands. It is malignant in 90% of occurrences in adults but only 20 to 30 per

cent of cases in children (Urdaneta, Yu and Wilson, 2011; Haag, Jungebluth and Macchiarini, 2013; Madariaga and Gaissert, 2018). Adult primary tracheal tumours are predominantly malignant, but paediatric primary tracheal tumours are typically benign. Squamous cell carcinoma (SCC) is the most prevalent type of tracheal cancer, followed by adenoid cystic carcinoma (ACC). SCC occurs more frequently in males in their sixth and seventh decades. Meanwhile, ACC is distributed equally between both sexes and occurs in the fourth and fifth decades (Junker, 2014; Madariaga and Gaissert, 2018).

The incidence of tracheal cancers is 2.6 new cases per one million people per year, accounting for 0.1 to 0.4 per cent of newly diagnosed cancers annually. Despite the incidence of primary tracheal cancer appearing low, most tracheal cancers are secondary endotracheal metastases, which arise from direct invasion from adjacent tissue or hematogenous distribution (Madariaga and Gaissert, 2018). It is an alarming condition since cancer of the trachea, bronchus, and lung has become the leading cause of mortality worldwide and the second leading cause of new cancer cases worldwide, based on the data published in the Global Burden of Disease Study 2019 (Abbafati *et al.*, 2020). Furthermore, primary tracheal cancer showed poor prognostic factors such as extension into the thyroid gland and lymphatic invasion. The progression is defined by tracheal wall extension, mediastinal extension, and lymph node metastases (Honings *et al.*, 2009; Madariaga and Gaissert, 2018)

2.2.2 Tracheoesophageal fistula

An abnormal connection between the trachea and the oesophagus is known as a tracheoesophageal fistula (Bacon, Patterson and Madden, 2014). Any aggressive tracheal disease can compromise the integrity of the tracheal wall, resulting in

mediastinum communication (Ke, Wu and Zeng, 2015). The most common occurrences are iatrogenic, traumatic, and malignant tumours. Infection from tuberculosis, HIV infection, and mediastinitis, as an aetiological risk, has decreased in recent years. Congenital tracheoesophageal fistulas are most common in neonatal patients, but they can also occur in adulthood (Bacon, Patterson and Madden, 2014).

2.2.3 Tracheomalacia

Tracheomalacia is a condition of weakness of the tracheal wall in which the cartilage rings are not rigid enough to support the trachea from respiratory forces leading to airway collapse (Hysinger and Panitch, 2016; Muthialu *et al.*, 2020; Guedes *et al.*, 2022). The trachea slightly dilates during inspiration and narrows during expiration during normal function. In a patient with tracheomalacia, the membranous component of the trachea compresses inwards, significantly limiting the airway lumen. In severe situations, this might cause the airway to completely collapse upon expiration (Muthialu *et al.*, 2020). Primary tracheomalacia is an uncommon congenital disease of the tracheal rings frequently found in preterm or neonatal neonates, with clinical presentations requiring ventilation to maintain the respiratory system (Hysinger and Panitch, 2016). On the other hand, acquired tracheomalacia is frequently caused by significant infection and inflammation. It is most commonly seen after a bout of tracheitis, such as croup or another viral infection (Muthialu *et al.*, 2020).

2.2.4 Trachea stenosis

Stenosis is the airway narrowing at any anatomic level that encompasses various conditions, either congenital or acquired defects. Tracheal stenosis frequently causes variable degrees of vertical plane distortion in addition to airway restriction

(Bacon, Patterson and Madden, 2014; Guedes *et al.*, 2022). When the trachea is pulled away from or twisted within its usual anatomical course, it increases airway turbulence and resistance. Acquired airway stenosis may arise from trauma, infection, inflammatory and autoimmune disease, tracheomalacia or other causes (Guedes *et al.*, 2022).

2.3 Clinical trachea replacement over time

The experimental and clinical tracheal repair dates back to the late 1800s, employing a spectrum of materials and techniques to improve the survival rate of the patients. However, no clinically effective tracheal replacement procedure has been established (Tan *et al.*, 2006; Bogan, Teoh and Birchall, 2016; Greaney and Niklason, 2021). The timeline of the graft or scaffold types used for trachea transplant is summarised in Figure 2.2.

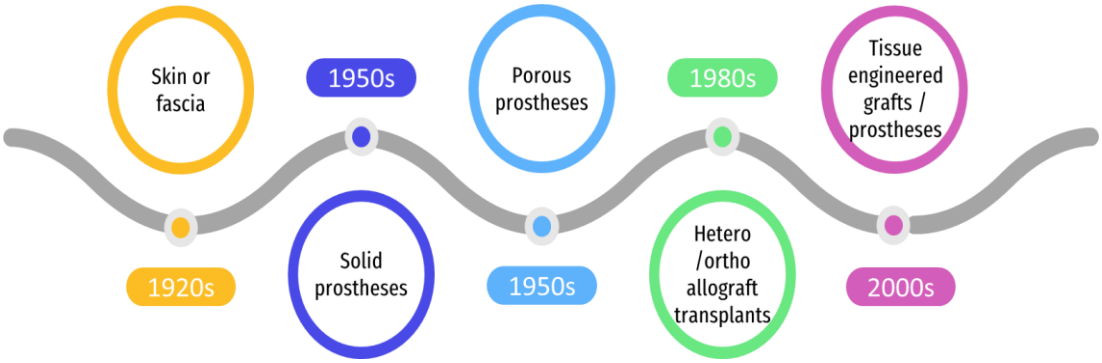

Figure 2.2 Summary of the grafts or scaffolds used in clinical trachea transplantation over the years. Skin and fascia were introduced in the 1920s, followed by solid, porous prostheses and allografts. Tissue-engineered prostheses are gaining attention in recent decades.

2.3.1 Skin or fascia only

Tracheal reconstruction became increasingly popular in the late 1890s and the 20th century. Initially, as with most surgical specialities, a knowledge base was developed primarily through case reports. At this time, the focus was on autogenous replacements, such as skin or skin and fascial grafts (Virk *et al.*, 2017; Greaney and Niklason, 2021). The graft was directly sutured to the remaining natural trachea, with the radial vessels being anastomosed to small neck vessels, thus restoring the graft's vascularization. While these grafts eliminate the requirement for foreign materials or immunosuppression and frequently revascularize effectively, they often necessitate treatments to remove secretions and stentings to treat blockage and collapse. This form of autologous scaffold has been utilised the longest of all graft types, from 1927 to 2018. Despite this, this technique has never acquired widespread popularity, most likely due to the difficulty of graft formation, the morbidity of several wound sites, and varied clinical outcomes (Greaney and Niklason, 2021).

2.3.2 Solid prostheses

From the 1950s to the 1970s, synthetic materials were extensively employed to build solid, tubular conduits as an alternative for resected trachea. Stainless steel (Gebauer, 1950; Sze, Samadi, & Conant, 1955), polymers such as polyethylene and polytetrafluoroethylene or Teflon, cobalt alloys, silicone rubbers, and methacrylates (Virk *et al.*, 2017; Greaney and Niklason, 2021) are among the materials used and tested in preclinical and clinical settings. Although success varied, all grafts ultimately migrated, dislodged, blocked, and promoted infection at the graft interface (Etienne *et al.*, 2018). Despite these problems, a stiff prosthesis can briefly maintain an open airway, even without a healing response. More attempts were undertaken using porous

materials to promote tissue ingrowth, reepithelization, and dislodgement (Tan *et al.*, 2006; Damiano *et al.*, 2021; Greaney and Niklason, 2021).

2.3.3 Porous prostheses

Concurrently during those decades, porous scaffolds such as meshes comprised of a mixture of materials were used in experimental and therapeutic settings (Damiano *et al.*, 2021; Greaney and Niklason, 2021). It was thought that host cell infiltration and connective tissue deposition would help epithelial migration. To create an airtight seal at the implant, fabric meshes were often reinforced by wire, plastic rings, or coils and covered with biopolymers such as fibrin or collagen (Bucher, Burnett, & Rosemond, 1951). Steel, stainless steel, titanium, polyethylene, Teflon, polyurethane, Dacron (polyester), and Polyvinyl acetal were examined experimentally (Greenberg and Willms, 1962). Scar tissue deposition in these grafts frequently resulted in blockage and stenosis. Large areas of mesh were often left unprotected, allowing bacteria to colonise.

2.3.4 Allograft transplantation and tissue-engineered scaffolds

Allograft transplants, either fresh, preserved or decellularized, were employed following the development of immunosuppressive therapies in the 1980s and 1990s, leading to significant improvements in survival for solid organ and composite tissue transplants (Cooper *et al.*, 1989; Strome *et al.*, 2001). Hollow structures such as tracheal and aortic grafts have been used in allograft transplantation (Delaere *et al.*, 1995). Fresh allografts must be transplanted immediately, and the recipient must be identified and prepared at almost the same time as harvest. Thus, it is usually not available in an emergency setting. In addition, the recipient usually requires life-long immunosuppression.

On the other hand, the harvested allograft tissue can be frozen or cryopreserved and stored when needed (Rich and Gullane, 2012). Tracheal allografts were treated with preservatives such as Eurocollins and formalin to enhance graft viability at the time of operation. However, preserved allograft transplants in patients ended with necrosis and collapse of the conduit rendering support from the stents (Jacobs *et al.*, 1996). Failure to promote revascularization in the nonviable grafts at the transplant site led to attempts of orthotopic transplantation. In this method, the allografts were transplanted in a highly vascularized region of the recipient before tracheal implantation (Delaere & Hermans, 2003). Yet, many complications were reported, and the most common are stricture, forearm fistula, and infection at both the forearm incision site and after orthotopic transplantation (Delaere *et al.*, 2012). There were also reports of the procedure failing when the graft in the forearm was necrosed (Loos *et al.*, 2016).

Another approach of allotransplantation employs contemporary decellularization methods to remove all cellular components from the cadaveric trachea, leaving primarily extracellular matrix proteins (Gilbert, Sellaro and Badylak, 2006). The cryopreserved tissues are decellularised through meticulous washing cycles using chemical detergents such as Triton-X-100, sodium dodecyl sulphate (SDS) and CHAPS detergent 3-[(3-cholamidopropyl) dimethylammonio]-1-propanesulfonate; enzymes such trypsin-ethylenediamine tetraacetic acid (EDTA) and pepsin; and physical means to remove the cellular and nuclear components (Conconi *et al.*, 2005; Scarritt, Pashos and Bunnell, 2015; White *et al.*, 2017; Mendibil *et al.*, 2020). These methods retain the ECM components comprising proteins, collagen subunits, proteoglycans, glycoproteins, and the mechanical properties of the tissues. Additionally, the decellularisation method reduces the antigenicity of the tissue matrix

and the possibility of immune-rejection risk by eliminating major histocompatibility complexes class I and II (Bader and Macchiarini, 2010). Thus, it provides a trachea scaffold with superior biomechanical qualities and a functional ECM and reduces the chance of immunological response (Gilbert, Sellaro and Badylak, 2006; Haykal *et al.*, 2012). While some of these grafts provided functional airways to patients for several years, significant morbidity was also associated with these methods. Surviving patients required multiple post-operative surgeries to repair the complications related to the grafts, such as stenosis, graft collapse or adverse host response (Haykal *et al.*, 2012; Partington *et al.*, 2013; Hamilton *et al.*, 2015). In particular, when applied to paediatric patients, the technical feasibility is obscured by a high incidence of major complications and long-term uncertainty (Jungebluth *et al.*, 2012).

Even though allograft transplantation is considered an effective method of replacing the trachea; however, it is limited. Vascularization at the heterotopic site requires some time to develop; thus, the graft is almost impossible to be placed in emergency cases (Haykal *et al.*, 2014; Kim *et al.*, 2017). Likewise, problems due to a shortage of tissues from suitable donors, unsuitable size of donor trachea and severe immune-rejection risks, as well as possible complications caused by infection or disease from the donor-to-patient, can be the limiting factors in allografts (Chiang *et al.*, 2016; Best *et al.*, 2018). Furthermore, a lack of structural integrity and consistency limit their applications which generally require support from patches, stents or T-tubes (Haykal *et al.*, 2014; Etienne *et al.*, 2018; Mercier, Kolb and Dartevelle, 2018). The grafts can be assisted by cartilage implantation derived from costal cartilage or other sites (Mercier, Kolb and Dartevelle, 2018; Udelsman, Mathisen and Ott, 2018). This technique does not involve immunosuppressive therapy, the harvested graft retains blood supply, and the procedure is swift. However, the process is technically

challenging and may result in mucociliary clearance of the patient. Furthermore, it is known that harvesting grafts are costly, traumatic, and anatomically restricted, with possible morbidity at the donor site because of infection.

Discontentment with conventional therapies and serious limitations to the availability of organ transplantation has shifted attention to tissue engineering (Law *et al.*, 2016; Damiano *et al.*, 2021). The artificial trachea is currently being investigated as a solution to these issues. In children, a biodegradable scaffold that replaces the damaged tracheal segment and facilitates tissue remodelling, cellular migration, and proliferation while disintegrating at an appropriate rate during growth would be ideal since it would alleviate the need for recurrent hospitalizations (Bogan, Teoh and Birchall, 2016; Damiano *et al.*, 2021). The purpose of fabricating an artificial trachea for tracheal regeneration is to achieve flexibility and strength similar to normal tracheas and ciliated epithelium. Various materials and technologies have been employed for decades to create artificial tracheas suited to these situations (Park *et al.*, 2019). Jungebluth and his team (2011) described the use of a tissue-engineered tracheobronchial graft made of a bioartificial nanocomposite (polyhedral oligomeric silsesquioxane poly(carbonate-urea) urethane) (POSS-PCU) seeded with autologous bone marrow mononuclear cells (Jungebluth *et al.*, 2011). POSS-PCU was favourable for tissue engineering applications since it is biocompatible, non-biodegradable, moderately immunogenic, mechanically robust, readily malleable, and non-toxic. However, the study's lead investigator, Paolo Macchiarini, was accused of scientific misconduct (The Lancet, 2018). The work of Dr Paolo Macchiarini, who has been involved in the clinical implantation of various decellularized and polymeric airway replacements since 2008, has sparked widespread discussion in the field of clinical tracheal replacements throughout the previous decade. Even though there is a lack of

preclinical proof of these procedures' safety and efficacy, Macchiarini's human implantations were widely embraced, and his reported results were adopted as the foundation for clinical application by other groups (Haykal *et al.*, 2014; Law *et al.*, 2016; Greaney and Niklason, 2021).

Despite Macchiarini's controversy, researchers have investigated and discovered various new data that could pave the way for the therapeutic use of tissue-engineered tracheae (Law *et al.*, 2016). The field of tracheal tissue engineering is still in its early stages, with most trials being experimental or preclinical. There are still many challenges to overcome before being applied clinically. The need for an ideal tracheal scaffold is in dire need in adult populations and particularly in paediatric populations due to the problems mentioned earlier.

2.4 Challenges in tracheal tissue replacement

Although a few experimental possibilities are mentioned in the literature, none have yet to be adopted into routine clinical practice. Long-segmental tracheal lesions are particularly challenging to treat due to inadequate blood supply to the trachea, persistent exposure of the airway lumen to the environment, and complicated mechanical demands on the tissue (Greaney and Niklason, 2021; Samat, Hamid and Yahaya, 2022). These factors are closely related to the anatomical features of the trachea itself.

One of the most serious obstacles in tissue engineering of the trachea is the delay in vascularization, which jeopardises the transplanted living cells. The trachea relies on segmental blood flow, which penetrates through the cartilage rings to nourish the luminal mucosal lining, while the transplanted tissue relies on blood vessels for