THE SYNTHESIS AND CHARACTERIZATION OF HYDROXYLAPATITE MICROTUBULES/POLYLACTIC ACID GTR MEMBRANES AND ITS EFFECT ON PERIODONTAL BONE REGENERATION

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LIST OF UNITS AND SYMBOLS

º	Degree
°C	Degree Celsius
g	Gram
h	Hour
-OH	Hydroxyl radicals
µl	Microliter
µm	Micrometer
mg	Milligram
ml	Milliliter
mm	Millimetre
nm	Nanometre
%	Percentage
rpm	Revolutions per minute
sec	Second
v/v	Volume over volume
w/w	Weight over weight

LIST OF ABBREVIATIONS

Au	gold
BSC	biosafety cabinet
CAL	clinical attachment level
CaO	calcium oxide
CCK-8	Cell Counting Kit-8
DMEM	Complete culture medium
ECM	Extracellular matrixp
ePTFE	expanded polytetrafluoroethylene
FDA	Food and Drug Administration
FTIR	Fourier transform infrared spectroscopy
GBD	Global Burden of Disease
GTR	guided tissue regeneration
HAp	hydroxyapatite
hPDLSCs	human periodontal ligament stem cells
KBr	potassium bromide
MSCs	mesenchymal stem cells
NCBI	National Library of Medicine
OCN	osteocalcin
OD	optical density
OPN	osteopontin

PBS	phosphate-buffered saline
PI	Principal Investigator
PLA	polylactic acid
PLLA	asymmetric poly (L-lactide)
PPD	probing pocket depth
PVDF	polyvinylidene fluoride
qPCR	quantitative PCR
RUNX2	runt-ralated transcription factor 2
SBF	simulated body fluid
SEM	canning electron microscope
TEM	transmission electron microscopy
UV	ultraviolet
XRD	X-ray powder diffraction
β-TCP	β-tricalcium phosphate

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SINTESIS DAN PENCIRIAN MEMBRAN GTR MIKROTUBUL HIDROKSIAPATIT/ ASID POLILAKTIK DAN KESANNYA TERHADAP REGENERASI TULANG PERIODONTAL

ABSTRAK

Objektif umum kajian ini adalah untuk membangunkan membran GTR mikrotubul hidroksilapatit/PLA yang novel dan menilai sifat-sifat mekanikal serta biologi membran tersebut. Mikrotubul HAp disediakan melalui proses sintesis hidrotermal. Komposisi dan morfologi bahan yang disintesis diperiksa menggunakan mikroskop elektron imbasan (SEM), mikroskop elektron transmisi (TEM), difraksi serbuk sinar-X (XRD), dan spektroskopi inframerah transformasi Fourier (FTIR). Kemudian, membran GTR mikrotubul hidroksilapatit/PLA yang novel telah dihasilkan. Membran tersebut disahkan sama ada memenuhi keperluan mekanikal klinikal dalam sifat-sifat degradasi dan regangan. Selain itu, kami mengkaji interaksi membran tersebut dengan sel induk ligamen periodontal manusia (hPDLSCs), yang diekstrak dari gigi yang sihat. Kesan osteogenik membran terhadap hPDLSCs diperiksa dengan menilai ekspresi gen dan protein RUNX2, OPN, dan OCN, masing - masing menggunakan Analisis PCR Kuantitatif (qPCR) dan serap Western. Dalam kajian ini, semua data untuk ujian sifat mekanikal, yang termasuk ujian sudut sentuh air, ujian tegangan, analisis pembengkakan dan analisis biodegradasi dinyatakan sebagai purata dan sisihan piawai. Untuk profilerasi sel dan eksperimen pembezaan osteogenik, data adalah berangka. Normalisasi pengedaran dinilai menggunakan analisis plot Q-Q. Dalam beberapa kes, transformasi log semulajadi digunakan untuk membantu mewakili data yang mempamerkan taburan normal. Tahap penting ditetapkan pada p = 0.05. Membran mikrotubul HAp/PLA menunjukkan kekuatan

regangan dan kekuatan patah yang lebih baik serta produktiviti fraktur yang lebih rendah berbanding dengan membran PLA. Kadar pengembangan mencapai 34% selepas 2 jam, dan kadar degradasi melebihi 25% selepas 40 hari. Hasil pengukuran sudut sentuhan air menunjukkan bahawa membran dwilapisan ini bersifat hidrofilik pada satu sisi dan hidrofobik pada sisi yang lain. Hasil eksperimen berkaitan sel juga menunjukkan bahawa membran tersebut tidak mempunyai sitotoksisiti. Bilangan sel dalam kumpulan membran mikrotubul HAp/PLA didapati jauh lebih tinggi daripada yang diperhatikan dalam kumpulan dengan membran PLA ($p<0.05$). Tambahan pula, ekspresi gen dan protein yang dikaitkan dengan komposisi tulang telah dinaikkan dengan ketara dalam kumpulan membran mikrotubul HAp/PLA berbanding kumpulan membran HAp/PLA dan hPDLSCS ($p<0.05$). Bahagian sisi mikrotubul HAp dapat menggalakkan ekspresi gen dan protein yang berkaitan dengan osteogenesis, manakala bahagian sisi PLA dapat menghalang lekatan sel. Membran mikrotubul HAp/PLA memiliki kestabilan dan sifat mekanikal yang baik. Struktur dwilapisan ini berfungsi sebagai penghalang biologi dan menggalakkan pembentukan tulang.

THE SYNTHESIS AND CHARACTERIZATION OF HYDROXYLAPATITE MICROTUBULES/POLYLACTIC ACID GTR MEMBRANES AND ITS EFFECT ON PERIODONTAL BONE REGENERATION

ABSTRACT

The general objective of the study is to develop novel hydroxylapatite microtubules/PLA GTR membranes and evaluate them for mechanical and biological properties. HAp microtubules were prepared through hydrothermal synthesis. The composition and morphology of the synthesized materials were examined by a scanning electron microscope (SEM), transmission electron microscopy (TEM), Xray powder diffraction (XRD), and Fourier transform infrared spectroscopy (FTIR). Then the novel hydroxylapatite microtubules/PLA GTR membranes were fabricated. The membranes were verified whether they met the clinical mechanical requirements in degradation and stretching properties. In addition, we examined its interaction with human periodontal ligament stem cells (hPDLSCs), harvested from healthy teeth. The osteogenic effect of the membrane on hPDLSCs was examined by evaluating the gene and protein expressions of the RUNX2, OPN, and OCN using Quantitative PCR (qPCR) and Western blotting analysis, respectively. In this study, all data for the mechanical property tests, which include water contact angle tests, tensile tests, swelling analysis and biodegradation analysis were expressed as mean and standard deviation. For cell proliferation and osteogenic differentiation experiments, the data were numerical. The normality of distribution was assessed using Q-Q plot analysis. In some cases, natural log transformation was employed to aid in representing data that exhibited normal distribution. The significance level was set at $p = 0.05$. The HAp microtubules/PLA membrane had better tensile and

breaking strengths and lower fracture productivity than the PLA membrane. The expansion rate reached 34% after 2 h, and the degradation rate exceeded 25% after 40 days. Water contact angle measurement results showed that the bilayer membrane was hydrophilic on one side and hydrophobic on the other side. The results of the cell experiments also showed that the membrane had no cytotoxicity. The number of cells in the HAp microtubule/PLA membrane group was found to be significantly higher than that observed in the group with a PLA membrane ($p<0.05$). Furthermore, the expression of genes and proteins associated with bone composition was significantly elevated in the HAp microtubule/PLA membrane group compared to the HAp powder/PLA membrane and hPDLSCs group ($p<0.05$). The HAp microtubule side can promote the expression of genes and proteins related to osteogenesis, while the PLA side can inhibit cell adhesion. The HAp microtubules/PLA membrane has stability and good mechanical properties. The double-layer structure has the function of a biological barrier and promotes bone formation.

CHAPTER 1

INTRODUCTION

1.1 Background

According to a 2003 World Health Organization report, oral diseases are considered a major public health problem because of their high prevalence and incidence (Niu et al., 2020). Periodontal disease is a prevalent oral disease that affects tooth-supporting structures and is considered the main cause of tooth loss among adults worldwide, affecting approximately 15% of the population (Petersen, 2003). The periodontitis refers to the evolution of the periodontal condition from gingivitis to a chronic, destructive, and irreversible inflammatory state. The bacteria penetrate the periodontal tissue, triggering a host response that defends against the invading bacteria. However, this defense mechanism also leads to the destruction of the periodontal tissue. Periodontitis results in the detachment of periodontal tissue, leading to the loss of alveolar bone and ultimately resulting in the loosening and loss of the affected tooth. (Ramesh et al., 2018).

The main goal of periodontal treatment is to fully regenerate the alveolar bone that supports the teeth, while preventing non-osteogenic tissues from impeding alveolar bone regeneration (Rakhmatia et al., 2013). To achieve the goal of efficient bone regeneration and reliable protection against the growth of adjacent soft tissues into the newly generated bone, guided tissue regeneration (GTR) technology has emerged as a solution to some of the adverse effects of periodontitis. The purpose of the GTR membrane is to act as a barrier to prevent soft tissue ingrowth into the defect and encourage bone regeneration through cellular exclusion, thereby enabling periodontal regeneration (Bottino et al., 2011).

The barrier membrane used for GTR therapy should possess several desirable characteristics, including nontoxicity, biocompatibility, bioactivity, osteoconductivity, integration with host tissues, clinical manageability, space-maintaining capability, and adequate mechanical and physical properties. These characteristics help to prevent the deposition of activated tissue and promote bone regeneration (Ueyama et al., 2002). Barrier membranes used in GTR surgery should have a degradation rate that allows for mechanical support during bone formation. Research on membranes is crucial for regenerating periodontal bone.

1.2 Problem Statement & Justification of Study

The desirable characteristics of a barrier membrane used for GTR therapy generally include preventing toxic reactions and inhibiting the deposition of activated tissue, which subsequently stimulates bone regeneration (Elgali et al., 2017). Additionally, the barrier membrane should have a degradation rate that is long enough to provide mechanical support during bone formation. At present, the traditional barrier membranes commonly used in clinical practice can be divided into two categories, namely non-absorbable membranes and absorbable membranes. The two types of membranes have different performances in terms of biological and mechanical properties, but the defects are also obvious. The non-absorbable film is represented by polytetrafluoroethylene, which can be used alone or together with other materials. The disadvantage is that the material is not absorbable, and the stiffness is high, so it usually needs to be removed by a second operation. The trauma of the second operation is bound to have a certain impact on the postoperative recovery of the patient and increase the risk of reinfection (Matichescu et al., 2020), so the non-absorbable membrane is generally only applied in operations with special needs. The

absorbable membrane, represented by synthetic polyester, has the advantage of good absorption and good biocompatibility, which can enable bone meal and osteoblast to better attach and grow (Miron et al., 2016). One disadvantage of using absorbable barrier membranes is their poor tensile stress, which often requires the use of bone graft materials. Furthermore, the currently available membranes lack suitable mechanical properties and controlled degradation rates. Additionally, most of the GTR membranes currently used are derived from humans or other mammals, which can be expensive, potentially infectious, and raise ethical concerns, as well as having high ecological impacts (Li et al., 2022). To address these issues, chemically synthesized scaffolds have been extensively researched.

Chemically synthesised GTR membranes can be given more functions by choosing different synthesis methods and materials due to the characteristics of the process. Conventional monolayer GTR membranes are capable of acting as cellular barriers, and in order to integrate more functions, a single design obviously cannot meet our needs. Many studies have shown that the design of bilayer or multilayer GTR membranes can be made more functional to meet clinical needs (Bee & Hamid, 2022; Li et al., 2020).

HAp is widely recognized as the primary inorganic component of human bones and teeth. It is a crucial material for bone repair and regeneration due to its exceptional biodegradability, biocompatibility, bone conductivity, and biological activity (Awasthi et al., 2021; Shen et al., 2017; Wang et al., 2018). HAp has been extensively researched in various fields, including tissue engineering scaffolds, cell imaging, and drug delivery (Li et al., 2014). It is widely recognized that HAp can enhance the osteogenic differentiation of stem cells by releasing calcium ions, which

gives it an advantage over other materials in bone repair (Shih et al., 2014). Other studies have confirmed that stem cells are highly sensitive to their cellular microenvironment (Scadden, 2006). When biomaterials are used as artificial stem cell niches, their surface morphology plays a crucial role in regulating stem cell fate, from morphology to directed differentiation. However, the efficiency of HAp in promoting osteogenic differentiation is low and most differentiation scenarios require additional growth factors, which is impractical in tissue engineering. Therefore, it is necessary to rely on structural strength to effectively promote osteogenic differentiation. One-dimensional tubular hydroxyapatite materials have potential applications in various fields due to their special properties. On the one hand, hydroxyapatite 1D tubular materials have higher aspect ratios and excellent mechanical properties compared to nanoparticles, which can be applied in the field of mechanical reinforcement. On the other hand, the unique internal cavity structure of one-dimensional tubular hydroxyapatite materials can be used for adsorption and loading of other molecules and nanoparticles. Therefore, it is very meaningful to study the effect of this special tubular structure hydroxyapatite on periodontal bone regeneration.

Due to its good biocompatibility, HAp can be combined with natural polymers (collagen, chitosan, alginate, fibroin, etc.) and synthetic polymers (polylactic acid, polylactic acid-glycolic acid copolymer, polycaprolactone, etc.) to prepare composite scaffolders. The combination of HAp and natural polymer can produce bone repair materials with excellent biocompatibility, mechanical properties and osteogenic activity (Mahmoud et al., 2020; Nga et al., 2020; Yao et al., 2022). Polylactic acid (PLA), a biocompatible and biodegradable material approved by the Food and Drug Administration (FDA), is also used popularly in tissue engineering (Shih et al., 2014). PLA typically has two applications: as a scaffold for tissue repair and as a barrier to

prevent postoperative adhesion (Avital et al., 2005; Ersoy et al., 2008). When used as a barrier membrane, PLA's natural hydrophobicity can help prevent adhesion between the defect area and surrounding tissue. HAp/PLA composites have become increasingly popular for bone regeneration (Kasuga et al., 2001). However, most hybrid materials aimed at repairing bone only use HAp as a calcium source, which cannot recruit cells or promote cell adhesion and growth (Danoux et al., 2014; Petricca et al., 2006). Combining the advantageous properties of HAp and PLA to achieve precise and controllable bone repair through novel structural design is a crucial issue.

Accordingly, in order to obtain the desired GTR membrane function, hydroxyapatite microtubules and PLA were selected as the materials for the synthesis of bilayer membranes, and the novel bilayer membranes were synthesised by the self-evaporation method. Their mechanical and biological properties were then tested to ascertain whether they meet the requisite clinical standards.

1.3 General Objectives

The general objective of the study is to develop novel hydroxylapatite microtubules/PLA GTR membranes and evaluate them for mechanical and biological properties.

1.4 Specific Objectives

1.To formulate hydroxylapatite microtubules and characterised their morphological characteristic using a scanning electron microscope (SEM), transmission electron microscopy (TEM), X-ray powder diffraction (XRD), and Fourier transform infrared spectroscopy (FTIR).

2.To fabricate novel hydroxyapatite microtubules/PLA membranes, characterise their surface morphology using SEM and evaluate their mechanical properties using tensile test, swelling analysis, biodegradation analysis and water contact angle.

3.To evaluate the bio-performance properties, including cytotoxicity test and cell proliferation of the novel hydroxylapatite microtubules/PLA membrane using primary human periodontal stem cells.

4.To evaluate the osteogenic differentiation of human periodontal ligament stem cells (hPDLSCs) on the novel hydroxylapatite microtubules/PLA membrane using quantitative PCR (qPCR) and western blot.

1.5 Research Question(s)

1.Does the novel hydroxylapatite microtubules/PLA membrane possess the required mechanical properties, particularly good surface morphology, tensile strength, swelling effects, and biodegradation?

2.Does the novel hydroxylapatite microtubules/PLA membrane fulfil the GTR membrane's desirable characteristics, such as non-toxicity, biocompatibility, and barrier integration?

3.Does the novel hydroxylapatite microtubules/PLA membrane have the ability to stimulate hPDLSC proliferation and osteogenesis?

1.6 Research Hypothesis

1.The novel hydroxylapatite microtubules/PLA membrane will have an ideal characteristic of the GTR membrane, particularly in surface morphology, tensile strength, swelling effects, and biodegradation.

2.The novel hydroxylapatite microtubules/PLA membrane will fulfil the GTR membrane's desirable characteristics, such as non-toxicity, biocompatibility, and barrier integration.

3.The novel hydroxylapatite microtubules/PLA membrane will have the ability to stimulate hPDLSC proliferation and osteogenesis.

CHAPTER 2

LITERATURE REVIEW

2.1 Periodontal Diseases

The chronic infectious disease of the gum tissues caused by plaque microorganisms is known as periodontal disease. It is the leading cause of tooth loss in adults and has a high prevalence worldwide (Peres et al., 2019). The periodontium is a special type of gum tissue that supports the teeth in the upper and lower jaws. The periodontium consists of four main tissues: alveolar bone, bone cementum, periodontal ligament and gums. Periodontitis is the most common chronic inflammatory non-communicable disease in humans. The Global Burden of Disease (GBD) database reports that there will be 1.1 billion cases of severe periodontitis worldwide in 2019. In addition, the age-standardised prevalence of severe periodontitis increased by 8.44% between 1990 and 2019. (Chen et al., 2021).

Periodontitis is a chronic process that begins with gingivitis, an inflammation of periodontal soft tissues that causes gingival bleeding and swelling. Gingivitis progresses to periodontitis in susceptible individuals with a low immune response, gradually destroying the alveolar bone and other periodontal tissues surrounding the teeth. Inflammation resulting from prolonged infection can cause varying degrees of alveolar bone loss, ultimately leading to tooth loss. Patients with moderate to severe periodontitis often experience varying degrees of alveolar bone loss, which may impact the restoration of tooth function in later stages of treatment (Papapanou et al., 2018).

2.2 Treatment of Periodontal Diseases

The primary objectives of periodontal therapy are to treat infections caused by pathogenic periodontal biofilms, to prevent or retard further attachment and bone loss, and ultimately to prevent tooth loss. Clinically, successful treatment is defined as a reduction in probing pocket depth (PPD), regression of inflammation (sepsis suppression or reduction in bleeding), and remodelling of the tooth and gingival environment to enable effective oral hygiene measures. It is desirable that improvements in clinical signs are accompanied by an increase in clinical attachment level (CAL) and radiographic bone volume. There is good evidence that these goals can often be achieved with nonsurgical treatment (Sanz et al., 2020).

Treatment of periodontitis consists of Initial therapy, surgical phase and supportive periodontal therapy. Initial therapy, also known as cause-related therapy, includes plaque control, supragingival scaling, subgingival scaling to remove calculus and subgingival plaque biofilm to prevent and control periodontitis (Betsy et al., 2014). After 4 weeks of review, if there are still periodontal pockets of more than 5mm and bleeding on probing, or if the gingivitis and bone are not in good shape, then a second phase of treatment is required, periodontal surgery. Periodontal surgery mainly consists of gingivectomy, gingivoplasty, flap surgery, etc. (Miller, 1993; Zucchelli et al., 2018), to reduce the depth of the periodontal pocket. These treatments only partially repair the periodontal tissue and do not achieve effective periodontal tissue regeneration. Periodontal tissue regeneration is the reconstruction of the periodontal tissue that has been lost due to periodontitis, with the formation of new osteoid and alveolar bone, which is connected by new periodontal fibres, and the newly formed bonding epithelium is located on the root side at the base of the periodontal pockets prior to treatment. This is the ideal result of periodontal treatment (Liu et al., 2019).

Regenerative surgery consists mainly of GTR and bone grafts, or a combination of both. The supportive periodontal therapy, which involves regular review and retreatment, is a prerequisite for the long-term maintenance of periodontal therapy.

The main objective of periodontal therapy is to enhance periodontal health and prevent attachment loss, meeting the patient's aesthetic and functional needs. In the last twenty years, there has been a significant interest in regenerating periodontal tissues to reverse the damage caused by the disease process to the periodontium. The reason for implementing this approach in practice is that it provides more advantages to the patient than traditional methods (Nibali et al., 2021). To achieve successful periodontal regeneration, several issues must be addressed. These include the formation of a functional epithelial seal, the insertion of new connective tissue fibers into the root, the formation of a new cell-free bone cement on the root surface, and the restoration of alveolar bone height (Shimono et al., 2003).

2.3 Guided Tissue Regeneration

Repair and regeneration are two ways of healing after tissue injury. In order to protect the organism after injury, higher organisms will quickly produce a repair response that is not conducive to tissue regeneration (Alqahtani, 2023). Take periodontal surgery as an example. In the process of periodontal tissue healing, gingival epithelium, gingival connective tissue, osteoblasts, and periodontal membrane cells compete for growth. Since epithelial cells have the fastest migration speed and strong proliferative ability, they quickly form a long adherent epithelium attached to the root surface, occupying the space of periodontal stem cells (PDLCs) and preventing the orderly regeneration of tissues. As a result, periodontal defects persist, and this healing process is called periodontal repair (Caton et al., 1987; Chen

& Jin, 2010). In addition, when fibroblasts in the gingival connective tissue contact the root surface, they form collagen fibres parallel to the root surface, which is also not conducive to periodontal attachment regeneration (Alqahtani, 2023). Therefore, the key to periodontal tissue regeneration is to block gingival epithelial cells and fibroblasts, maintain the space of the periodontal defect, and precisely coordinate in time and space the various cells and extracellular matrix remaining in the periodontal wound tissue (Lackler et al., 2000).

In the 1980s, Karring and Nyman et al. proposed the concept of GTR. GTR utilizes a membrane around the periodontal defect as a physical barrier to prevent gingival epithelial cells and fibroblasts from invading the diseased area, thus creating a relatively closed environment. Progenitor cells with regenerative potential from the remaining periodontal membrane, adjacent alveolar bone, or blood are directed to recolonize the damaged area. They then proliferate and differentiate into new periodontal supporting tissues, achieving periodontal regeneration (Bosshardt & Sculean, 2009; Bottino et al., 2012; Chen et al., 2010). GTR technology has been used in periodontal therapy for almost 30 years and has been effective in promoting regeneration of periodontal and bone tissue around natural teeth and dental implants. One potential advantage of applying the GTR principle to the treatment of periodontal defects is that it allows for periodontal regeneration rather than connective tissue repair of the exposed root surface (Jepsen et al., 2002).

2.4 Classification and Existing Problems of GTR Membrane

The GTR technique has been employed in periodontal therapy for approximately 30 years, during which time it has been demonstrated to be an effective method of promoting the regeneration of periodontal and bone tissue around natural

teeth and dental implants. One potential advantage of applying the GTR principle to the treatment of periodontal defects is that it allows periodontal regeneration rather than connective tissue restoration of exposed root surfaces (Rakhmatia et al., 2013). Furthermore, periodontal tissue that has been regenerated with non-biocompatible membranes is not more susceptible to periodontitis than natural periodontal tissue. In numerous animal and human studies, specially designed expanded polytetrafluoroethylene (ePTFE) membranes have been proven biocompatible, primarily for the reconstruction of periodontal defects. Defects treated with these membranes heal faster and have a higher quality and quantity of regenerated bone than defects not treated with these membranes (Piattelli et al., 1996). In an experimental setting, a porous ePTFE membrane is placed over the exposed tooth surface and sutured to the soft tissue. Once the newly formed tissue has filled the space, the non-resorbable ePTFE membrane is removed in a second surgical procedure. This additional surgical trauma causes discomfort for both the patient and the healthcare provider. Furthermore, it can increase the risk of patient infection and other adverse events, such as disruption of the newly regenerated tissue (Trombelli et al., 2005; Aybar et al., 2003).

Therefore, to avoid membrane removal after healing and to achieve more efficient periodontal regeneration, many researchers have evaluated the value of biodegradable membranes, which have attracted considerable interest in clinical trials in recent years (Dupoirieux et al., 2001). Most GTR membranes currently in use are produced by humans or other mammals; they are, therefore, expensive, have a risk of infection, and their use is ethically questionable and has significant ecological implications (Li et al., 2022). Chemically engineered scaffolds have been extensively researched as a potential solution to some of these problems, and the development and

fabrication of bioresorbable GTR devices have been spurred by the aforementioned concerns, using natural derivatives and synthetic organic polymer technologies. A variety of biomaterials have been proposed and used, most commonly type I collagen, PLA and poly (lactic-co-glycolic acid) (PLGA), which mimic the chemical and physical properties of the natural extracellular matrix (ECM) (Kubo et al., 1998). Collagen membranes have excellent cell affinity and biocompatibility to regenerate tissue (Abou Neel et al., 2013). In addition, negatively charged collagen membranes have been reported to be able to influence cell behaviour and the distribution of ECM components to allow rapid defect healing without delay (Veríssimo et al., 2010). Immobilisation of alkaline phosphatase on collagen membranes was effective in further enhancing the osteogenic potential of the membranes (Oortgiesen et al., 2012). However, there may be disadvantages to the use of collagen membranes. The mechanical strength of collagen membranes is usually low, making them difficult to manipulate, and they can induce local chronic inflammation and rapid degradation (Milella & Ramires, 2001). Enzymes, bacterial proteases, and even immobilised alkaline phosphatase (Kozlovsky et al., 2009) had been demonstrated that the application of this substance can accelerate the degradation of collagen membranes. Copolymers utilising PLA membranes and glycolides have emerged as a potential alternative to GTR technology. PLA-based polymers exhibit sufficient mechanical properties and controllable biodegradation rates. By controlling the chemical composition, the membrane can be maintained for a period of time sufficient to achieve tissue regeneration before it degrades (Nieminen et al., 2006). However, PLA-based membranes are inherently hydrophobic and have poor cell affinity, so finding an ideal barrier membrane material remains the key to advancing GTR technology.

2.5 GTR Membrane Development Trend

2.5.1 Basic Function of GTR Membrane

The barrier membranes used in the GTR process should meet several requirements. The barrier function of the GTR membrane is the basis for periodontal tissue regeneration. Due to the contact inhibition effect of the GTR membrane on epithelial growth, the healing of periodontal connective tissue is slowed down, while PDLCs are stimulated by periodontal inflammation for a long time, and their healing ability is also damaged. Therefore, the barrier function of the GTR membrane must be maintained for at least 4-6 weeks (Rodriguez et al., 2018; Zheng et al., 2015). At the same time, the GTR membrane should meet the following five main criteria (Figure 2.1). First and foremost, it has to be biocompatible. A membrane must ensure that the interaction between the membrane and the tissue has a positive impact on the microenvironment and does not affect the healing of the defect area. The degradation products of absorbable membranes should be non-cytotoxic and able to integrate into the host tissue (Caballé-Serrano et al., 2018). In addition, the membrane needs to have the ability to guide tissue regeneration. This requires that the membrane needs to have space maintainer and occlusive. A membrane can withstand pressure from the gingiva to avoid soft tissue collapse into the damaged area and prevent soft tissue from growing into the defective area, but at the same time, allow the oxygen, fluid and bioactive substances needed for cell growth to reach the defect area (Hämmerle & Jung 2003). In order to prevent gingiva cracking, the hardness of the film should not be too strong, and the ductility of the film should not be too strong, otherwise it is not conducive to clinical operation (Won et al., 2016). This requires a membrane to be easy to handle. In addition to the aforementioned fundamental requisites, the membranes must also be bioactivation-friendly. However, this feature is not currently

a consideration in the development of membranes. New strategies for bone regeneration are being developed, which take membranes to the next level, where they play not only a passive role, but an active role at the regeneration site. (Hoornaert et al., 2016).

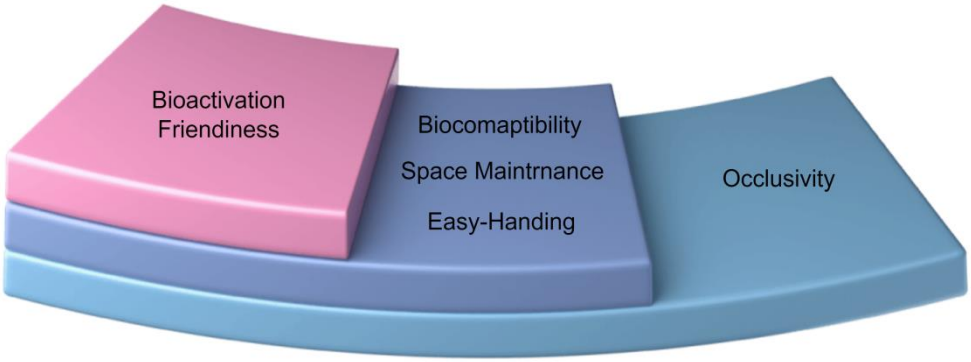

Figure 2.1 Pyramid with the 5 main criteria that barrier membranes should fulfil. The figure is adapted from Caballé-Serrano et al. 2018 (Caballé-Serrano et al., 2018).

2.5.2 GTR Membrane Osteogenic Modification

Promoting periodontal bone regeneration is essential for rebuilding periodontal support tissue. Although commercially available GTR membranes are used as barrier objects to provide and maintain the appropriate osteogenic space for periodontal regeneration, the therapeutic effect is often unsatisfactory. The limitation of bone tissue regeneration is one of the main reasons. At the stage of basic periodontal treatment, damaged periodontal tissues, including periodontal ligament stem cells, have been removed, resulting in a small amount of residual PDLCs, and it is difficult to achieve complete periodontal regeneration. In addition, under the long-term stimulation of chronic periodontitis, the differentiation ability of PDLCs is significantly reduced, and it is difficult to coordinate the harmonious regeneration of

periodontal tissues (Zheng et al., 2015). Therefore, periodontal regeneration mainly depends on the successful recruitment of locally derived regenerative progenitor cells to the lesion site to maintain tissue homeostasis and subsequently differentiate into osteoblasts, PDLCs, and cementum mesenchymal stem cells (MSCs) (Lin et al., 2008), including resident MSCs or circulating MSCs in periodontal tissues as a source of progenitor cells, can be directed to periodontal tissues to play an important role in regeneration and immune regulation (Andreas et al., 2014; Ouchi & Nakagawa, 2020) and become the "source power" of periodontal bone tissue regeneration. It can be seen that periodontal bone tissue regeneration can be promoted by regulating the cellular activity of MSCs and stimulating cell differentiation. However, bone tissue regeneration requires a long cycle, and long-term systematic administration of osteoinductive drugs will often be accompanied by some side effects and even lead to systemic bone metabolism abnormalities (Laurencin et al., 2014). Therefore, bone-inducing substances are added to the GTR membrane and continuously released at a fixed point during the continuous degradation of the membrane. This helps to initiate the cascade reaction of osteoblastic molecular signals and stimulate the regenerative ability of the body's own MSCs, further improving the effect of periodontal bone regeneration (Salgado et al., 2004).

2.6 Hydroxyapatite and Application

2.6.1 Osteogenic Properties of Hydroxyapatite

The chemical properties of hydroxyapatite [HAp, Ca_{10} $(OH)_2$ $(PO_4)_6$] is a commonly used bone graft material in clinical practice, and its calcium-phosphorus ratio of 1.67 is similar to that of bone. HAp accounts for about 69 wt% in bone tissue and 96 wt% in teeth (Ripamonti et al., 2012; Zhan et al., 2005; Zhao et al., 2011).

HAp is a kind of autogenous material and can recruit cells to grow on its surface. Due to its excellent biocompatibility, low inflammatory response, and similar structure to natural bone tissue, synthetic hydroxyapatite has been extensively studied by researchers and used as a bone substitute in biomedical applications. (Habraken et al., 2007; Hutmacher et al., 2007). When the HA scaffold is implanted in the human body, it can promote the movement and proliferation of osteoblasts at the scaffold/body interface, resulting in the formation of a large number of new bones and guiding the new bone tissue to form a strong and firm mechanical bond with the scaffold. A large number of clinical cases have shown that after bone tissue implants are implanted in the human body, a large number of fibrotic soft tissues are formed around them, causing the interface combination between the implant and the surrounding tissue to move and fall off easily, eventually leading to implant failure. HAp can be used as a coating material to help the implant and surrounding tissue form a strong mechanical bond, greatly reducing the possibility of implant failure (Ratner et al., 2013). A large body of literature has confirmed that HA has good application prospects in drug release, protein and gene transport (Ginebra et al., 2006). The results showed that sintered hydroxyapatite has good biocompatibility with soft tissues such as skin, muscle and gingiva. This capability makes HAp an ideal candidate for orthopaedic and dental implants or implant components. Synthetic hydroxyapatite is widely used for hard tissue repair. Common applications include bone repair, bone grafting and coating or filling of implants (Bohner, 2010; Wagoner Johnson & Herschler, 2011).

Simple HAp materials have problems such as insufficient fracture toughness, fatigue failure and brittle failure (Proussaefs et al., 2002) and the degradation time is too long (Proussaefs et al., 2002), making it impossible to achieve the ideal effect of the material degradation rate matching the bone regeneration rate. Furthermore, HAp

exhibits reduced bone conductivity, and in most cases of differentiation, additional growth agents are required, which presents a significant challenge for practical tissue engineering. These performance deficiencies therefore significantly restrict the applicability of HAp.

2.6.2 Preparation of Hydroxyapatite

Hydroxyapatite can be synthesized by industrial synthesis and extracted from natural resources such as fish, shells, eggshells, etc. (Cüneyt Taş et al., 1997). The synthesis of HA with different structures and morphologies has greatly stimulated the research interest of many academic and industrial research institutions. Over the past three decades, many synthetic routes have been investigated for the preparation of HA powder. The synthesis method of HA powder can be divided into the dry method and the wet method.

The dry route of HAp production is based on heat treatment of finely ground mixed precursors. The basic requirement for preparation by the dry method is a completely uniform mixture of reactants. The purity of the final product depends on accurate weighing during the preparation process, the size and mixing effect of the synthetic precursor material and the control of external impurities. These variables can influence the formation of the optimal final compound. Tromel et al. identified the optimal conditions for HAp formation during calcination of a mixture of calcium phosphate and calcium oxide (CaO) at 1050 °C in air (Kaneda & Mizugaki, 2009; Wei & Yates, 2012).

Wet methods of HA include double decomposition or co-immersion, emulsion, hydrolysis, sol-gel, and hydrothermal methods. These methods have been widely used due to their simplicity of procedure, as well as their ability to synthesise HAp in high

yields with complete control over structure and morphology. Compared with the dry method, the experimental parameters in the preparation process of the wet method have a greater influence on the product. Therefore, the morphology, size and structure of the hydroxyapatite nanomaterial can be adjusted by adjusting the temperature, heating method, solvent ratio, solvent type, reactant concentration, pH and other experimental conditions during the experiment. Among the preparation methods, the hydrothermal reaction has been the most studied, as it can be synthesised in large quantities with good reproducibility. Specifically, the reactant is dissolved in an aqueous solution and then placed in a closed hydrothermal reactor under high temperature and pressure to react, and finally, hydroxyapatite material is obtained (Cao et al., 2004; Liu et al., 1997; Suchanek et al., 2004). Hydroxyapatite synthesized by hydrothermal method has high crystallinity, no need for calcination crystallisation treatment, uniform particle size and relatively regular shape, and can be synthesized by changing the conditions of hydrothermal reaction with different shapes and sizes.

2.6.3 Hydroxyphospapatite Topography

Based on the above methods, the synthesized hydroxyapatite has nanolinear shapes (Li et al., 2020; Yu et al., 2018), nanorods (Li et al., 2020; Singh et al., 2020), microspheres (Liu et al., 2020; Mondal et al., 2019), micron structural flowers and micron sheet shapes (Zhang et al., 2011). The surface topography of biomaterials serves as a niche for artificial stem cells and is of critical importance for regulating stem cell density, from morphology to the induction of differentiation (Marques et al., 2023). One-dimensional HAp tube materials have considerable potential for application due to their distinctive structure. On the one hand, they exhibit a higher aspect ratio and superior mechanical properties compared to nanoparticles, which can be utilised in the field of mechanical reinforcement. However, in vitro studies have

indicated that microtubule scaffolds may be more efficacious in promoting the adhesion and differentiation of bone marrow mesenchymal stem cells to the osteoblast lineage (Won et al., 2020).

2.7 Microtubule Structure

2.7.1 The Acts of Tubular Structures on Cells

Anatomical studies of natural bone reveal the existence of complex, interconnected microtubule structures within natural bone, which at the macrostructural level can be divided into cancellous bone and cortical bone. Cancellous bone is a porous network structure composed of a large number of interconnected needle-like and leaf-like bone trabeculae, and the network is filled with bone marrow. Cortical bone is composed of a large number of interconnected tiny tubes, blood vessels and nerves, and the bone plates are regularly arranged in concentric circles. There are a large number of micron hierarchical structures with different scales in human tissues, such as bones, tendons, etc., which play a very important role in tissue growth and development (Zhang et al., 2015). Studies have shown that micron hierarchical structure materials can effectively regulate cell adhesion, migration, proliferation and differentiation and other behaviours (Holthaus et al., 2012). This is because living cells are very sensitive to the microstructural properties of the extracellular matrix, and cells undergo structural rearrangement with changes in the microstructure of the material's surface. At this time, the adhesion of cells on the surface of the matrix is through the mutual aggregation of many receptor molecules way to achieve (Zhang et al., 2015).

In vitro, studies showed that the microtubule scaffold could better stimulate the adhesion and differentiation of bone marrow mesenchymal stem cells into the

osteoblast lineage (Won et al., 2020). The pores and diameters of the different sizes of bone scaffolds have different functions. Small-scale microtubules (size <10μm) are more easily impregnated by tissue fluid and provide more adsorption sites for cells, larger surface area, and higher ionic solubility, supporting and even directing mesenchymal stem cells towards osteogenesis (Pei et al., 2017). The regulation of osteogenic differentiation of mesenchymal stem cells provides more sites and more chemical stimulants; in addition, the large surface area can promote the adhesion of macrophages and accelerate the migration and proliferation of cells from the surface to the inside of the scaffold. Medium-sized microtubules (20-40 μm) favour the polarisation of primitive macrophages to the M2 type and can upregulate the expression of anti-inflammatory genes to suppress the host immune response to the graft, which is important for the ingrowth of host cells, especially mesenchymal stem cells. Large-scale microtubules (diameter >100μm) provide space and channels for cell adhesion, neovascularisation and bone ingrowth (Wang et al., 2016), facilitating cell colonisation, engraftment and homing. Therefore, both microtubule scales are important for bone scaffolds (Pei et al., 2017).

2.7.2 Tubular Structure of Hydroxyapatite

In the past few decades, one-dimensional tubular materials such as carbon nanotubes, WS_2 nanotubes (Remìkar et al., 1998) and Nb_2O_5 nanotubes (Liu et al., 2011) have rapidly attracted wide attention due to their unique physical and chemical properties. However, the aforementioned tubular materials are not biologically active and even have some toxicity. For example, carbon nanotubes can enter people's lungs and cause cancer, respiratory diseases and other health problems.

HAp one-dimensional tubular materials have the potential to be applied in a number of different fields due to their unique properties. On the one hand, hydroxyapatite one-dimensional tubular materials have a high aspect ratio and excellent mechanical properties, which can be applied in the field of mechanical reinforcement. On the other hand, the unique internal cavity structure of hydroxyapatite one-dimensional tubular materials can be used to adsorb and load other molecules and nanoparticles. So far, hydroxyapatite nanomaterials with different morphologies and sizes have been synthesised by hydrothermal/solvothermal, templating and precipitation methods (Chandanshive et al., 2013). However, synthesizing and preparing one-dimensional tubular hydroxyapatite materials with long lengths, high crystallinity, good dispersibility, and tunable size still face certain challenges.

2.8 Polylactic Acid Bioabsorbable Material

PLA is a biocompatible and biodegradable material whose use is approved by the Food and Drug Administration (Surrao et al., 2012). PLA can be broken down into harmless small molecules and excreted into the bloodstream. Due to its degradability, environmental friendliness and good physical and mechanical properties, it has been widely used in the biomedical field. PLA is a type of aliphatic polyester polymer obtained by polymerising fermentation products from renewable resources and is one of the most widely used biodegradable biomaterials. In the mid-20th century, DuPont (an American scientific research enterprise founded in 1802) researchers produced high molecular weight PLA by ring-opening polymerisation and began the industrial application of PLA (Gupta & Kumar, 2007; Jeevitha & Amarnath, 2013; Wildemann et al., 2004). PLA is a member of the polyester family, mainly formed by

condensation of lactic acid by dehydration; lactic acid can be obtained by fermentation of natural products, and PLA produced from lactic acid can be degraded into small molecule products for natural circulation, therefore PLA is an ideal green polymer material to meet the development concept of green environmental protection in today's world. PLA has excellent mechanical properties, physical properties, degradability, biocompatibility, bioabsorbability and ductility. Additionally, PLA can effectively prevent the adhesion of adherent cells and act as a barrier protection (Eren Ersoy et al., 2008).

PLA is a biodegradable biomaterial, the degradation product is a small molecule that can be eliminated from the bloodstream. It has good mechanical and physical properties, biocompatibility and biological activity to meet medical requirements and is widely used in the medical field. Therefore, PLA, with excellent properties, is used as the matrix material of the composite. However, the composite material made of PLA alone cannot meet the comprehensive and systematic requirements of the tissue for the composite material, and its degradation product lactic acid will cause the local pH to drop, resulting in aseptic inflammation and foreign body reaction (Kim et al., 2006). The incorporation of HAp has been demonstrated to enhance the mechanical properties and osteogenic activity of the material in question, a phenomenon that has been extensively researched (Chen et al., 2017; Diez-Escudero et al., 2021). HAp and PLA composites have good compatibility with fibroblasts, periodontal stem cells and osteoblast progenitor cells (Fang et al., 2010)

2.9 Layer-Designed Membranes for GTR

2.9.1 Designing Surface Layers for Interface Tissues

The design of GTR membranes is intended to meet the criteria of acting as a barrier to prevent gingival tissue from contacting the root surface and creating a protective space at the defect site. In the treatment of bone defects and alveolar ridge augmentation, GTR membranes must allow regenerative bone cells to migrate to the defect site and exclude epithelium and connective tissue from growing into the defect site. The aforementioned criteria, including biocompatibility, cell occlusion, space creation, tissue integration, osteoconductivity, and clinical manageability, are challenging to reconcile in a single layer without progressive alterations to the structure and composition. It is becoming increasingly important to design barriers that can promote the regeneration of different boundary tissues. Recent research trends have favoured the fabrication of GTR barriers in such a way that they can serve a dual function. Ideally, the membrane should act as a physical barrier to prevent epithelial cells and fibroblasts from migrating to the bone defect site, while its surface morphology and membrane composition should promote protein adsorption and support a variety of cellular activities, including membrane-cell adhesion, cell migration, proliferation, and differentiation (Li et al., 2020; Lian et al., 2019; Sheikh et al., 2016). In order to meet the diverse functional requirements of these criteria, a layer design membrane with a distinct porosity morphology, surface roughness and composition on both sides of the surface was developed and is currently being considered for use in soft tissue and periodontal defects in GTR.

A variety of inorganic materials, including bioactive glass, silica, HAp, β-tricalcium phosphate (β-TCP), and calcium silicate, were incorporated into the polymer system with the objective of achieving enhanced surface roughness,