PREPARATION AND CHARACTERIZATION OF ASYMMETRIC CHITOSAN/BIOGENIC HYDROXYAPATITE COMPOSITE MEMBRANE FOR GUIDED BONE REGENERATION

by

BEE SOO LING

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

AFM	Atomic force microscopy
ALP	Alkaline phosphatase
ASTM	American Society of Testing and Materials
ATR	Attenuated total reflectance
BCA	Bicinchoninic acid
BET	Brunauer-Emmett-Teller
BG	Bioactive glass
BJH	Barrett, Joyner and Halenda
BMP	Bone morphogenetic protein
BSA	Bovine serum albumin
Ca^{2+}	Calcium ion
$CaCl_2$	Calcium chloride
$CaCO_3$	Calcium carbonate
CaO	Calcium oxide
CaP	Calcium phosphate
CHNS-O	Carbon, hydrogen, nitrogen, sulphur-oxygen
Co	Cobalt
CS	Chitosan
CT	Chitin
DDA	Degree of deacetylation
DTG	Differential thermal gravimetry
e-PTFE	Expanded-polytetrafluoroethylene
ECM	Extracellular matrix
EMA	European medicine agency

FDA	Food and drug administration
FESEM	Field emission scanning electron microscopy
FTIR	Fourier transform infrared
GA	Glutaraldehyde
Ga	Great Amberjack
GBR	Guided bone regeneration
GSK3	Glycogen synthase kinase 3
GTR	Guided tissue regeneration
HA	Hydroxyapatite
HCl	Hydrochloric acid
HIF	Hypoxia-inducible factors
HIF-1-α	Hypoxia-inducible factors-1-α
Hm	Hoarse mackerel
HRTEM	High resolution transmission electron microscopy
IR	Infrared
ICP-OES	Inductively coupled plasma-optical emission spectroscopy
JCPDS	Joint committee on powder diffraction standards
K	Potassium
KBr	Potassium bromide
KCl	Potassium chloride
K_2CO_3	Potassium carbonate
$K_2HPO_4 \cdot 3H_2O$	Di-potassium hydrogen phosphate trihydrate
Mg	Magnesium
$MgCl_2 \cdot 6H_2O$	Magnesium chloride hexahydrate
MSCs	Mesenchymal stem cell

Na	Sodium
NaCl	Sodium chloride
Na_2CO_3	Sodium carbonate
$NaHCO_3$	Sodium hydrogen carbonate
NaOH	Sodium hydroxide
Na_2SO_3	Sodium sulfite
Na_2SO_4	Sodium sulfate
NaTPP	Sodium tripolyphosphate
NOS	Nitric oxide synthase
OP	Osteopontin
PO_4^{3-}	Phosphate ion
PBS	Phosphate buffer saline
PDL	Periodontal ligament
PGA	Poly(glycolic acid)
PLA	Poly(lactic acid)
PLGA	Poly(lactic-co-glycolic acid)
RMS	Root mean square roughness
Runx2	Runt-related transcription factor-2
SAED	Selected area electron diffraction
SBF	Simulated body fluid
SCA	Static contact angle
SEM	Scanning electron microscopy
Si	Silicon
Sr	Strontium
SSA	Specific surface area

STA	Simultaneous thermal analyzer
TCD	Thermal conductivity detector
TEM	Transmission electron microscopy
TGA	Thermogravimetric analysis
TGF-β1	Transforming growth factor- β1
Tn	Tuna
TRIS	Tris-hydroxymethyl aminomethane
VEGF	Vascular endothelial growth factor
WR	Working reagent
XRD	X-ray diffraction
XRF	X-ray fluorescence
Yt	Yellow tail
Zn	Zinc
2D	Two dimensional
3D	Three dimensional
β-TCP	β-tricalcium phosphate

LIST OF SYMBOLS

CFU/ml	colony-forming unit per milliliter
cm^{-1}	per centimeter
cP	centipoise
g	gram
h	hour
Hz	Hertz
kDa	kilodalton
kV	kilovolt
L	liter
mA	milliampere
mg	milligram
mg/ml	milligram per milliliter\
min	minute
mL	milliliter
mm	millimeter
mm^2	square millimeter
mmol	millimole
mol	mole
MPa	megapascal
M_w	molecular weight
N	newton
nm	nanometer
pH	potential of hydrogen
phr	part per hundred resin

s	second
SR	swelling ratio
T_{max}	maximum decomposition rate temperature
TSM	total soluble matter
W_d	dry weight of the membrane before swollen in PBS
W_{deg}	weight of membrane after degradation at predetermined time interval
W_f	final weight of the membrane after solubilized
W_i	initial weight of the membrane before solubilized
wt.%	weight percent
W_w	wet weight of the membrane after swollen in PBS
x	amount of HA added
µl	microliter
µm	micrometer
μm^2	square micrometer
^{o}	degree
^{o}C	degree Celsius
%	percent

PENYEDIAAN DAN PENCIRIAN MEMBRAN KOMPOSIT ASIMETRI KITOSAN/BIOGENIK HIDROKSIAPATIT UNTUK REGENERASI TULANG BERPANDU

ABSTRAK

Membran yang digunakan untuk aplikasi regenerasi tulang berpandu (GBR) berfungsi sebagai penghalang fizikal untuk mengelakkan migrasi sel epitelium ke dalam tapak kecacatan sebelum pembentukkan tulang baru berlaku. Objektif kajian ini adalah untuk menghasilkan membran asimetik berdasarkan kombinasi kitosan (CS) dan biogenik hidroksiapatit (HA) untuk aplikasi regenerasi tulang berpandu (GBR). Secara ringkasnya, HA yang mempunyai sifat komposisi, struktur dan morfologi yang optimum telah berjaya diekstrak daripada sisa tulang ayam melalui proses pengkalsinan pada suhu 600 ^{o}C dan tempoh pengkalsinan selama 20 h. Seterusnya, HA yang disediakan telah dicampur dengan CS untuk menghasilkan CS/HA membran komposit, di mana kesan penambahan pengisi (10-50 phr HA) dan rawatan dengan natrium hidroksida (NaOH) terhadap ciri-ciri membran yang dihasilkan akan dinilai berkenaan dengan perubahan dari segi morfologi permukaan, struktur, mekanikal, hidrofilik permukaan, antimikrobial, pembengkakkan, penjerapan protein, degradasi dan bioaktiviti *in vitro*. Mikroskopi Imbasan Elektron (SEM) menunjukkan bahawa semua membran komposit mempunyai permukaan asimetik "licin-kasar", di mana tahap kekasaran tersebut semakin meningkat apabila jumlah kandungan pengisian HA semakin bertambah. Selain itu, penambahan HA turut meningkatkan keupayaan CS/HA membran untuk menjerap protein. Berbanding dengan membran CS tulen, membran komposit dengan kandungan pengisian HA yang melebihi 10 phr turut menunjukkan peningkatan drastik dari segi sifat bioaktiviti. Tambahan lagi, rawatan membran dengan NaOH juga menambahbaikan daya tahan air dan sifat mekanik,

namun, ia turut menghilangkan keupayaan antimikrobial CS yang sedia ada. Secara keseluruhannya, semua membran menunjukkkan degradasi kurang daripada 22% daripada berat asal selepas tempoh inkubasi selama 2 bulan, di mana kadar degradasi membran menurun apabila penambahan HA meningkat. Kesimpulannya, hasil penyelidikan ini menunjukkan kebolehlaksanaan HA yang diekstrak dari sisa tulang ayam untuk digunakan sebagai pengisi bioaktif bagi meningkatkan sifat biologi dan juga untuk melaraskan kadar degradasi membran CS untuk aplikasi GBR.

PREPARATION AND CHARACTERIZATION OF ASYMMETRIC CHITOSAN/BIOGENIC HYDROXYAPATITE COMPOSITE MEMBRANE FOR GUIDED BONE REGENERATION

ABSTRACT

Membranes used in guided bone regeneration (GBR) application serve as a physical barrier to prevent the migration of epithelial cell into the defected site before new bone formation occurs. The objective of this research is to prepare asymmetric membrane based on the combination of chitosan (CS) and biogenic hydroxyapatite (HA) for GBR. Briefly, HA with optimum compositional, structural and morphological properties was successfully extracted from chicken bone waste via calcination process at temperature of 600 ^{o}C and 20 h of calcination time. Thereafter, the prepared HA was incorporated into CS to form CS/HA composite membrane, where the impact of filler loading (10-50 phr of HA) and NaOH treatment on the characteristics of resulting membranes were evaluated with respect to surface morphology, structural change, hydrophilicity, mechanical property, antimicrobial property, swelling behavior, protein adsorption, degradation and *in vitro* bioactivity. Scanning electron microscopy (SEM) revealed that all composite membranes displayed an asymmetric smooth-rough surface, in which the coarseness of the rough surface increased when the HA content was increased. Furthermore, increasing HA loading also enhance the protein adsorption capability of the resulting membranes. Meanwhile, HA-incorporated membrane exceeding 10 phr loading exhibited improved bioactivity in comparison with pristine CS sample, which able of developing apatitic layer after 4 weeks of soaking in simulated body fluid. On the other hand, it was revealed that NaOH treatment improve water resistance and mechanical properties of all membranes, however, it had unfavourably diminish their antimicrobial ability. Overall, all membranes degraded

less than 22 % of the initial weight after 2 months of incubation period, where their degradation rate decrease further as HA loading increase. These findings demonstrate the feasibility of chicken bone-derived HA to be employed as bioactive filler to augment and tailor the biological characteristics and degradation behavior of CS membrane for GBR application.

CHAPTER ONE

INTRODUCTION

1.1 Research Background

Tooth is a prominent organ for our daily basic activity. However, periodontal diseases which is mainly initiated and perpetuated by a dysbiotic microbiota can greatly impair the structure of the periodontal system that supporting the teeth and thus affecting the fate of tooth health. Periodontitis, which is considered to be one of the most destructive oral pathologies, can adversely affects the integrity of periodontium, and, in severe cases can lead to tooth mobility or tooth loss (Zhang et. al., 2016, Bottino et. al., 2012). According to the survey carried out by the Centers for Disease Control and Prevention, periodontitis affect almost half of the U.S. adult population, and 64% of adults over the age of 65 have moderate to severe forms of periodontitis, which is the major factor contributing to tooth loss (Eke et. al., 2012). Indeed, numerous scientific researches have also suggest that chronic periodontitis can influence systemic health and increase the risk for cardiovascular disease, diabetes, rheumatoid arthritis and possibly kidney disease (Hajishengallis et. al., 2013).

In the past decades, the understanding of tissue engineering and regenerative medicine in dental biomaterials is becoming essential in the effort of developing therapies to re-establish lost, damaged and aging tissues for restoring their biological function. Among numerous approaches, guided bone regeneration (GBR), which was pioneered by Nyman and co-workers in 1982, has emerged as a promising technique to restore the architecture and functionality of the damaged periodontal system caused by periodontitis (Nyman et. al., 1982, Xianmiao et. al., 2009). This technique involves

the insertion of a biocompatible barrier membrane to cover the periodontal bone defect site, which intended to hinder the migration of epithelial and connective tissue from the gingiva into the wound region. In this way, it creates space for the slow-migrating innate cells with regenerative potential to repopulate the wound site and slowly differentiate into a new periodontal bone tissues (Horst et. al., 2012).

Generally, there are two types of barrier membranes used in GBR therapy: non-resorbable (e.g. expanded polytetrafluoroethylene (e-PTFE)) and resorbable (e.g. collagen) membranes. Non-resorbable e-PTFE membrane, despite having good clinical outcome compared to resorbable membrane, has been receiving drawback as barrier membrane due to the necessity for a secondary surgery to retrieve membrane, which therefore increase the likelihood for post-surgical bacterial infection and surgical trauma to occur (Norowski et. al., 2015). Nowadays, the use of resorbable membrane based on collagen has replaced the conventional non-resorbable membrane in clinical GBR therapy due to their biodegradability properties that can avoid additional surgery procedure (Cai et. al., 2017). Nonetheless, the relatively high cost and uncontrollable degradation rate of collagen-based membrane has become a bottleneck for its further application in GBR (Xianmiao et. al., 2009). Due to these limitations, many researchers are seeking for alternative resorbable material with better properties in order to prepare ideal membrane for GBR application.

Among numerous biodegradable polymeric materials, chitosan (CS), which is mainly extracted from marine bio-wastes such as shrimp and crab shells, has become increasingly attractive in subject of bone tissue regeneration application. This is due to its promising biological properties such as biocompatibility, biodegradability, non-toxicity, antimicrobial and wound healing activity (Ahmed et. al., 2018). Furthermore, the structure of CS which resembles those of the extracellular matrix of bone

component e.g. glycosaminoglycans also favor their application for GBR (Teng et. al., 2009). Nonetheless, poor bioactivity exhibited by pure CS has becoming the biggest stumbling block for its application in GBR.

Hydroxyapatite (HA) is a type of calcium orthophosphate based-bioceramic which can be substantially found in the mineralized tissue of vertebrate such as bones and teeth. It has been regarded as one of the most potent implant material or bioactive filler for dental and orthopedic regeneration application due to their ability to physiochemically bonded to the bone and capability to augment the osteoconductivity to promote bone formation (Dorozhkin, 2010). However, it is nearly unattainable for HA to be used alone in soft tissue/ bone defect interface as GBR membrane due to its inherent brittleness in nature.

Since pristine CS lacks bioactivity to promote bone regeneration, its combination with bioactive ceramics such as HA in term of composite system has becoming a popular research trend to fabricate GBR barrier membrane. This composite exploits the flexibility of CS with the bioactive property of the bioceramic fillers to expand the possibility to be used in soft tissue/bone defect interface for periodontal regeneration (Turnbull et. al., 2017, Qasim et. al., 2015). According to the past literatures, it was reported that the incorporation of HA into polymer matrix is capable of favoring apatite mineralization which in turn offering better integration of biomaterials with host bone tissue and further improves osteoblastic activity. For example, Kong and her co-workers (2014) demonstrated that the addition of HA could impart osteo-differentiation, attachment as well as proliferation of mesenchymal stem (MSCs) cell as compared to pristine CS sample. In another work, it was proved that the CS/HA composites scaffold possessed better bioactivity as compared to pristine

CS scaffold, which is evidenced by the formation of more apatite on the composite scaffold surface during biomimetic process (Kong et. al., 2006). Frohbergh and colleagues had revealed that the CS composite scaffold reinforced with HA showed improved osteogenic differentiation of osteoblast-like cells as compared to pure CS scaffold (Frohbergh et. al., 2012). A recent study related to glycol-chitosan/HA composites also showed osteoinductivity toward human bone marrow mesenchymal stem cells, which is promising for bone tissue engineering application (Dumont et. al., 2016). Similar to scaffolding materials mentioned above, it is also envisaged that CS/HA composite can be good candidature material for the fabrication of resorbable barrier membrane for GBR application.

1.2 Problem Statements

Food industries by-products, specifically chicken bone is usually regarded as waste and will be discarded without fully utilizing it. From the perspective of waste to wealth, this by-product can be employed as a cheap bio-resource to form HA. Indeed, naturally-derived HA is more preferred over synthetic stoichiometric HA due to their resemblance properties to the natural bone that is more bioactive. This naturally-derived HA can thus serve as an alternative bioactive filler to improve the biological properties of pristine CS membrane for periodontal regeneration. Based on the past literatures, direct thermal calcination approach is usually performed at various temperature which ranged from 600 to 1200 ^{o}C to form HA (Niakan et. al., 2015, Pal et. al., 2017). Indeed, calcination parameters including heating temperature and soaking time plays a crucial role in affecting the final properties of HA, such as crystallinity, composition and phase impurity (Terzioğlu et. al., 2018). It should be

noted that the calcination parameters should be carefully tailored, since recrystallization can cause crystallite growth which can affect the biological properties of the resulting HA (Ooi et. al., 2007, Zhu et. al., 2017). Since the extraction of biogenic HA from chicken bone waste has not yet been studied, the scrutiny of its optimum thermal calcination parameter is therefore becoming crucial in order to obtain HA with promising biological properties.

Despite diversity of existing CS membrane with different constitutions that have been fabricated, their limited success in clinical GBR procedures have been reported, particularly due to underestimation of morphological aspect for cell growth and tissue integration (Fu et. al., 2017). This is because most of the barrier membrane are constructed in non-porous and dense morphology. Thus, the surface of the membrane which is facing the bone defected region lacks of desirable surface topography to promote cell growth. In order to address this limitation, membrane with distinct morphology on both side of surfaces, namely asymmetrical membrane appeared to be promising to fulfil the requirement of GBR application (Ma et. .al., 2014). On the other hand, pristine CS-based membrane has poor water resistance property which make their functionality as barrier membrane to be hardly attained in clinical practice.

So far, a few literatures have been reported regarding the fabrication of barrier membrane based on the combination of CS and HA for GBR application (Xianmiao et. al., 2009, Song et. al., 2014). Despite this, to date, no research related to the formation of asymmetric CS-based membrane that is incorporated with chicken bone-derived HA have been studied. In this study, novel asymmetric smooth-rough barrier membrane based on biodegradable CS and naturally-derived HA were developed using solvent casting and evaporation method. The rough side of the barrier membrane

contain topographic features that is promising for cell growth on the surface while the non-porous smooth side serve as physical barrier to hinder the invasion of redundant cells (from gingiva) into defected site. Base neutralization treatment was then used to modify and offer the CS/HA membrane with water resistance property to make their application in GBR attainable in clinical practice.

1.3 Objectives

The aim of this research is to fabricate asymmetric resorbable CS/biogenic HA composites membrane by solvent casting and evaporation method followed by sodium hydroxide (NaOH) neutralization treatment. In order to accomplish this goal, the following specific objectives are considered:

i. To investigate the effect of calcination temperature and soaking time on the crystallinity, compositional and morphological properties of biogenic HA derived from chicken bone wastes.
ii. To evaluate the effect of increasing HA loading and NaOH neutralization on the solubility, surface, mechanical and antimicrobial properties of CS/HA composites membrane.
iii. To evaluate the effect of increasing HA loading on the surface wettability, protein adsorption, swelling behavior, degradation and *in vitro* bioactivity of the base-treated CS/HA composites membrane.

1.4 Research Scope

The research scope of this study can be divided into three major stages. The first stage of research involves the extraction of biogenic HA from chicken bone

wastes via thermal calcination approach, where the crystallinity, morphology and composition of the calcined samples were assessed as a function of different calcination temperature (i.e. 600-1000 ^{o}C) and soaking time (4, 12 and 20 h).

The second stage of the research involves the formation of asymmetric resorbable composites barrier membrane based on CS and various amount of HA (10-50 phr of HA loading) by solvent casting and evaporation method. Subsequently, the prepared membranes were treated with NaOH solution to make them water-resistance. Thereafter, the impact of HA loading and NaOH treatment on the solubility, morphological, mechanical and antimicrobial properties were investigated by means of total soluble matter, fourier transform infrared spectroscopy, x-ray diffraction, scanning electron microscopy, atomic force microscopy, tensile and agar diffusion testing.

For the final stage of the research, swelling behavior and *in vitro* evaluation including protein adsorption, bioactivity and degradation were further scrutinized for all NaOH -treated membranes.

1.5 Thesis Outline

This thesis is organized as follows. Chapter one discuss about the research background, problem statement, objectives and research scopes of the study. Chapter two begins with a concise review of periodontal guided bone regeneration alongside with the important criteria for designing ideal barrier membrane for guided bone regeneration. A brief overview regarding the pros and cons of commercially available barrier membrane was also covered in this section. The final part of this chapter highlights the promising characteristics of CS and naturally-derived HA to be serve as

alternative materials to form barrier membrane. Various modification approach and fabrication techniques for CS-based membrane were also embraced in this section. Meanwhile, Chapter three describes the experimental procedure for the material preparation and the characterization of the final products. The experimental results for the prepared HA as well as all the composites membrane fabricated were extensively discussed in Chapter four. Lastly, Chapter five elucidate the quality findings of the research by summarizing all the important output obtained and the suggestions for future studies were also provided.

CHAPTER TWO

LITERATURE REVIEW

2.1 Periodontium

Periodontium is an intricate supporting apparatus that anchoring teeth to the jaw bones while withstanding and dissipating external forces originated by the chewing process. It composed of four essential components including gingiva (gum), alveolar bone, periodontal ligament (PDL) and cementum. Both gingiva and PDL are the soft tissues whereas the alveolar bone and cementum are the mineralized hard tissues. Gingiva is part of the mucous membrane lining the inside of the mouth that firmly bound to the underlying bone supporting the teeth and protect them from oral environment. The alveolar bone is part of the jaw bone that forms the tooth sockets to support and hold teeth in place. Cementum is the mineralized connective tissues that binds to the root of the teeth. PDL is a connective tissue that connects the cementum to the surrounding alveolar bone. Basically, the structure and functionality of the periodontium can be greatly compromised by bacterial-infected periodontal diseases such as gingivitis, gingival recession and periodontitis (Babo et. al., 2014, Sowmya et. al., 2013). Figure 2.1 shows the anatomy of the periodontium.

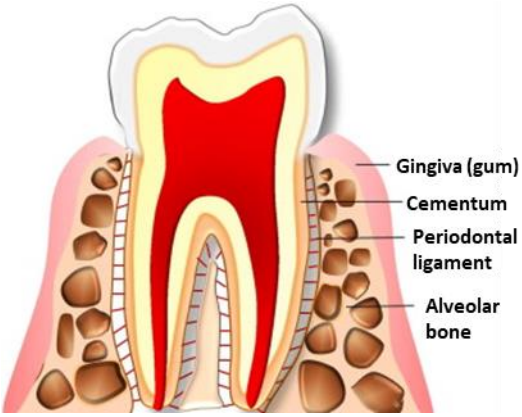

Figure 2.1: Anatomy of the periodontium (Sowmya et. al., 2013).

2.2 Periodontal Guided Bone Regeneration

Periodontitis is a chronic inflammation disease of the periodontal system triggered by dental plaque and microorganism accumulation on the oral cavity. It affects the structural integrity of the periodontal system by progressively damaging the tooth-supporting tissues (including gingiva, PDL, cementum as well as the alveolar bone) around the affected region, thus forming pocket around the teeth. This periodontal pocket will serve as a reservoir for further bacterial growth which ultimately causing tooth loss if left untreated (Zhang et. al., 2016, Sheikh et. al., 2016, Sam and Pillai, 2014).

Traditional treatment of this disease such as open flap surgery often includes the elimination of infection factor from the inflamed areas by exposing the supporting alveolar bone and root surfaces of teeth for intense cleaning procedure. However, this approach offers limited potential in regenerating the damaged periodontal tissue, as the invasion of fast-growing epithelial and gingival tissues into the empty periodontal defect will result in long junctional epithelium which suppress the subsequent healing of alveolar bone and PDL (Mota et. al., 2012). Indeed, periodontal damage can be reversed as the osteoprogenitor cells residing in periodontal tissues (including cementoblasts, fibroblasts, and osteoblasts) can differentiate into new PDL, cementum and alveolar bone, provided they have the chance to recolonize the periodontal wound ahead of epithelial tissues (Bottino et. al., 2012, Horst et. al., 2012).

In recent years, guided bone regeneration (GBR) technique has emerged as the most widely practiced regeneration therapy for periodontitis treatment. GBR, which is a regeneration treatment originated from guided tissue regeneration (GTR), is primarily based on the concept of partitioning defected bone from soft tissue. In

GBR therapy, a barrier membrane is placed in the interface between soft tissue and restoration areas which aimed to occlude the migration of fast-growing epithelial tissues from soft tissue toward the bone defected region. In this way, it creates a secluded space for the slow-migrating innate cells with regenerative potential to repopulate the wound site and slowly differentiate into a new periodontal bone tissues (Cai et. al., 2017). Barrier membrane usually work in conjunction with bone graft in GBR treatment to preserve the applied grafting material by reducing their resorption rate (Figure 2.2) (Sheikh et. al., 2017).

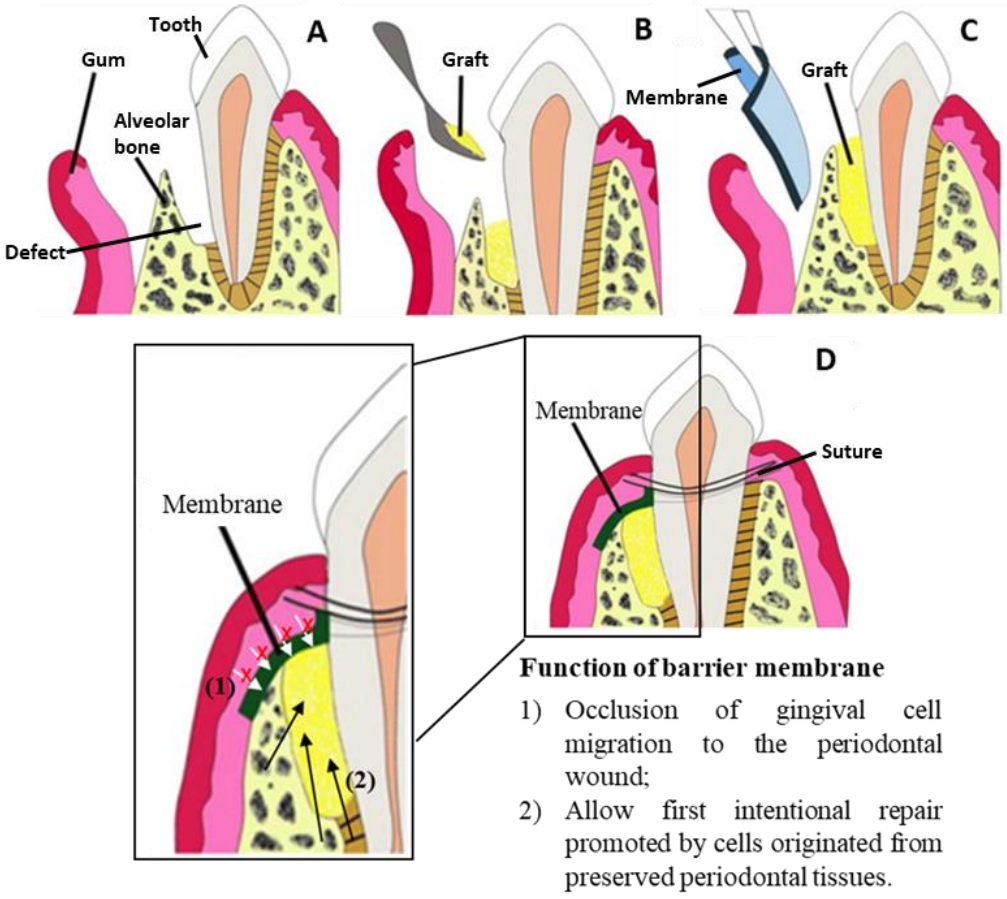

Figure 2.2: Schematic diagram illustrating the application principle of a combined therapy encompassing barrier membrane and bone graft for periodontal regeneration: (A) Retraction of gum and debridement of periodontal wound, (B) packing of graft material into osseous defect, (C) placement of barrier membrane providing the space necessary for new tissues ingrowth; (D) colonization of the intraosseous wound with progenitor cells from PDL and alveolar bone (Sowmya et. al., 2013).

2.3 GBR Barrier Membranes

2.3.1 Ideal Requirements for Barrier Membrane

Barrier membrane is one of the key elements in GBR therapy. For a barrier membrane to be utilized in GBR application, there are certain ideal design criteria and requirements that should be taken into consideration. These include biocompatibility, cell occlusivity, bioresorption, mechanical strength, surface property, biological activity and clinical manageability (Qasim et. al., 2015, Hitti and Kerns, 2011, Liu and Kerns, 2014, Sam and Pillai, 2014, Rakhmatia et. al., 2013).

i. **Biocompatibility**. The material used to fabricate barrier membrane should be well-integrated with host tissue without eliciting immune response or chronic inflammation after implantation, as material incompatibility will adversely affect the subsequent healing and cause rejection by the patient body.

ii. **Cell occlusivity**. Basically, migration of gingival cell is ten times faster than osteoprogenitor cell (innate cell responsible for bone formation). Therefore, the barrier membrane should function as physical barrier against invasion of redundant cells or tissues (gingival epithelium and connective tissue) into bony defect, thus providing sufficient time for periodontal bone tissue to regenerate. Membrane design, such as porosity is therefore closely related to the cell occlusivity and should be well-tailored as this have major impact for the potential cell invasion. Noted that the size of gingival fibroblast cell ranged from 10-15 μm (Ma et. al., 2014).

iii. **Bioresorption**. The membrane should degrade after its role as a temporary cellular barrier has been attained, and the degraded by-product should be non-toxic and can be excreted from the body through natural pathway without

interfering with other organ. This aspect is pivotal for current development of barrier membrane as it eliminates the need of second stage surgery intervention to remove the implant. It is also crucial for the membrane disintegration rate to synchronize with the new tissue formation rate. Usually, barrier membrane should maintain its structural integrity for at least 4-6 weeks to ensure uninterrupted regeneration of new periodontal tissue (Sowmya et. al., 2013).

iv. **Mechanical properties**. The designed membranes should possess adequate sustained mechanical strength to protect underlying blood clot against pressures originated from overlaying gingival flaps and chewing process, until clot forming underneath barrier membrane has matured enough to serve as scaffold for the ingrowth of progenitor cells. GBR barrier membrane also require flexibility to enable membrane to optimally cover over the defected area (Xue et. al., 2015). A barrier membrane that is too stiff can be hard to mold to the shape of the defected region. Moreover, the sharp edges from a membrane that is too stiff might perforate the gingival tissue and leads to issue such as membrane exposure.

v. **Biological activity**. Ideally, the barrier membrane should be bioactive (i.e. able to interact with the surrounding living tissues) and able of promoting the growth and differentiation of progenitor cells towards PDL cell, osteoblast and cementoblast lineages, which is responsible for the regeneration of PDL, alveolar bone and cementum. Additionally, barrier membrane with antimicrobial properties can be useful to hamper bacterial colonization in the wound area that might afflict the bone regeneration.

vi. **Surface properties**. Surface wettability and topographic characteristics of the barrier membrane should be designed in such a way to promote the attachment

and proliferation of osteogenic cell. Generally, biomaterial with hydrophilic surface is more propitious for the attachment of cells. The rationale behind can be elucidated by the adsorption of proteins on the biomaterial surface. Upon implantation of the periodontal membrane at the defected sites, protein adsorption is the first biological response that will takes place on the membrane surface before cell can adhere to the surface. Serum protein, which constituted of both hydrophilic and hydrophobic ends, can be adsorbed to both hydrophilic and hydrophobic surfaces. Nevertheless, protein adsorbed on different surfaces can leads to different conformation changes that can affect subsequent cell adhesion. Usually, hydrophilic surfaces which interact closely with aqueous biological fluids can enabling a normal protein adsorption on the biomaterials surface, where the protein will transform into active conformation that enable subsequent interactions with cell. Meanwhile, adsorbed protein on hydrophobic surfaces tends to denature partially and in turn making their cell-binding sites to become less accessible (Figure 2.3) (Gittens et. al., 2014, Chen et. al., 2018).

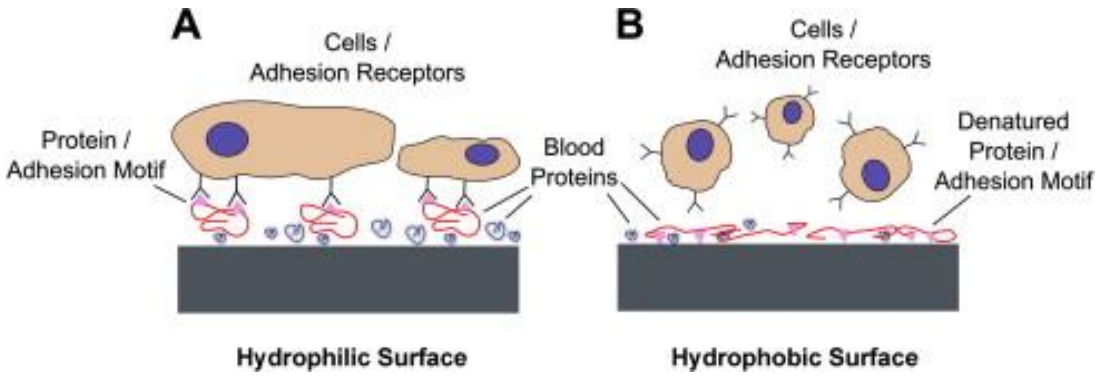

Figure 2.3: Schematic of the possible interactions with (A) hydrophilic and (B) hydrophobic surfaces on protein adsorption, conformation as well as cell adhesion (Gittens et. al., 2014).

Furthermore, membrane with hydrophilic surface is more favorable as compared to hydrophobic surface due to the likelihood of the latter to trigger immune

response at the implanted site (Ghasemi-Mobarakeh et. al., 2015). Surface topography and roughness of the biomaterial also play a vital roles in affecting the bone regeneration process. Nano-structural surface with high surface area is conducive for mediating attachment, growth and differentiation of cells necessary for bone regeneration. In comparison with smooth surface, membrane with roughened surfaces are able to induce osteointegration more by increasing the apposition of osseous tissue. (Fidalgo et. al., 2018).

vii. **Clinical manageability**. Barrier membrane should be fabricated in configuration which is easy to handle and manipulated, trim and to implant during surgical procedure.

2.3.2 Classification of GBR Barrier Membrane

In general, the barrier membranes utilized in GBR therapy can be divided into two categories from the point view of materials stability in body, i.e. non-resorbable and resorbable membranes.

2.3.2(a) Non-resorbable Barrier Membrane

The first attempt to regenerate periodontal tissue using guided regeneration concept is based on the non-resorbable barrier membrane which is constructed from cellulose acetate (Millipore® filter membrane) (Nyman et. al., 1982). A series of animal studies showed that the integration of this membrane in GBR resulted in regeneration of alveolar bone, PDL and cementum. Since then, continuous studies of barrier membrane using various types of materials have been developed. Currently, the most prevalently used non-resorbable membrane is based on a fluorocarbon

polymer called expanded-polytetrafluoroethylene (e-PTFE, Teflon®). Over the past decades, e-PTFE has been used in various fields of biomedical applications such as abdominal reconstruction, cerebral, cardiovascular and cosmetic facial surgeries, and many studies also highlights its potential in tissue-guided regeneration application (Rakhmatia et. al., 2013).

In case of e-PTFE-based membrane, their promising characteristics such as biocompatibility, chemical stability and biological inertness to the implanted site has driven to their utilization in GBR application (Retzepi and Donos, 2010). They does not encounters any microbiological and enzymatic degradation upon implantation and are able to maintain their structural stability for as long as they are left in the implanted site, thus offering periodontist with complete control over the time of application (Elgali et. al., 2017). Furthermore, they also possess superior cell occlusion and space-maintaining properties as compared to resorbable membrane due to the rather poor mechanical properties exhibited by the resorbable membrane (Dimitriou et. al., 2012). For the condition where bone formation is desired in large defects in the oral cavity, e-PTFE-based membrane can be reinforced with titanium to enhance mechanical properties to ensure their space-maintaining capability for bone regeneration. Examples of commercially available e-PTFE-based membrane that have been widely employed in clinical treatment for GBR including Gore-Tex® Periodontal Material, Cytoplast® TXT-200 and Cytoplast® Ti-250 membrane (Hitti and Kerns, 2011, Tayebi et. al., 2018). With their numerous success in past clinical studies, e-PTFE-based membrane is becoming the so-called "gold standard" for future development of barrier membrane.

Due to their non-resorbable property, a second surgical procedure to retrieve membrane become indispensable, which represents a shortcoming for non-resorbable

membrane as it raises concerns of patient discomfort, increased cost and possibility of postoperative morbidity (Bottino et. al., 2012, Turri et. al., 2016). Additionally, the newly regenerated tissues are susceptible to post-surgical bacterial infection and/or trauma which can compromise and decrease the likelihood of complete bone regeneration (Dimitriou et. al., 2012). Another complication of non-resorbable membrane is their potential soft tissue dehiscence and membrane exposure engendering by variable amount of flap sloughing during regeneration process. Once they become exposed to the oral environment, bacterial penetration toward the on-going regenerated tissues might occurs (Neel et. al., 2013).

2.3.2(b) Resorbable Barrier Membrane

Due to the necessity of second-stage surgical procedure to remove non-resorbable membrane, current research has been focused upon the development of resorbable membrane. As the name suggests, resorbable materials possess the capability of being resorbed and eliminated by the body, thus avoiding the requirement for additional surgery procedure to remove membrane. Hence, this conquers all the limitations associated with membrane-removing procedure, such as additional pain, economic burden, potential risk of patient morbidity and tissue damage. Given those benefits, the conventional non-resorbable e-PTFE membrane has been largely substituted by resorbable membranes and becoming the standard for most clinical situations. Currently, there are two types of resorbable-based materials used to fabricate barrier membrane: naturally-derived and synthetic polymers. The best-known natural polymer used for this purpose is collagen whereas synthetic membranes are mainly made up of ubiquitous aliphatic polyesters family such as poly (lactic acid) (PLA) and its copolymers (Sheikh et. al., 2017).

2.3.2(b)(i) Naturally-derived Resorbable Barrier Membranes

Natural polymers, which are either components of, or are similar to the natural extracellular matrix (ECM) display the advantageous point of being able to promote cell adhesion without provoking significant immune response (Bai et. al., 2018). Collagen membrane is one of the most widely scrutinized natural origin resorbable barrier membrane with comparable success rate relative to conventional non-resorbable e-PTFE membrane. Collagen protein, which is the major component of ECM, has garnered enormous attention in GBR application due to its low immunogenicity, biocompatibility, bioresorbability, haemostatic property and good osteoblast adherence ability (Ferreira et. al., 2012). Moreover, collagen membrane has been shown to be chemotactic for PDL and gingival fibroblasts *in vitro*, which subsequently improve cell migration and thus facilitating bone regeneration process (Bunyaratavej and Wang, 2001). Marinucci et. al. (2001) evaluated the impact of non-resorbable e-PTFE and collagen membranes on human osteoblast culture in order to investigate their ability to induce cell proliferation and collagen synthesis. It was revealed that collagen membrane shown augmented alkaline phosphatase (ALP) activity and collagen synthesis compared to e-PTFE membrane. Higher secretion of Transforming Growth Factor-β1 (TGF-β1, a growth factor involve in bone remodeling) as observed in collagen membrane also depict the ability of collagen membrane to promote bone regeneration (Marinucci et. al., 2001). Since collagen is one of the most abundant protein in mammals, commercially available collagen is usually acquired from animal tissues such as skin and tendons, which is mainly derived from bovine and porcine origins (Neel et. al., 2013). Currently, several types of GBR barrier membrane based on collagen have been brought to the market and some of the

commercially available products are Bio-Gide®, BioMend Extend®, Neomem^{TM} and OsseoGuard® (Rakhmatia et. al., 2013).

Despite the promising and peculiar properties of collagen-based membrane for GBR application, its relatively high cost and poor mechanical properties have become a bottleneck for its application in GBR. Other than that, the use of animal-derived collagen membranes (e.g. porcine or bovine derived collagen) also raise issues related to religious belief. Moreover, the rapid and unpredictable rate of collagen membrane resorption engendering by enzymatic action of macrophages and polymorphonuclear leucocytes tends to permit the undesirable cell migration into the deficient region (Yoshimoto et. al., 2018). This can adversely debilitate the maturation of regenerated bone tissue (Qasim et. al., 2015).

2.3.2(b)(ii) Synthetic Resorbable Barrier Membrane

Apart from collagen membranes, poly (α-hydroxy esters) such as poly (lactic acid) PLA, poly (glycolic acid) (PGA) and its copolymers poly (lactic-co-glycolic acid) (PLGA) are the most common biomaterials used to replace traditional non-resorbable materials. These polymers are approved by the U.S. Food and Drug Administration (FDA) and European Medicine Agency (EMA) and have already pave it way to commercial market in various medical application such as orthopedic devices (Biotrak® pins, Osteotrans-MX® and SmartNail®), drug delivery carriers (Lupron® Depot, Zoladex® and Arestin®) and periodontal GBR membranes (Resolute®, Atrisorb® and Epi-Guide®) (Narayanan et. al., 2016). One of the advantages of these polymers for GBR is their biodegradable properties that can be hydrolyzed into smaller oligomers or monomers, which will be further metabolized and eliminated from body

via Krebs Cycle as carbon dioxide and water (Yoshimoto et. al., 2018). Moreover, their degradation rate can be tailored to match the new periodontal tissue regeneration rate by adjusting their chemical composition (e.g. lactide: glycolide ratio), crystallinity and molecular weight (Lee et. al., 2016). In addition, their relatively low cost and higher mechanical strength as compared to collagen membrane also make them viable for such application. Figure 2.4 shows the chemical structures of the common synthetic polyester used for this purpose.

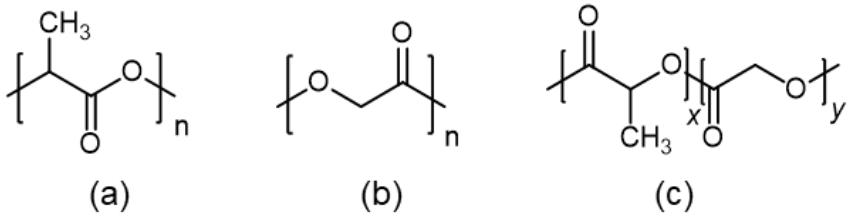

Figure 2.4: Chemical structures of (a) PLA, (b) PGA and (c) PLGA.

Nevertheless, low cellular adhesion ascribed to their inherent hydrophobicity, poor wetting and biologically inert surface have become limitations for synthetic polyester-based barrier membrane in GBR application (Janik and Marzec, 2015). Unlike collagen membrane, pristine polyester-based barrier membrane lacks of osteoinduction property. Hence, their ability to induce new bone formation at the defected region is considerably lower as compared to collagen-based membrane (Lee and Kim, 2014). Besides, accumulation of high concentration of acidic degraded by-products (i.e. lactic acid or glycolic acid) at the implantation site will cause inflammation which can be toxic to the cells. In addition, polyester-based membrane tends to become stiffer and more brittle in hydrated physiological environment which can easily collapsed and thus compromising its role as barrier membrane (Zhang et. al., 2016).

2.4 Chitosan-based GBR Barrier Membrane

Despite a numbers of barrier membranes that have already pave it ways in clinical practice (including e-PTFE, collagen, PLA and PLGA based membranes), novel barrier membranes based on new biomaterials are continuously developed and studied in an effort to overcome the shortcomings of the currently available membranes. Among numerous biopolymers such as chitosan, alginate and silk fibroin, barrier membrane based on chitosan is the most widely scrutinized alternate membrane materials that envisage to meet ideal membrane requirements and current market needs.

2.4.1 Chitosan (CS)

CS, (1-4)-2-amino-2-deoxy-β-D-glucosamine, a partial deacetylated analog of chitin (CT), is a natural polyaminosaccharide constituting N-acetyl glucosamine and glucosamine units linked by β-(1-4)-glycosidic bonds. It is obtained from alkaline hydrolysis of CT, which is a structural biopolymer that can be extracted from the exoskeleton of crustaceans (e.g. shrimp, crabs and lobsters), insects, molluscs and cell walls of fungi (Ahmed and Ikram, 2016, Anitha et. al., 2014). CS and CT are the second most ubiquitous natural biopolymer after cellulose, which possess a glucose-based linear backbone that is very similar to cellulose structure (Patrulea et. al., 2015). As shown in Figure 2.5, they differ from each other by the presence of amine ($–NH_2$) or acetamido ($–NHCOCH_3$) group at C-2 position instead of hydroxyl (–OH) group that available in cellulose (Pighinelli and Kucharska, 2013). Noted that when the degree of deacetylation (DDA) is >50% the deacetylated polymer is usually known as CS (DDA is defined as the number of glucosamine units presence in the

macromolecular chain). The β-1,4-linkage offers a rigid and unbranched structure to CS (Alves and Mano, 2008).

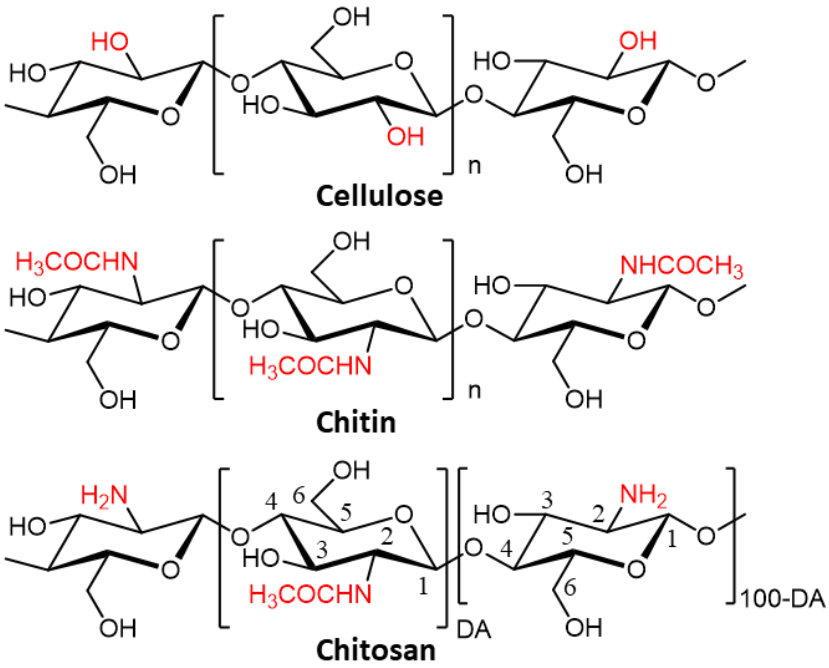

Figure 2.5: Chemical structures of cellulose, CT and CS.

The myriad of hydrophilic groups: –OH groups (primary and secondary –OH group at C-3 and C-6 position respectively), $–NH_2$ or $–NHCOCH_3$ groups along the CS chain offers the ability to form intra- and intermolecular hydrogen bonding. This results in the insolubility of CS in water and organic solvent. Nonetheless, in dilute aqueous acidic medium (pH< 6.3), protonation of free $–NH_2$ groups on the glucosamine repeating unit into $–NH_3^+$ will hinder their intermolecular interaction, thus allows solubilization of CS to occur (Escudero-Oñate and Martínez-Francés, 2018, Hamedi et. al., 2018).

In recent years, CS and its derivative-based biomaterials have gained much attention in periodontal GBR application due to its intriguing biological properties such as biodegradability, tissue compatibility, low foreign body reactions, wound

healing activity and non-toxicity. Furthermore, the structure of CS which resembles those of the ECM of bone component e.g. glycosaminoglycan as well as its non-toxic degraded products that can be easily excreted from the body also made them a suitable candidate for such application (Soundarya et. al., 2018, Tamburaci and Tihminlioglu, 2018). Moreover, CS has shown to possess good affinity to human PDL cells and able of facilitating the osteoblast formation which is responsible for new bone formation (Fakhry et. al., 2004). In dentistry, CS has shown a broad spectrum of antifungal and antibacterial action against numerous oral microorganisms suspected in plaque formation such as *Candida albicans*, *Streptococcus Mutans*, *Aggregatibacter actinomycetemcomitans* and *Porphyromonas gingivalis*. (Hosseinnejad and Jafari, 2016, Hamedi et. al., 2018). Unlike synthetic biopolymeric materials such as PLA that remain rigid in hydrated environment, the flexibility of hydrated CS-based membrane is indeed useful as it ease the handling of membrane during surgical procedure while enabling the membrane surface to adapt to the defected region optimally during implantation (Yamaguchi et. al., 2001). CS also possess excellent membrane-forming property owning to their ability to form intra-and intermolecular hydrogen bonding (Ma et. al., 2017). Unlike collagen that involves a high cost of production, the utilization of CS as starting material is economically feasible and ecologically desirable due to the relatively low cost of production from natural shell source that can be obtained from food industry waste.

One key challenge of pristine biodegradable CS membrane to be used as matrix for the fabrication of GBR membrane is their poor water resistance property. Upon dissolution of CS in aqueous acid medium (e.g. acetic acid) to form membrane, the ionic interaction between protonated $–NH_3^+$ groups and CH_3COO^- ions will form salt residues ($NH_3^+CH_3COO^-$ salt) that is readily soluble in aqueous medium, as their

intermolecular hydrogen interaction become greatly debilitated (Sangsanoh and Supaphol, 2006). This limitation is detrimental as the membrane tends to deform and degrade upon contact with water, which is becoming unrealistic and impossible for them to perform its function as barrier membrane. In addition, pure CS lacks osteoconductivity and osteoinductivity to promote bone regeneration process and displayed relatively low stiffness, especially in moist environment (Zuo et. al., 2010). Therefore, modification of CS membrane is necessary in order to expand its applications.

2.4.2 Modification of CS Membrane

As previously mentioned, biodegradable CS membrane need to be modified in order to last long enough in hydrated physiological environment to be able to perform its barrier functionality over a desired period of time. Various approaches have been taken into consideration to augment the water resistance property of pristine CS membrane, such as crosslinking using chemical agents and alkaline treatment. It is noteworthy to mention that the ideal modification approach should be able to create a cell friendly environment to favor cell viability without impairing the inherent biocompatibility of the CS membrane.

2.4.2(a) Crosslinking using Chemical Agents

Fundamentally, the chemical crosslinking of CS is a reaction with a cross-linker that leads to biopolymer preservation through the formation of linkages between macromolecular chains. Depending on the types of cross-linker employed, the chemical crosslinking process can be divided into two types: covalent and ionic