PRODUCTION OF TEMPORARY BONE SCAFFOLD REINFORCED WITH OPEFB-CMC FROM OIL PALM EMPTY FRUIT BUNCH

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For my parents, husband and children:

Hj. M. Yusup B. Hj. Ridhwan,

Hjh. Basimah Bte. Hj. Pahing,

Noor Nasriq Bin Selamat,

Nur Balqis Yazmin Binti Yazmi,

Muhammad Adib Fahmi Bin Yazmi,

Muhamad Alif Iskandar Bin Noor Nasriq,

Nur Awatif Afiah Binti Noor Nasriq

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ABSTRACT

Bone fracture is a common injury because of its nature position that is mostly closest to skin and exposed to excessive compression and depression. Current treatment for bone fracture employs the scaffolding approaches which are specifically positioned for a certain period of time. These allow the defective bones to undergo proper healing processes. However, these scaffolds have two issues that need to be addressed; the material's compatibility and degradability. Previously, there was poor interaction between the Chitosan (CS) and Hydroxyapatite (HA)/nano HA (nHA) phases causing the composite to have poor physico-chemical properties. This research used Carboxymethylcellulose (CMC) as the reinforcement material for CS / HA or nHA composite scaffold. The main objective is to produce CMC from Oil Palm Empty Fruit Bunch (OPEFB) for temporary biodegradable bone scaffold from a combination of CMC, CS and HA/nHA. Series of experiments were done including extracting CMC from the OPEFB, fabricating composite scaffold by a co-solution method followed by freeze-drying approach to produce a porous bone implant. The final procedure was to analyse the CMC and scaffold produced by various analyses and tests including FTIR, SEM, EDX, TGA and compressive-modulus for its mechanical characteristics. The findings indicated that the strength has increased within 32 – 50 kPa with CMC content compared to chitosan scaffold alone which was only recorded at 0.042 – 0.7 kPa. With the additional of Calcium Phosphate the results only recorded from 0.024 kPa until 2 kPa. The composite scaffold was also successfully constructed with lots of pores, allowing the scaffold to demonstrate preferential proliferation and extracellular matrices and generate mineralised bones. The investigation was extended to in-vitro test involving Simulated Body Fluid (SBF) solution to evaluate the biodegradation rate and

the growing of apatite layer during immersion. The implant had exhibited biodegradation feature parallel to new bone formation. The ability in attracting Calcium (Ca) and Phosphate (P) elements for apatite layer development on its surface was also proven with the calculated value of Ca/P ratio that has identical value with the theory, at 1.67.

ABSTRAK

Patah tulang adalah kecederaan biasa kerana kebiasaannya, ianya terletak paling dekat dengan kulit menyebabkan pendedahan yang melampau pada tekanan yang tidak disengajakan. Rawatan terkini untuk patah tulang menggunakan pendekatan perancah yang berada pada kedudukan yang khusus untuk tempoh masa yang tertentu. Ini membolehkan tulang yang rosak untuk menjalani proses penyembuhan semula. Walau bagaimanapun, perancah ini mempunyai dua isu yang perlu ditangani; keserasian bahan dan degradasi. Sebelum ini, wujud interkasi yang lemah di dalam fasa antara Chitosan (CS) dan Hydroxyapatite (HA) / nano HA (nHA) menyebabkan komposit mempunyai ciri-ciri fiziko-kimia yang lemah. Kajian ini menggunakan carboxymethylcellulose (CMC) sebagai pengukuh untuk CS / HA atau nHA perancah komposit. Objektif utama adalah untuk menghasilkan CMC dari Minyak Sawit Tandan Buah Kosong (OPEFB) untuk perancah tulang sementara yang boleh terbiodegradasi sendiri daripada gabungan CMC, CS dan HA / nHA. Beberapa siri eksperimen telah dilakukan termasuk mengekstrak CMC dari OPEFB, merekabentuk perancah komposit dengan kaedah *co-solution* diikuti oleh pendekatan beku-pengeringan untuk menghasilkan implan tulang yang berliang. Prosedur akhir adalah untuk menganalisis CMC dan perancah komposit yang dihasilkan melalui pelbagai analisis dan ujian termasuk FTIR, SEM, EDX, TGA dan mampatan-modulus untuk ciri-ciri mekanikal. Dapatan kajian menunjukkan bahawa kekuatan ini telah meningkat di antara 32-50 kPa bersama kandungan CMC berbanding perancah chitosan sahaja hanya direkodkan pada 0,042-,7 kPa. Dengan tambahan Kalsium fosfat keputusan hanya direkodkan daripada 0,024 kPa sehingga 2 kPa. Perancah komposit ini juga telah berjaya dibina dengan banyak liang, membolehkan sel-sel tulang untuk memulakan percambahan dan matriks extracellular

dan menjana semula tulang yang baru. Siasatan itu telah dilanjutkan kepada ujian in-*vitro* yang melibatkan larutan *Simulated Body Fluid (SBF),* kaedah untuk menilai kadar biodegradasi dan pertumbuhan lapisan apatite semasa rendaman. Implan tersebut telah menunjukkan cirri-ciri biodegradasi selari dengan pembentukan tulang baru. Keupayaan dalam menarik Kalsium (Ca) dan fosfat (P) elemen untuk pembangunan lapisan apatite di permukaannya juga dibuktikan dengan mengira nisbah Ca/P yang mempunyai nilai yang sama dengan teori, pada 1.67.

TABLE OF CONTENT

LIST OF TABLES

LIST OF FIGURES

LIST OF SYMBOLS AND ABBREVIATIONS

Abs	Absorbance of the peak sample at a particular wavelength
AGU	Anhydroglucose units
ad	air dried
ASTM	American Society of Testing and Materials
B	Scaffold fabricated from CMC commercial, CS and HA
B40	Scaffold with 40% CMC conventional content, CS and HA
CA	Cellulose Acetate
$CaCl_2$	Calcium Chloride
CED	Cupriethylenediamine
CH_2COOH	Carboxymethyl groups
CH_4O	Methanol
$C_2H_4O_2$	Acetic acid
C_2H_6O	Ethanol
$ClCH_2CO_2H$	Monochloroacetic acid
CMC	Carboxymethyl cellulose
CP	Cellulose Phosphate
CS	Chitosan
D	Scaffold fabricated from CMC conventional, CS and nHA
D40	Scaffold with 40% CMC conventional content, CS and nHA
DMSO	Dimethyl Sulfoxide
DP	Dissolving pulp

DP	*Degree of Polymerization*
DP – O	Oxygen dissolving pulp
DP – OZ	Oxygen-Ozone dissolving pulp
DP – OZP	Oxygen-Ozone-Peroxide dissolving pulp
DS	Degree of Substitution
DTG	Derivative of TGA analysis
E	Scaffold fabricated from CMC improvement, CS and HA
E30	Scaffold with 30% CMC improvement content, CS and HA
F	Scaffold fabricated from CMC improvement, CS and nHA
F40	Scaffold with 40% CMC improvement content, CS and nHA
FTIR	Fourier Transform Infrared
GAGs	Glucosaminoglycans
$(HOCH_2)_3CNH_2$ Tris	Tris-hydroxymethylaminomethane
HA	Hydroxyapatite
HCl	Hydrochloric
HDPE	High Density Polyethylene
H_2O_2	Hydrogen Peroxide
H_2O_{volume}	Volume of water
H_2SO_4	Sulphuric acid
KCl	Potassium Chloride
$K_2HPO_4.3H_2O$	Di-potassium hydrogen phosphate trihydrate
MSCs	Mesenchymal stem cells
M_{avg}	Average moisture content
$MgCl_2.6H_2O$	Magnesium Chloride hexahydrate
$MgSO_4.7H_2O$	Magnesium Sulphate aqueous solution
NaCl	Sodium Chloride
nHA	Nano Hydroxyapatite
$NaHCO_3$	Sodium Carbonate
NMR	Nuclear Magnetic Resonance
NaOH	Sodium Hydroxide
Na_2SO_4	Sodium Sulphate

od	oven dried
OPEFB	Oil Palm Empty Fruit Bunch
OZP	Oxygen-Ozone-Peroxide
PP	Polypropylene
SBF	Simulated Body Fluid
SEM	Scanning Electron Microscope
TAPPI	Technical Association of the Pulp and Paper Industry
TBAF	Tetrabutylammonium Flouride
TCF	Totally Chlorine-Free
TE	Tissue Engineering
TGA	Thermogravimetric Analysis
V	Volume
V_{NaOH}	Volume of aqueous NaOH
$V_{overall}$	Volume of overall liquid
w_a	Initial weight of scaffold (dry weight)
w_b	Weight after dried in an oven
w_d	Dry weight of scaffold before immersed in ethanol
W_o	Weight after immersed in SBF
W_w	Dry weight of scaffold
w_w	Weight of scaffold after immersed in ethanol
XRD	X-Ray diffraction

Greek letters

$\rho_{ethanol}$	Density of ethanol

CHAPTER 1

INTRODUCTION

1.1 Background

Bone is notably created to support and protect various organs in a body. It produces red and white blood cells and also stores minerals for living things; humans and animals. Mechanical functions of bones are for protection where bones protect internal organs. For instance, the skull is protecting the brain and the ribs are protecting the heart and lungs. In addition, bones also provide a structural body frame to keep the body supported.

Dynamically, as referred to the web from The Cleveland Clinic Foundation (2013), bones trigger movement for the body, where it provides a leverage system for skeletal muscles, tendons, ligaments, and joints function together to generate and transfer forces. So, individual body parts or the whole body can be manipulated in three dimensional spaces. It obviously shows that bones are an eventful structure for all living things for survival to execute daily and routine activities.

The characteristics of bones are very interesting and unique. It bends when it receives sudden, unpredictable forces up to its own limitation (Riggs & Melton, 1995). However, bones are prone to impact from unwanted forces. If the forces exerted against a bone exceeded its limit, bones could not withstand the forces and starts to break. This phenomenon occurs as bones are only covered with very thin

skin and less fat surrounding them, hence provide them with little absorption during higher impact events. Despite easily crack problems, bones are able to regenerate and redeveloped (Yamamuro, 1995). The newly generated bones provide the same functions and strength as normal bones. Bone regeneration is a continuous process and happens for an entire life. Unfortunately, the regeneration process decreases slowly with the addition of age.

Bone healing is a complex process. The time required for ossification or process of bone healing are dependent and can be affected by many factors including types of bone fracture and dependent on the patient's age and their nutritional status (Alvarez & Nakajima, 2009) . Since bone healing is a natural process, the period of time taken to cure is of concern. Therefore, several proactive curings are taken to assist the process of bone healing.

Autograft and allograft techniques are frequently used in order to overcome the bone fracture problem. Autograft is a technique of replacing the fractured bone with the healthy bone from the same person. The advantages of autograft are it provides bone cells and growth factors that are essential for healing and bone regeneration, no risk of disease transfer and no risk of rejection (Silber et. al., 2003; Myeroff & Archdeacon, 2011; Oppenheim, Segal & Spitzer, 2002). Despite the advantages of autograft, the patients are required to have double surgical operations from two different sites in the same body host. This caused double pain to the patients as well as increasing the traumatic experiences of the patients (Valliant & Jones, 2011).

As for options, allograft technique is introduced. This technique involves the bone transplant from different host or a bone bank. Allograft provides safer alternative to patients who are at higher risk of complications under anesthesia. The surgeon would not take a long time to harvest and prepare the autograft, complete the reconstruction faster thus avoid having longer period of surgery (Mahony & Jones, 2008).

Synthetic bone graft substitution brings new phenomena in orthopaedic and tissue engineering after more findings were discovered as an effort in curing the bone defect. Moore, Graves & Bain (2001) quoted that a variety of synthetic bone graft substitutes have been developed during the past 30 years with the aim to minimize the risk of postoperative infection and fractures as well as the potential risk of disease

transmission as it is from synthetic origin. Moreover, synthetic bone grafts also contribute in osteoinductive and osteostimulative (osteointegration) (Moore et. al., 2001) which is an essential attribute for bone regeneration stage, offering biodegradable properties, an ample supply for bone substitute and available in a wide range of size and shape. Unfortunately, most synthetic bone grafts do not provide sufficient mechanical strength like ceramics and they are not osteogenic.

Another type of bone treatment is by metallic implants. In this process, metal plates were used rather than the actual bones. Normally, metal plates used were stainless steel and titanium and Cobalt based alloys (Schmutz, Quach-Vu & Gerber, 2008). They show a high corrosion resistance due to their stable passive layer. However, they also have some benefits; superior in mechanical properties such as hardness and stiffness compared to other materials such as polymer and visible during x-ray (Schmutz et. al., 2008). Metallic implants were used in many treatments and were fairly successful, but problems related to stress shielding during post-healing and fatigue and loosening of the implant limit its function. Moreover, second surgery is usually required in order to remove the metallic implant after healing, and it increases the risk of the operation and the expense to the patient (Middleton & Tipton, 2000).

The above treatments have mentioned several benefits and drawbacks of the treatments. It has been a desire for biodegradable implants to be developed that will eventually biodegrade itself. Upon degradation process, ion releases are able to encourage surrounding cells to form new bone formation more rapid at a preferred rate. According to Pilliar et. al. (2001), the controllable rate of new bone formation is necessary in order for the defect site to eventually be replaced by a newly formed natural bone and strong enough to fulfil required load-bearing. The new bone can at least be functional during the early stage of the post - implantation period, before significant bone ingrowth and the replacement has occurred.

Most metallic materials are not biodegradable, which bring polymeric materials more benefits than the metal implants because it eliminates the need for a second operation and can prevent some problems associated with stress shielding. Sundararajan, Ma & Howard (1999), Pilliar et. al. (2001), Langer & Vacanti (1993), Hubbel (1995), Hellman (1997) and Niklason & Langer (1997) have stated that the tissue engineering

approach to repair and regenerate is founded upon the use of polymer scaffolding which serve to support, reinforce and in some cases organize the regenerating tissue. So, the reconstruction of new bone is more effective and well organized.

There is a need for the development of new biodegradable materials to be used in orthopaedics and as scaffolding for hard tissue enginnering (Mano et. al., 1999). Polymers are often used as matrix in bone scaffold composite. For example, lignocellulosic fibers obtained from renewable resources where it is composed from carbohydrate polymers is one of the example of natural polymer. An example of carbohydrate polymer is cellulose. It is the abundant renewable resource that has become of more and more interest as reinforcement in composites. This is because they are biodegradable and harmless for the ecological system. Furthermore, they have promising mechanical properties and are less expensive than conventional synthetic polymers (Zimmermann, Pohler & Geiger, 2004).

1.2 Problem Statement

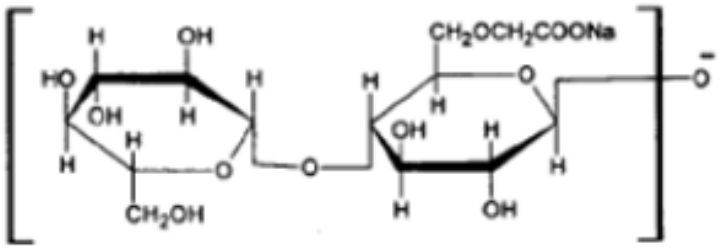
Bones are important organs to ensure smooth movement for daily activities but it is prone to get fractured since it is surrounded by thin skin and less fat. That makes it easily exposed to get harmed. Bone implant is the second most replaced organ in the body after blood where approximately 2.2 million bone graft procedures are performed worldwide each year (Giannoudis, Dinopoulos & Tsiridis, 2005). Moreover, the estimated cost of these procedures approaches $2.5 billion per year (Desai, 2007). While bone transplantation and tissue reconstruction are highly successful therapies for a variety of bone diseases and fracture problems, a shortage of donor bone tissue limits their application (Jones & Hench, 2001).

Due to the serious circumstances, the vital alternative is to create an implant fabricated from synthetic and also natural sources. Extracellular matrices (ECMs) of hard tissue are composed of organic (collagen type I and small amount of GAGs-glycosaminoglycans) and inorganic phases (mainly nano hydroxyapatite crystals – nHA) (Zhao et. al., 2002).

Nano scale HA is known to own excellent biocompatibility based on its close chemical and crystal resemblance to bone material (Hench, 1998; Suchanek & Yoshimura, 1998; Gomez-Vega et. al., 2000). While that, chitosan (CS) can accelerate the bone formation because of the similarity to GAGs in structure (Seol et. al., 2004; Di, Sittinger & Risbud, 2005; Madihally & Matthew, 1999; Yamane et. al., 2005; Loke et. al., 2000).

However, there is a poor interaction between CS and HA/nHA phases causing the composite to have poor physico-chemical properties. Due to the fact that normally, for interface improvement between HA/nHA and CS, the second organic polymer acts as reinforced phase in HA/nHA-based composite is essential (Jiang, Li & Xiong, 2009b). Carboxymethyl cellulose (CMC) possesses very similar structure to CS structure which creates strong ionic cross-linking action between CMC and CS (Xiao et. al., 2006; Qiu & Li, 2005). This evidence has been supported by Latif, Anwar & Noor (2007) as shown in Fig. 1.1.

(a)



(b)

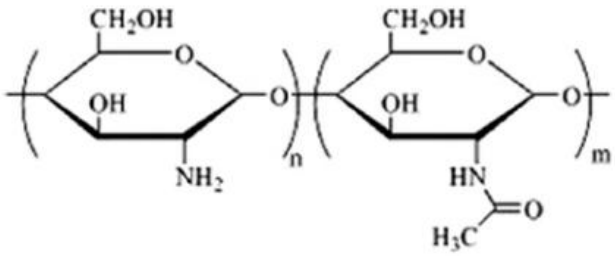


Figure 1.1: The chemical structure of (a) CMC, and (b) Chitosan (Latif et. al., 2007)

Briefly, CMC, also known as cellulose gum, is a cellulose derivative with carboxymethyl groups (-CH_2-COOH). The functional group is bound to some of the hydroxyl groups (-OH) of the glucose monomers that make up the cellulose backbone.

The availability of CMC sources is undoubted. In this research, it was extracted from Oil Palm Empty Fruit Bunch (OPEFB). Empty Fruit Bunch (EFB) from palm oil waste is a potential raw material. This is because palm oil has made an impressive and sustained growth in the global market over the past four decades, and it is projected that in 2016-2020, the average annual production of palm oil in Malaysia will reach 15.4 million tonnes (Teoh, 2000; Abdullah & Sulaiman, 2013).

Sulaiman et. al. (2010) indicated that large amount of oil palm residues that can be re-utilised were dumped. This resulted in millions of ringgit energy value wasted each year with approximate loss of about 6,379 million ringgit (Sulaiman et. al., 2010). Due to the environmental concerns over properly disposing the waste, OPEFB could be converted into useful material in biomedical engineering.

Therefore, a novel approach of the composite with the additional of CMC as a natural polymer in order to reinforce CS and HA was created to address the limitations of the previous sample. For the scaffold to integrate with surrounding tissue, it should imitate the structure and morphology of the natural bone tissue (Stevens et. al., 2007). Thus, there is strong ionic cross-linking action between CMC and chitosan and it is able to produce better composite for bone scaffold. The strong ionic cross-linking between CMC and chitosan is possible to occur because chitosan is a cationic polymer whereas CMC is an anionic polymer where by their combination, a strong ionic bond is created to produce stronger composite.

1.3 Objective

The aim of this research is to produce CMC from OPEFB as biomaterial for temporary bone scaffold reinforced with chitosan and HA/nHA. In order to achieve the aim, several objectives have been highlighted as follow:

(1) To evaluate and analyse the performance of the OPEFB-CMC as the reinforcement material to strengthen chitosan and HA/nHA, as a porous composite scaffold,

(2) To investigate the strength of composite by compression test and physical characteristics,

(3) To evaluate the degradation time, apatite layer formation, porosity measurement and swelling ability through in-vitro test simulation.

1.4 Scope of Research

The scope of this research includes:

(1) To produce CMC that was synthesized from oil palm waste, the empty fruit bunch. It was chosen because it dissolved easily in water because in order to utilize cellulose widely in any application, cellulose must be converted to soluble derivatives. The fabrication process is also at lower cost, easy and safe to produce. Analyses involved are FTIR and XRD.

(2) To produce porous scaffold fabricated from natural polymer and HA/nHA with the attendance of chitosan for better physico-chemical properties.

(3) To investigate the mechanical properties and focus only on compression test in evaluating the effectiveness of CMC as a potential material in bone scaffold. The analysis involved is TGA analysis.

(4) To analyse the morphology of the scaffold including its porosity content either at the surface or inside the scaffold. FTIR, SEM and EDX analyses will be implemented to examine this.

(5) To simulate the biodegradation rate of bone scaffold and the growing of apatite layer by immersion of samples in Simulated Body Fluid (SBF) liquid for in-vitro test.

1.5 Contribution to Knowledge

CMC in this research was produced through two different methods from OPEFB. The first method was followed by conventional and commercial product processes. It used aqueous Sodium Hydroxide (NaOH) for cellulose activation. Improvement in fabricating process of CMC using solid NaOH approach for cellulose activation with the polysaccharide was priorly dissolved in Tetrabutylammonium Flouride (TBAF)/Dimethyl Sulfoxide (DMSO). So, there is no requirement to use aqueous NaOH to obtain CMC with higher Degree of Substitution (DS).

Conventionally, CMC wide application was in fields of membrane separation, coating, film and textile (Fauzi, Wan Daud & Mohamad Ibrahim, 2014) while CMC is famous in the application of food, pharmaceuticals, toothpaste, detergents, oil drilling mud, paper coating (Ambjornsson, Schenzel & Germgard, 2013; Stigsson, Kloow & Germgard, 2001) and others. This research attempts to use the material in orthopaedic applications for temporary bone scaffold. The manufacturing of the product itself was very environmentally friendly since no chlorine was involved during bleaching process, easy and safe to produce in the laboratory.

The scaffold fabrication by previous co-solution method added all the materials together in distilled water before uniformly agitated on hotplate. Since OPEFB-CMC state is not identical to commercial products, a new method has been developed. In order to dissolve and uniformly disperse the OPEFB-CMC in distilled water, there is a requirement to sonicate it before mixed with other materials. This resulted in more strength composite.

1.6 Organization of Thesis

This thesis contains six chapters. Chapter 1 is the introduction explaining briefly about the background, problem statement, objective, scope and contribution of the research.

Chapter 2 explains the literature review of current and latest studies. This includes explanation about bone structure, problems involving bones, the materials commonly used for bone tissue engineering, fabricating of bone implant and some review about the analyses and tests for bone scaffold.

Chapter 3 describes the methodology carried out during the experimental work. It comprises of generally two main parts which are materials used and procedures in fabricating bone scaffolds.

Chapter 4, provide the results and discussions of characterization of CMC from oil palm waste, the empty fruit bunch.

Chapter 5 is the analysis results for porous bone scaffold fabricated from the combination of organic materials which are chitosan and CMC and inorganic material, hydroxyapatite and its nano size.

Chapter 6 is the final chapter of the thesis consisting of the conclusion and future recommendations. It summarizes the overall findings from the experiments done. The conclusions reflected the achievements of the listed objectives obtained throughout the study. Finally, future recommendations for the research are listed for improvement of future study in the same field of study.

CHAPTER 2

LITERATURE REVIEW

This chapter discusses some important features related to previous works done by other researchers. This includes the literature reviews on tissue engineering, bone fractures, materials used for bone replacements as well as the procedures of bone generations. This chapter gives some information for readers on the research conducted herein.

2.1 Introduction

The conjunction of a combination of cells, engineering and materials methods, together with suitable biochemical and physico-chemical features to improve or replace biological functions are known as Tissue Engineering (TE) technology. It has gained so much attention since it used the combination of biology, engineering and material science in providing suitable biochemical and physiochemical factors to achieve better improvement while replacing biological functions. TE involves attempts to mimic specific biochemical and physical functions combining cells within artificially-created support systems (Carrico et. al., 2008).

Engineering factors are commonly associated with the repair or replacement of tissue portions or the whole tissue itself, for example, bone, cartilage, blood vessel, etc. It includes the mechanical and structural properties to operate properly. The concept is that tissues and organs can be "engineered" to be used for transplantation, which could provide revolutionary and stimulating for self healing.

All inclusive explanation involving TE applied to bone based on natural sources is the main focus of this research. This begins with a brief explanation on bone constitution and characteristics, followed by more detailed clarification on material selection, production of scaffolds and important features necessary to produce good scaffold to be used as temporary bone implant. The scaffold will be evaluated through several analyses and tests to clarify its effectiveness.

2.2 Bone and Bone Tissue

Bones are the rigid organs that form parts of the endoskeleton of vertebrates that function to move, support and protect such vital organs. It is an excellent and inimitable structural composite (Mobini et. al., 2012) composed of two major phases; collagen fibers, serves as an organic matrix with biological apatite precipitated along the collagen fibrils as reinforcing constituent (Park & Larks, 2007; Murugan & Ramakrishna, 2005; Mobini et. al., 2012; Ramakrishna et. al., 2001; Olah et. al., 2006) as shown in Fig. 2.1, designed by nature. The high elastic modulus hydroxyapatite mineral comprises approximately 70% of the dry bone mass and contributes significantly to the bone stiffness (Olah et. al., 2006).

Since bones are original composite, it has also come in a variety of shapes and have a complex internal and external structure. They possess special attributes such as lightweight, yet strong and hard. Moreover they have to fulfil many other functions including mineral storage, acid-base balance, detoxification and sound transduction (Carrico et. al., 2008).

(a) (b)

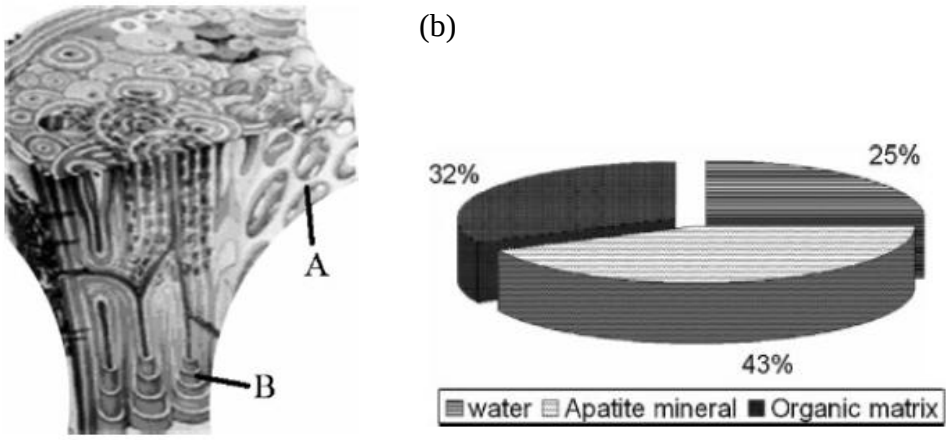


Figure 2.1: (a) Macroscopic features of bone structure: (A) cancellous bone; (B) cortical bone, (b) Composition of the bone in volume percent (Ramakrishna et. al., 2001; Olah et. al., 2006).

The tissue that constructs the bone is a mineralized tissue constituted by hydroxycarbonate apatite (HCA); the mineral that gives it the necessary rigidity, some other proteins and a matrix structured protein (collagen fibrils) that provides tensile strength and toughness (Mahony & Jones, 2008; Stevens & George, 2005).

About 99% calcium content makes bone as a reservoir for calcium in the body. Bone mineral is mostly (85%) in the form of hydroxyapatite (HA, $Ca_{10}(PO_4)_6(OH)_2$), with calcium carbonate (10%), calcium fluoride (2%–3%) and magnesium fluoride (2%–3%) (Polo-Corrales, Latorre-Esteves & Ramirez-Vick, 2014). The chemical composition in bone is as exhibited in Table 2.1 (Liebschner & Wettergreen, 2003).

Table 2.1: Composition of major chemicals in bone (Liebschner & Wettergreen, 2003)

Chemical composition of bone	Percentage (%)
Calcium	26.7
Phosphorus	12.47
Carbonate	3.48
Sodium	0.731
Magnesium	0.436

2.2.1 Compact and spongy bone

The cross section of bones shown in Fig. 2.2 presents the transparent area of bone (Stevens, 2008). Compact bone is tightly packed, situated at the outer layer which nearly approaches the solid state of bone. It contributes 80% of the total bone mass of an adult skeleton and contains 5% – 30% of pores. This tissue, also known as a dense bone due to its minimal gaps and spaces, gives bones their smooth, white and solid appearance.

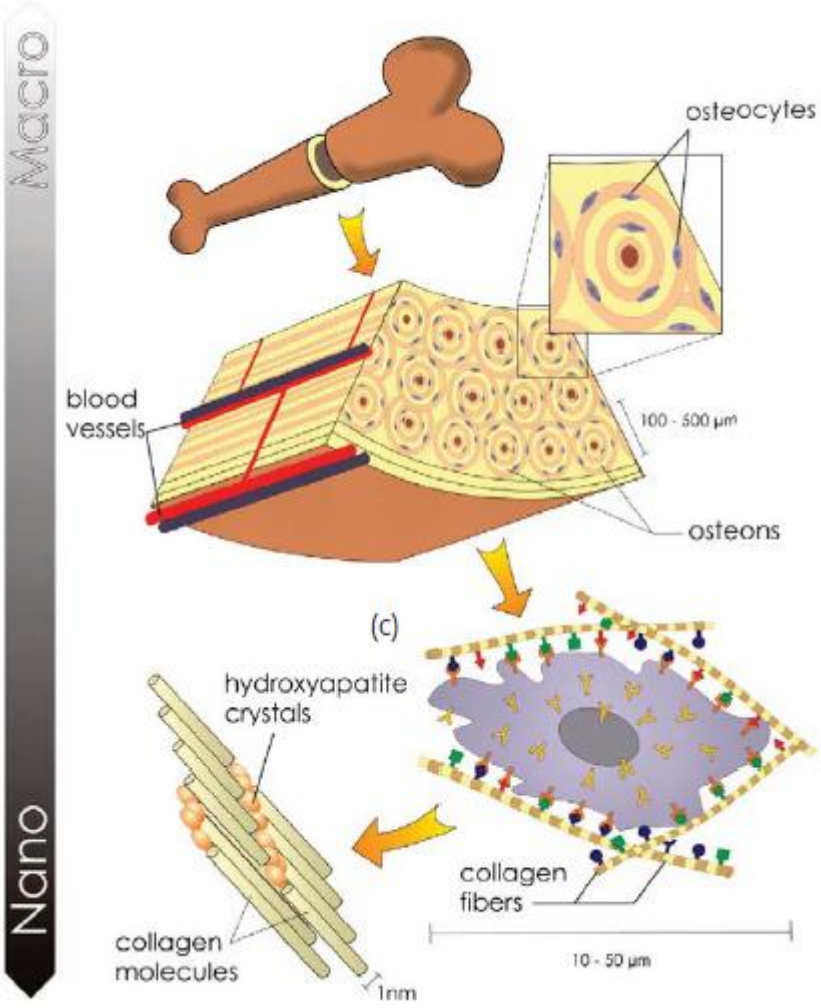


Figure 2.2: Cross-section of bone (Stevens, 2008)

The remaining 20% bone mass is from spongy bones since it contains a lot of pores (sponge look-alike), estimated between 30% – 90%, with nano size where the regeneration of new bone formation begins here. In trabeculae, it corresponds to the areas with the lacuna (void or space) and contains osteocyte, the mature bone cells besides having nerves and veins for blood circularization and providing for cell seeding too (Clarke, 2008; Erikson, Axelrod & Melsen, 1994).

2.2.1.1 Constitution

The matrix builds up the major constituent of bone surrounding the cells. The inorganic matter in the matrix represents about 50% of its dry weight. Crystalline mineral salts and calcium are almost constituted of hydroxyapatite. The organic part of matrix is Type I collagen. In terms of mimicking natural tissue, an ideal polymer for tissue scaffolds would be type I collagen as it has excellent mechanical properties and comprises over 90% of the organic component of bone (Iler, 1979; Pereira et. al., 2005; Valliant & Jones, 2011).

The main difference that distinguishes the matrix of a bone from other cell is its hardness. The matrix is initially laid down as non-mineralized osteoid (manufactured by osteoblasts). Mineralization involves the secretion of alkaline phosphatase by osteoblasts vesicles. As referred to web of Boundless, Born (2015), regarding to the cells, there are three different types: osteocytes (internal bone cells), osteoblasts (bone creation) and osteoclasts (bone resorption).

Osteocytes, which synthesize the organic components of the matrix (type I collagen, proteoglycans and glycoproteins), are exclusively located at the surfaces of bone tissue. Some of them, when surrounded by newly formed matrix become osteocytes and create an empty space, named lacuna as referred to Fig. 2.2. These osteocytes, found each one in a different lacuna, have a kind of extensions – a network of thin canaliculi – able to pass molecules from cell to cell. Osteoclasts, which are multilnucleated giant cells involved in the resorption and remodeling of bone tissue, are derived from the fusion of bone marrow – derived cells, which secret specific enzymes

that promote the digestion of collagen and dissolution of calcium salt crystals (Carneiro, 2005).

2.2.1.2 Histogenesis and bone growth

Bone can be formed in two different ways, there are by direct mineralization of matrix secreted by osteoblasts (intramembraneous ossification) or by deposition of bone matrix on a pre-existing cartilage matrix (endochondral ossification). In both processes, the bone tissue that appears first is primary, or woven. It is a temporary tissue and is soon replaced by the definitive lamellar secondary tissue, in a process that allows maintaining the bone shape while it grows. The rate of bone remodelling (bone turnover) is very active in young children, where it can be 200 times faster than that in adults. Bone remodelling in adults is a dynamic physiologic process that occurs simultaneously in multiple locations of the skeleton, not related to bone growth (Carneiro, 2005).

The relevance of bone TE lies on replacing the organism in a function it cannot naturally perform. The body's bone regenerative capacity is insufficient to heal severely injured bone portions.

2.3 Bone Fracture

In medical, bone failure always refers to bone fracture, a condition of which the existence of small cracks or break in the continuous bones. In general, the common cause for bone fracture can be summarized as follows (Krucik, 2012):

1) Traumatized incidents such as a sports injury, vehicle accidents and falls.
2) Acquired diseases of bone such as osteoporosis.
3) Anomalies formation of bone in a congenital disease such as osteogenesis imperfection and brittle bone disease.

Traumatized incidents contributed to more than half problems in the whole world. It is commonly caused by activities that place stresses on bones and joints like harsh and overwhelming sports and tremendous vehicle accidents. Disease such as osteoporosis is also main contributor for bone fracture problem too.

Jacob et. al. (2008) clarify that osteoporosis is a disease characterized by low bone mass and deterioration of bone structure that increases the risk of fracture. It effects directly from the unhealthy lifestyle such as lack of daily nutrition taken needed by bones to keep healthy.

Jacob et. al. (2008) also mentioned a rate of 26 in 100 women and 4 in 100 men had been told by their doctors they had osteoporosis where the rate show an increment than those found a decade earlier due to the increasing of bone mass testing and extensive educational and awareness effort. Nevertheless, in 2004, only 16 percent of persons admitted to the hospital were diagnosed with osteoporosis proved that osteoporosis is significantly under-diagnosed (Jacob et. al., 2008).

Moreover, a brittle bone disease also known as Osteogenesis Imperfecta presents at birth and occur commonly among babies who have a family history of the disease. The worst case, it could cause hearing loss, respiratory or heart failure, spinal cord and brain stem problems and permanent deformities (Kivi & Solan, 2012).

Furthermore, the nutritional status is usually directly related to the age of the patient. The absorption of nutrient for elderly patient is decreased because the bone is going through aging, which resulted in low collagen in the bone that finally causing low bone formation. Bonjour, Schurch & Rizzoli (1996) stated that deficiency in both micronutrients for example calcium and vitamin D and macronutrients for example protein can accelerate age-dependent bone lose, increase the propensity to fall by impairing movement coordination and affect protective mechanisms that reduce the impact of falling.

2.4 Bone Formation

Clearly, bone regeneration naturally occur is continuously for entire life. Fig. 2.3 shows general bone formation which clearly shows four stages in bone fracture healing process. The stage (1) is the hematoma formation which describes the formation of blood clots from blood leakages. During this stage, bone cells without nutrition died. Stage (2) is the fibrocartilage formation. During this stage, fibrocartilage callus forms a splint. Tissues with new capillaries are granulated whereas dead tissue is disposed. The fibrocartilage callous forms connective tissue fibres, cartilage and some bony matrix. Stage (3) is called the bony callus formation. Here, the osteoblasts and osteoclasts migrate into the tissues and divide, replace the cartilage with bony callus. Finally, in the stage (4), remodelling of bony matrix according to the stresses placed on bone takes place. Permanent patch finally is produced resulted in bone healing completely.

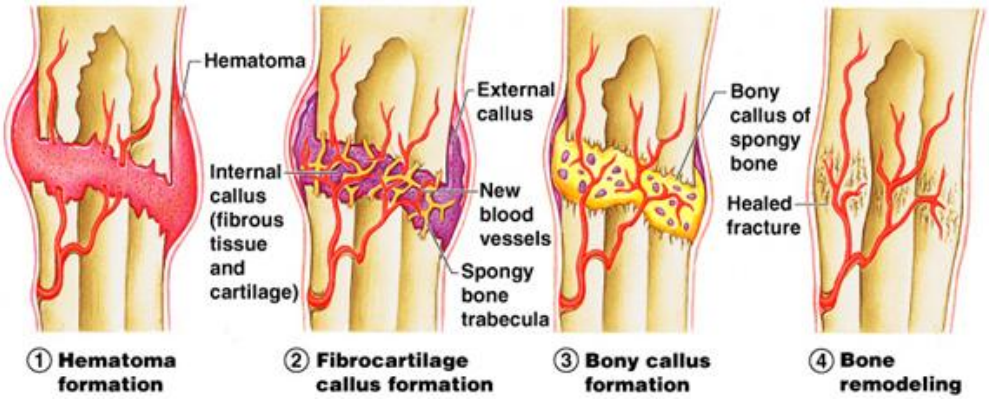


Figure 2.3: Stages in the healing of a bone fracture. (1) Hematoma formation, (2) Fibrocartilage callus formation, (3) Bony callus formation and finally (4) Bone remodelling (Kivi & Solan, 2012)

Due to the advancement in medical treatments, several attempts have been made to encourage bone formation during healing processes. This involves with bone graft, bone synthetic graft with the assistant of foreign things such as metallic implants, and the most recent by using biodegradable and bioactive materials such as polymers and ceramics (Hench, 1991).

2.5 Bone Graft

Bone graft is one of the famous grafts after blood and skin implant (Valliant & Jones, 2011). This is another valuable and successful alternative when facing bone failure, such as fracture and break. Bone graft is a surgical procedure needed when someone suffered bone fracture related problems. It has been confronted with an unlimited amount of challenges since the first successful graft had been performed in the 1870's (Hing, Wilson & Buckland, 2007). There are two prominent techniques in bone grafting including allograft and autograft. Apart from these two techniques, synthetic bone graft is also of interest when dealing with bone fractures (Bauer, 2007).

2.5.1 Allograft

Allograft possesses osteoinductive properties where few mature osteoblasts survive the transplantation (Cypher & Grossman, 1996) but adequate numbers of precursor cells do (Triffit, 1996). It is from these precursor cells that the osteogenic potential is derived. Though it only could be recognized when the graft is utilized in either a morsellized or dimineralized form (Moore et. al., 2001). This is because allograft is a surgical operation which takes bone from another person, commonly from elderly, or also from a bone bank as the main supplier. However, taking bone from an elderly is quite a risky as the mechanical properties are rather low, as a result of decreased protein and collagen contained.

Betz (2002) also has outlined several difficulties arises from having this treatment. Since the defect bone was substituted with bone retrieved from the bone bank, there are several issues related to minor immunogenic rejection and disease transmissions, which unfortunately still unresolved. These bones have higher risk contain less nutrition because in order to lower the risk of disease transmission and also rejection, all bones are irradiated to kill all cells, leaving only the bone matrices (Valliant & Jones, 2011).

Although the risk is low, the probability the bones to get infected (Moore et. al., 2001) are still there. The irradiation process also damages the collagen structure, which causes a severe reduction in terms of the fractures (about 64%) with rate up to 19 percent (Moore et. al., 2001) and fatigue loading (about 87%) (Costain & Crawford, 2009). However, the failure percentage depends on the dosage during the irradiation process. The bones are also commonly harvested from elderly that cause bone density to be low and cause poor mechanical properties (Seebach et. al., 2010).

Bone bank is more risky with complication such as non-union problem (Moore et. al., 2001). Coalition or union of allograft is difficult to assess and inconsistency has been found between clinical, radiological and histological union. Costly, time consuming bone banking as well as the possible danger of viral and bacterial transmitted diseases are disadvantages that were also concluded by Schieker et. al. (2006).

2.5.2 Autograft

In avoiding problems created by allograft, autograft is seen as a better curing for its availability and avoidance of morbidity by autogenous graft. Autograft is largely useful for large bone defects, which require structural support, or when insufficient autogenous graft volume is available. The operation provides all three elements for generating and maintaining bone tissue, namely osteogenic progenitor cells, osteoinductive growth factors, and osteoconductive matrices (Schieker et. al., 2006). However, Schieker and his co-workers also mentioned that this method is still restricted due to limited quantity and an additional secondary operative procedure.

Autograft is limited in terms of more operative time, limited availability and significant morbidity related to blood loss, wound complications, local sensory loss and most importantly, chronic pain as the technique involves double surgery on the same host– on the defected and donor sites. Pain persists more than three months on the donor site, causing trauma to the patients and it seems proportional to the extent of dissection required to obtain the graft. The second site of surgery, which is at the donor site usually requires more time to heal as it needs further treatment under maximum supervision.

Fig. 2.4 illustrated an example of the autograft treatment for tracheal autograft technique (Backer, 2005). Technically, the bone transplant for autograft is identical with this procedure. Moreover, rehabilitation process needs more time, essentially necessary to fasten the bone curing and encouraging new bone to quickly suit usual activities. These are costly which majority of the patients could not afford to cover.

As described in previous section, both operations related to bone graft have their own benefits and drawbacks. Nevertheless, it is essential to create another possible alternative for curing bone defect to avoid any problems that would arise later instead of being dependant to only these two techniques. Constantino & Freidman (1994) altogether with Cypher & Grossman (1996) have stated four characteristics that an ideal bone graft material should exhibit which include:

(1) Osteointegration – the ability to chemically bond to the surface of bone without an intervening layer of fibrous tissue,

(2) Osteoconduction – the ability to support the growth of bone over its surface,

(3) Osteoinduction – the ability to induce differentiation of pluripotential stem cells from surrounding tissue to an osteoblastic phenotype, and

(4) Osteogenesis – the formation of new bone by osteoblastic cells present within the graft material.

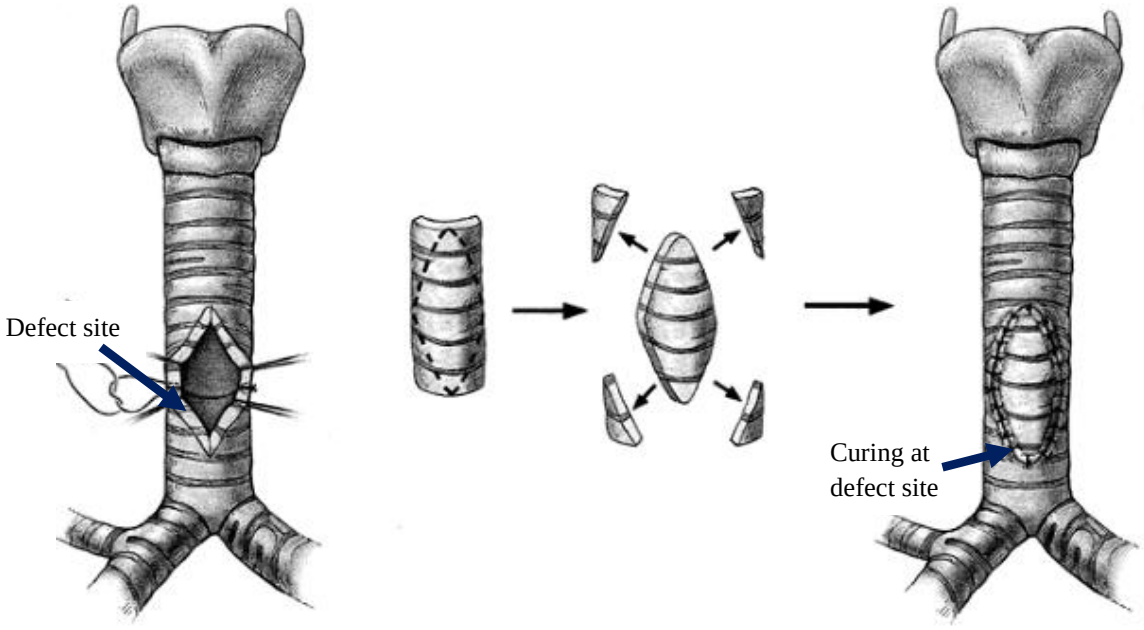


Figure 2.4: Autograft procedure for tracheal (Backer, 2005)

2.5.3 Synthetic Bone Graft

The awareness of bone shortage by applying either allograft or autograft technique in curing bone defect is making researches around the world to figure out several alternatives. The most effective one is by creating a material (other than human sources), that could imitate the ability of natural bone. Synthetic is an unnatural substantial made artificially from materials like ceramic and glass materials. There is also natural or synthetic polymer and the combination of both polymer and ceramic producing stronger composites and lighter which are similar to natural bones (Zimmermann et. al., 2011).

Bauer (2007) said that the material involved with the fabrication of synthetic bone graft should have biological properties like osteointegration which has the ability to chemically bond with the bone surface. In order to implement the task perfectly, osteoinduction which promotes bone formation along its surface when it is placed into the bone would take part followed by differentiation of stem cells from surrounding tissues into osteoblast cells. Osteoblasts are unique cells designed to support the formation of new bones. This is known as osteogenesis.

Synthetic bone substituting graft appearances are diversified such as in the form of bone cement filling, membrane and among them, bone scaffold is the most preferred because Chen, Roether & Boccaccini (2008) highlighted that bone scaffold is highly porous, three-dimensional (3D) which exhibit tailored porosity, pore size and interconnectivity for vascularization. Fig. 2.5 exhibited μCT image of a silica/γ-PGA hybrid foam scaffold from previous works (Valliant & Jones, 2011).

Moreover, porous bone scaffold could priorly seeded with cell culture during in vitro activity. The implementation is related to the application of a tissue-engineered implant surface to permanently stabilize implants by coating the prosthesis with cells or tissue before implantation (Burg, Porter & Kellam, 2000). Reconstructive orthopaedic surgeries took credit with this benefit because it always have high possibility incidences of failure secondary to large bone defect (Burg et. al., 2000; Dekker et. al., 1998).

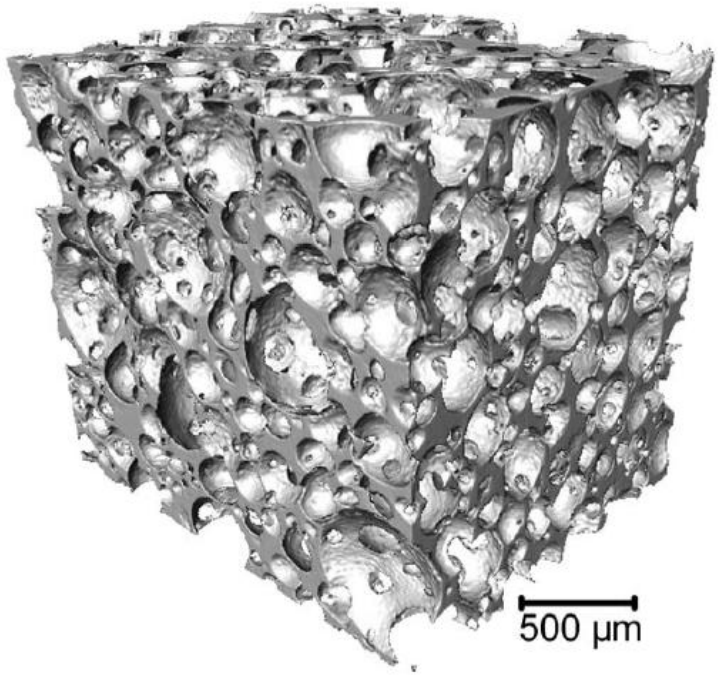


Figure 2.5: Porous bone scaffold (Valliant & Jones, 2011)

However, porosity and pore structure are the key parameters which are determining the properties and the applicability of scaffolds for tissue engineering. In Fig. 2.6 shows a summary of different functions related to the pore structure that must be tailored to the particular tissue under consideration (Boccaccini & Maquet, 2003).

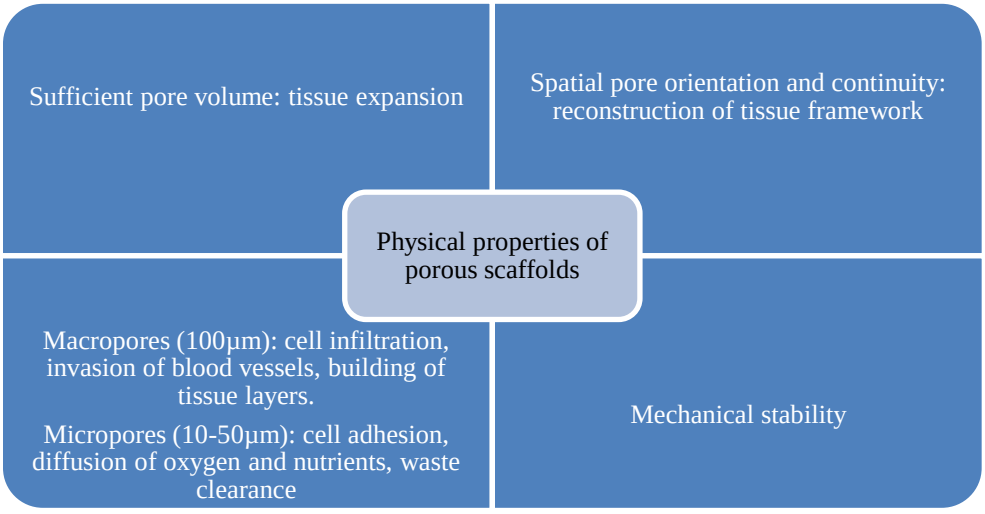


Figure 2.6: Schematic diagram showing the different functions of a tissue engineering scaffold depending on its porosity and pore structure (Boccaccini & Maquet, 2003)

It is a tricky and challenging task to fulfil all the essential requirements in producing good porous scaffold. All important aspects should take into consideration that would related from the materials chosen, fabricating process, the design and not to forget the characteristics in developing a scaffold that will be explained in detail in the next section.

2.6 Development of Scaffolds

The development in fabricating scaffolds indicate positive improvement since it was first invented decades ago (Gösta, 2000). Several important attributes are taken into consideration such as the characteristics of the bone scaffold, the materials chosen, scaffold architecture and its fabrication with the main aim is to produce a tough even having high porosity content.

2.6.1 Characteristics

Vascularization is essentially necessary because large scaffolds need blood vessels for successful bone regeneration. Blood vessels can be grown in vitro prior to implantation so they will connect the host vessels after implantation. To achieve that, stem cells and endothelial cells were seeded inside a gel and scaffolds were soaked in it for about one day for veins to be traced.

An ideal bone scaffold should at least exhibit some of these general attributes (Jones, Lee & Hench, 2006; Jones, Gentlemen & Polak, 2007; Jones et. al., 2009):

1) Act as a template for bone growth in 3D and has an interconnected pore structure to allow 3D bone ingrowth.
2) Resorbs at the same rate as the bone is repaired, producing degradation products that are non-toxic and that can be excreted easily by the body.
3) Biocompatible (non-toxic) and promotes cell adhesion, stimulating new bone growth (osteogenesis).

4) Bonds to the host bone without the formation of scar tissue, creating a stable interface.
5) Exhibit mechanical properties matching those of the host bone after in vitro tissue culture.
6) Made from a processing technique that can produce irregular shapes to match that of the bone defect.
7) Has the potential to produce commercially and sterilized to the required international standards for clinical use.

2.6.2 Biomaterials: Synthetic and Natural

Synthetic is defined as something that chemically or sometimes naturally made exclusively to imitate the natural product. The terminology of biomaterials are product that interacts with biological systems. Synthetic biomaterials could be simplified as something that synthesized chemically to mimic the natural product so the products produced are able to interact with biological system. Materials that synthetically synthesized in order to fabricate bone scaffold commonly from metals, ceramics, glass, chemically synthesized polymers, natural polymers and combinations of these materials to form composites (Karageorgiou & Kaplan, 2005).

2.6.2.1 Metal-based biomaterials

In attempting to repair the skeletal systems, surgeons must endeavor to replicate the static and dynamic responses of the bone. Bone exhibits a higher flexural strength and flexural modulus than polymeric materials but is weaker and more deformable than metals (Dunn & Casper, 1985). Therefore, there is vital circumstances in searching the most suitable material for temporarily substitute defect bone.

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