BIOCOMPATIBILITY AND OSTEOGENESIS OF LOCALLY PRODUCED β-TRICALCIUM PHOSPHATE

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by

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LIST OF ABBREVIATIONS

β-TCP	β-Tricalcium Phosphate
MTS	[3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt; MTS].
H_3PO_4	Phosphoric acid
$Ca(OH)_2$	Calcium hydroxide
FDA	Food and Drug Administration
MTT	(3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, a yellow tetrazole)
CLSM	Confocal laser scanning microscope
AFM	Atomic force microscope
FTIR	Fourier transform infrared spectroscopy
DMSO	Dimethyl Sulphoxide
RIPA	Radio Immuno Precipitation Assay
SDS	Sodium Dodecyl Sulfate
PAGE	Polyacrylamide Gel Electrophoresis,
PVDF	Polymer of Vinylidene Fluoride
HRP	Horseradish Peroxidase
NHOst	Normal Human Osteoblast Cell Line
hFOB	Human Fetal Osteoblast Cell
ATCC	American Type Culture Collection
DMEM/F12	Dulbecco's Modified Eagle Medium and Ham's F12
FBS	Foetal Bovine Serum
EDTA	EthyleneDiamineTetraAceticacid

PBS	Phosphate Buffered Saline
OGM	Osteoblast Growth Medium
PMSF	Phenylmethylsulfonyl fluoride
BSA	Bovine Serum Albumin
APS	Ammonium Persulphate
TEMED	N,N,N',N'-Tetramethylethylene-diamine
NaCl	Sodium Chloride
KCl	Pottasium Chloride
IgG	Immunoglobulin G
ECL	Enhanced chemiluminescence
CLSM	Confocal Laser Scanning Microscope
AFM	Atomic Force Microscope
FT-IR	Fourier Transform Infrared Spectroscopy
TBS	Tris Buffer Saline
TBST	Tris Buffer Saline Tween-20
ASTM	American Society for Testing Materials
CO_2	carbon dioxide
IC_{50}	Inhibitory Concentration 50%
O.D	Optical Density

KEBIOSERASIAN DAN OSTEOGENESIS KEATAS

β-TRIKALSIUM FOSFAT KELUARAN TEMPATAN

ABSTRAK

β-Trikalsium Fosfat (β-TCP) dengan formula molekul [β-$Ca_3(PO_4)_2$] ialah biobahan sintetik seramik yang telah digunakan secara meluas sebagai bahan graf tulang dalam bidang otopedik dan pergigian kerana kebioserasiannya dan sifatnya. Tujuan kajian ini adalah untuk menentukan kebiokeserasian dan osteogenesis β-TCP yang dihasilkan secara tempatan dengan menggunakan sel selanjar osteoblas manusia. β-TCP seramik dihasilkan secara tempatan menggunakan bahan permula yang sama iaitu $Ca(OH)_2$ dan H_3PO_4 dengan nisbah 1.5 tetapi disintesis dengan dua kaedah berbeza iaitu hidroterma dan pemendakan.

Sifat sampel β-TCP diperhatikan menggunakan angkup vernier, spektroskopi inframerah transformasi Fourier (FTIR) dan mikroskop daya atom (AFM) bagi dimensi, kumpulan berfungsi dan kekasaran permukaan. Sel osteoblas dikultur dengan ekstrak β-TCP menggunakan kepekatan berbeza untuk menentukan sitotoksik menggunakan asai alamar blue dan MTS. Pelekatan dan penghidupan sel osteoblast pada β-TCP diperhatikan menggunakan mikroskop konfokal sinar listrik (CLSM) selepas diwarna dengan calcein-AM dan ethidium homodimer untuk menghasilkan warna berfluoresen. Kehadiran kalsium mendapan dan protein matriks ekstrasellular (ECM) seperti kolagen jenis 1 dan fibronektin yang bertanggungjawab semasa osteogenesis diperhatikan menggunakan pewarnaan alizarin merah dan

analisa pemblotan western. Chronos β-TCP keluaran komersil digunakan sebagai pengawal.

β-TCP hidroterma dan pemendakan menunjukkan persamaan sifat pada dimensi, kumpulan berfungsi dan kekasaran permukaan. Hasil ujian sitotoksik menunjukkan tiada sitotoksik oleh β-TCP hidroterma dan pemendakan pada kepekatan yang diuji (6.25 mg/ml sehingga 200 mg/ml) dan imej dari mikroskop cahaya menunjukkan kemampuan pertumbuhan sel dan pengekalkan morfologi selepas diinkubasi dengan ekstrak. Imej dari CLSM menunjukkan sel hidup bertumbuh dan mampu melekat pada permukaan β-TCP hidroterma dan pemendakan. Pemerhatian mendakan kalsium dan ekspresi protin ECM menunjukkan osteogenesis yang positif dari sel osteoblas yang dikultur dengan β-TCP hidroterma dan pemendakan.

Keputusan menunjukkan bahawa β-TCP keluaran tempatan menggunakan kaedah hidroterma atau pemendakan adalah tidak sitotoksik dan bioserasi. Biobahan ini menyokong pelekatan sel, pertumbuhan sel, mengekspresi protin yang berkait dengan osteogenesis dan mempunyai potensi untuk digunakan sebagai biobahan pergigian dan ortopedik.

BIOCOMPATIBILITY AND OSTEOGENESIS

OF LOCALLY PRODUCED β-TRICALCIUM PHOSPHATE

ABSTRACT

β-Tricalcium Phosphate (β-TCP) with molecular formula [β-$Ca_3(PO_4)_2$] is a synthetic ceramic biomaterial that have been extensively used as a bone graft material in orthopaedic and dental field due to its biocompatibility and properties. The purpose of this study was to evaluate the biocompatibility and osteogenesis of locally produced β-TCP in-vitro using osteoblast cell line. β-TCP ceramic was locally prepared using same starting material of Ca(OH)2 and H3PO4 with Ca/P ratio of 1.5 but synthesis by two different methods namely, hydrothermal and precipitation.

The characteristics of β-TCP samples were observed using vernier caliper, Fourier Transform Infrared Spectroscope (FTIR) and Atomic Force Microscope (AFM) for its dimension, functional group and surface roughness. Osteoblast cells were cultured with extracts of β-TCP at various concentration to determine the cytotoxicity using alamar blue and MTS assay. Cell attachment and viability of osteoblast cell with β-TCP were observed using Confocal Laser Scanning Microscope (CLSM) after staining with calcein-AM and ethidium homodimer to produce fluoresecence colour. The presence of calcium deposition and extracellular matrix (ECM) protein such as Collagen 1 and fibronectin that responsible during osteogenesis were determine using alizarin red staining and western blot analysis. Chronos β-TCP as a comercial produced β-TCP was used as controls.

Hydrothermal and precipitation β-TCP showed the similar characteristic in dimension, functional group and surface roughness. The results of in-vitro studies indicated the non-cytotoxicity of hydrothermal and precipitation β-TCP at the tested concentrations (6.25 mg/ml to 200 mg/ml) and images from optical microscopy showed that the cells were able to proliferate and retain its morphology after incubation with the extracts. Images from CLSM showed the viable cells had grown and able to attach on the surface of hydrothermal and precipitation β-TCP. Calcium deposition observation and ECM protein expression showed the positive osteogenesis from osteoblast cell cultured with hydrothermal and precipitation β-TCP.

The results showed that the locally produced β-TCP using hydrothermal or precipitation method are non-cytotoxic and biocompatible. This biomaterials support cells attachment and cells proliferation, expressing protein related to osteogenesis and have the potential to be used as biomaterial in dental and orthopedic.

CHAPTER ONE

INTRODUCTION

1.1 Background of the study

Biomaterial is defined as a natural or synthetic material that intended to be used as medical devices inside the human body and need to be biocompatible. Biocompatibility is the ability of a material to perform with an appropriate host response in specific application (William, 1987). Materials have four main classes which are; metal, ceramic, polymer and composites. Biomaterials which used as implant can be divided into three main types based on their tissue response. Bioinert material defined as material that give no or minimum tissue reaction. Bioactive material is a material that can stimulate bone tissue formation around it and biodegradable is materials that may dissolve completely overtime after being incorporated inside the body. These materials have various applications in orthopaedic, medical and dental fields such as bone graft substitutes, artificial heart valves, artificial hip joint, catheter and many more. (Kokubo, 2008)

One of ceramics materials that extensively used as bone graft in orthopaedic and dental filled is β-Tricalcium Phosphate (β-TCP). β-Tricalcium Phosphate is considered as ideal bone graft material due to its excellent biocompatibility, osteoconductive, osteoinductive, biodegradable and unlimited supply quantity (Kondo *et al.*, 2005). It used extensively in orthopaedic and dental field for bone fractures repair and replacement, as void filler after tumors removal and also used as coating material for metal implants. (Barrere *et al.*, 2006)

Calcium phosphate have special characteristic as bone graft material compared to others materials. This is due to its chemical composition that is similar with natural human bone (Descamps *et al.*, 2008). The most widely used synthetic bone graft is Tricalcium Phosphate. There are two types of Tricalcium Phosphate (TCP), $Ca_3(PO_4)_2$, which are alpha (α) and beta (β) phases. Both phases have different crystallographic structure. The α-TCP crystallizes in monoclinic space group while the β-TCP crystallizes in rhombohedral space group (Anselme, 2000). This features lead to different resorption rate, the α-TCP is more soluble than β-TCP. The β-TCP phase is stable below 1125°C while the α phase is only stable in the range of 1125-1430° C. Since α-TCP is very reactive and degrades rapidly in-vitro, therefore the present study only focused on β-TCP (dos Santos *et al.*, 2002).

Due to the broad range potential uses of the material, attempt has been done in School of Materials and Minerals Resources Engineering, Nibong Tebal, USM to produce the material locally. Synthesization of β-TCP has been achieved by two different methods, which were precipitation and the hydrothermal. The synthesization of both β-TCP using the same starting material that is H_3PO_4 solution and $Ca(OH)_2$ solution but with different synthesization method. Hydrothermal method is a wet-chemical process using high thermal pressure while precipitation method involved reaction of mixture under control condition. The density of β-TCP obtained by the precipitation method is around 3.1 g/cm^3 whilst the hydrothermal method produces powders of higher density (ρ=3.7 g/cm^3).

An ideal implant material is expected to be able to mimic the mechanical and biological properties of natural bone that being replaced. It also should possess a good bone remodelling with rapid completion of new bone formation. The search of ideal biomaterial for bone graft substitutes not only focus on mechanical properties required but include excellent biocompatibility and bioactivity. Biocompatibility is important to ensure the materials that incorporated inside the human body did not give any adverse effects and can receive well by the body. (Burg *et al.*, 2000)

Biocompatibility is closely related with the behaviour of cells interact with biomaterial. The attachment of the cells with biomaterial is crucial because it is the first interaction that occurs and will lead to the next biological process. The cell attachment phase occurs rapidly between cells and biomaterials and involves ionic forces and van de Walls forces. While the cell adhesion phase occur in longer time and involves variety of biological molecules such as extracellular matrix protein, cell membrane protein and cytoskeleton proteins (Anselme, 2000). The interaction between these proteins will induce signal transduction and therefore stimulate the cell growth and behaviour. The quality of cells attachment will guide to extracellular matrix organization and facilitate the cells proliferation and differentiation phase.

In bone, the extracellular matrix is composed of collagenic protein and non collagenic protein such as fibronectin, collagen type I and others glycoprotein. The extracellular protein is important because its production will modulate the osteogenesis process. Collagen type I is expressed during the initial period of proliferation and synthesize of extracellular matrix while fibronectin is important for osteoblast differentiation and mineralization. (Sharma and Snedeker, 2010)

For biocompatibility testing, normal human osteoblast cell were chosen as an in-vitro model cell because its match the cell population of implants sites. Since β-TCP were intended to be used as a bone graft substitutes, osteoblast cells is the ideal cells for *in-vitro* testing because its responsible during the whole process of bone tissue formation (osteogenesis). Many researchers have done research on osteoblast cells interaction with biomaterials such as titanium, zirconia, hydroxyapatite and novel biomaterials to understand the parameter that influences the osteoblast cell behaviour whether at cellular or molecular level.

1.2 Problem Statement

The use of synthetic material as a bone graft substitutes has gained wide acceptance in dental and orthopaedic surgery. This synthetic material have advantages than allograft, autograft and xenograft because its availability, biocompatibility, bioactivity and lack of disease transmission. Due to demand of β-TCP application as synthetic bone graft in dental and orthopaedic field, research has been made to produce the material locally. There are several synthesization methods have been used to commercially produced β-TCP. In this study two synthesization method which are hydrothermal and precipitation were successfully produced the material locally. Although the newly produced material has been followed the mechanical properties requirements for medical application, it is necessary to undergo certain biological test to ensure it safety. No previous research were done to evaluate the biocompatibility of locally produced β-TCP using hydrothermal and precipitation method.

1.3 Justification of study

The quests for excellent biomaterial to be used as implant material were continuously explored by many scientists. The best biomaterial should exhibit mechanical and chemical properties similar to original bone and have excellent biocompatibility, bioactivity and can mimic the natural biological process during bone regeneration without any negative effects. However no materials even titanium has been totally free from any adverse reactions.

Extensive research work is ongoing to understand the behaviour of osteoblast cell with biomaterials. Finding from this research is useful to understand the osteoblast cells during interaction with β-TCP which produces using two different methods that are hydrothermal and precipitation. It provides a valuable data that can be used to design and modify the β-TCP to obtain optimum performance. In-vitro testing results can be used to predict the outcome during interaction of β-TCP before proceed to in-vivo study.

1.4 Objectives

1.4.1 General objectives

To evaluate the biocompatibility of locally produced β-TCP by using hydrothermal and precipitation methods.

1.4.2 Specific objectives

1. To evaluate the physical properties and characterisation of β-TCP samples for dimension, functional group and surface roughness using vernier calliper, AFM and FTIR.

2. To evaluate the effects of hydrothermal and precipitation produced β-TCP on cytotoxicity using osteoblast cell.

3. To evaluate the osteoblast cell morphology and attachment on hydrothermal and precipitation produced β-TCP using optical and confocal microscope.

4. To determine the presence of calcium deposition by osteoblast cell cultured with hydrothermal and precipitation β-TCP using alizarin red staining.

5. To determine the expressions of collagen type I and fibronectin proteins by osteoblast cell cultured with hydrothermal and precipitation produced β-TCP using western blot analysis.

CHAPTER TWO

LITERATURE REVIEW

2.1 β-Tricalcium phosphate

Figure 2.1 Images of β-TCP in granules, powder and pellet form.

β-Tricalcium phosphate (β-TCP) is the most frequently used tricalcium phosphate compounds in implant materials due to its biodegradability. β-TCP is a stable anhydrous tricalcium phosphate phase which crystallises in the rhombohedral system with 21 formula units $Ca_3(PO_4)_2$ per hexagonal unit cell. Unlike the hydroxyapatite that still remains even after a long time implantation, the β-TCP will eventually degrade and replaced by natural tissue (Ogose *et al.*, 2005).

β-TCP have been commercialised for a long time ago and have been used in medical and dental field until today because its stability at high temperature and easy to fabricated. These materials can be in the form of porous ceramic pieces and granules which are used in the reconstruction of bone and dentals defects (Ogose *et al.*, 2006) and also used as mineral ion-releasing substances in polymer materials (Kokubo, 2008). Examples of β-TCP which are available in market are Osseoconduct™ granules produced by Steiner Laboratories,USA, Bonesigma™ TCP produced by Sigma Graft,USA and ChronOs produced by Synthes,USA.

2.2 Synthesization method

Method of synthesization calcium phosphate has been widely studied due to its excellent biocompatibility as biomaterial. The synthesization method include, wet precipitation method (Mirhadi *et al*., 2011), hydrothermal synthesis (Liu *et al*, 1997), sol-gel procedures (Chen *et al*., 2011), solid state reaction (Rao *et al*., 1997), mechanochemical synthesis (Yeong *et al*., 2001), combustion synthesis (Tas, 2000) and microwave irradiation (Kumar *et al*., 2000).

Hydrothermal method is a wet-chemical process promoted under high-pressured water above 100℃. This method employs the use of temperature, pressure and a controlled atmosphere to convert a substance into another via exchange reaction. Even though hydrothermal method can be extremely time consuming, this method is particularly effective in the crystalization of poorly soluble compounds. (Sunarso *et al*., 2013, Kokubo, 2008)

Precipitation method is most widely synthesization method used due to the simplicity of the equipment required and the potential to obtain a material with controlled chemical composition and morphology. This method involved the reaction of mixture under control condition of pH, temperature and atmosphere. (Kokubo, 2008)

2.3 Bone

The skeletal system is consisting of bones and cartilage which provide biomechanical support and metabolic supply for the whole body (Gokhale *et al.*, 2001). Cartilage is a tough and flexible connective tissue consisting cells called chondrocytes, while bone is a highly specialized connective tissue which composed of cells and

extracellular matrix that can be calcified. Normal bone is lamellar and has two types of structures which are cortical bone and cancellous bone. Immature and pathologic bone is woven that contains more osteocytes and is not stress-oriented. Cortical bone (compact bone) is a semi-solid shell that covers the entire bone. It is characterized by a slow turnover rate, a high Young's modulus (E) and higher resistance to torsion and bending than cancellous bone. Cancellous bone (spongy or trabecular bone) is made up of sponge-like trabular network structures that contain blood vessel and marrow which is less dense and undergoes more remodelling according to lines of stress (Wolff's law). It has a smaller Young modulus and is more elastic than cortical bone. Bone functions involve in vital organ protection, body motion, support, and also play an important role in maintaining mineral homeostasis.

Bone consists of organic matrix (20-40%), inorganic mineral (50-70%), cellular elements (5-10%) and lipids (3%) (Deng and Liu, 2005). The organic component of bone matrix is highly consist of type I collagen with small amount of non-collagenous proteins such as osteopontin, osteonectin and osteocalcin. The inorganic composition of bone is hydroxyapatite and calcium carbonate. It contains many impurities such as carbonate, citrate, magnesium, fluoride and strontium. The cellular elements of bone include osteoclast, osteoblast, osteocytes and bone lining cells (Aubin *et al.*, 2003). Osteoclasts are multinucleated giant cells which act as bone resorbing cells and containing numerous vesicles. Osteoblasts are involved during entire bone formation process while osteocytes are the cell that embedded within the bone matrix and eventually undergo apoptosis (Bonewald and Edward, 2003). Bone lining cells are layer of elongated flat cells and also known as resting osteoblast

which derived from surface osteoblast when completion role as bone forming cells (Deng and Liu, 2005).

2.4 Osteoblast

Osteoblasts are originated from stromal marrow cells which derived from mesenchymal progenitors. Osteoblast has a large nucleus and typical osteoblast sizes are 15-30 microns cuboidal-shaped cells surrounding the entire formation of bone surface. They have much cellular process, endoplasmic reticulum, Golgi apparatus, mitochondria and collagen-containing secretory vesicles. Osteoblast known as bone-forming cells that involved in the whole bone formation process. They secrete and synthesize collagen and some carbohydrate protein complexes which make up osteoid as unmineralized bone matrix. Osteoblast involved in osteoid calcification by regulating the deposition and exchange of calcium and phosphate (Deng and Liu, 2005).

Osteogenesis or ossification is the process of new bone tissue formation by osteoblasts cells. There are two types of osteogenesis which are intramembranous ossification and endochondral ossification (Thompson *et al.*, 2002). Both processes produce the normal and healthy bone tissue.

Intramembranous ossification is known as membrane bone formation (Tortelli *et al.*, 2010). It is osteogenic process which bone is formed directly without involvement of cartilage (Qu *et al.*, 2004). Intramembranous ossification is begins with mesenchymal stem cells. When the number of cells and fibers increase to a certain amount, the mesenchymal stem cells will differentiate into osteoblast. Osteoblasts

generate many thin primary woven trabecular spicules, which thicken and become connected by continued osteoblastic formation and gradually forming a spongy-like woven cancellous network. Then the primary osteons will finally fill porosity within the cancellous network to become compact corticol bone. Endochondral ossification known as cartilaginous bone formation is an osteogenic process of cancellous bone formation which involves cartilage (Mackie *et al.*, 2008). The cartilage template is gradually replaced by a bone matrix. In the beginning, chondrocytes will proliferate and deposit cartilage matrix. Osteoblasts arise in the regions of calcified cartilage as scaffold and then develop into osteocytes which form trabecula. (Deng and Liu, 2005)

2.5 Osteogenesis

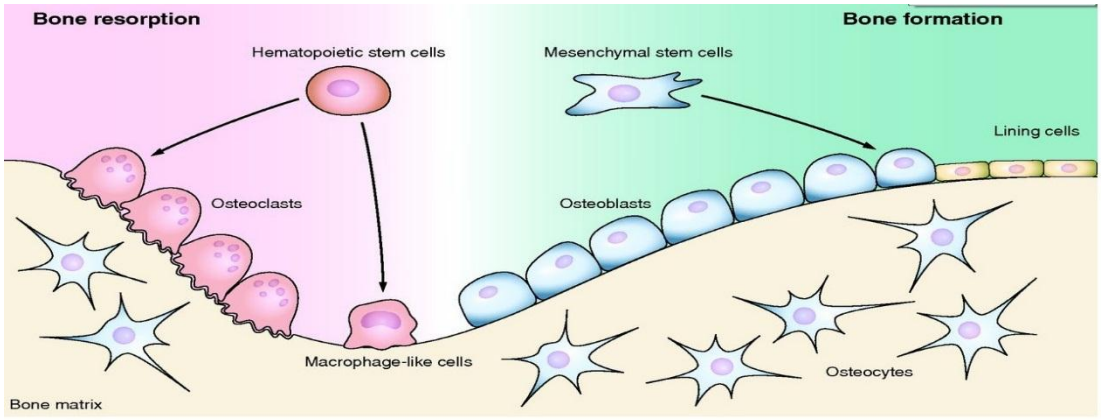

Figure 2.2 Schematic diagram of osteoblast during bone formation. (Imai *et al*, 2013)

Osteogenesis process regulated by bone formation marker such as collagen type 1, bone sialoprotein, glycoprotein, osteopontin and osteocalcin. Alkaline phosphates and collagen type 1 are expressed during early osteogenesis. Osteopontin, osteonectin and bone sialoprotein are involved during middle phase of osteogenesis while the late phase of osteogenesis involve osteocalcin and glycoprotein that are important during bone mineralization. (Setzer et al., 2009)

2.6 Cell attachment

Interaction between cells and material involved chemical bonding, such as electrostatic, hydrogen bonding, polar or ionic interactions between various molecules on cell membrane and functional chemical groups. Cell attachment of anchorage-dependent cell with material involved the presence of extracellular matrix proteins such as collagen type I to ensure the signalling from extracellular environments into cells and survival of anchorage-dependent cells. The ability of cells to synthesize and producing their extracellular matrix proteins within 24 to 48 hours after seeding with material is important to promote cell attachment on cell membrane. However, cells will undergo apoptosis if the material inhibit the synthesization process of ECM protein for the cell attachment and viability. (Moiseeva 2001, Garcia *et al*. 1999, Groth *et al*. 1999)

2.7 Collagen Type I

Collagen is the most abundant protein in human body. Tissues that are rich in collagens are bone, skin, tendon, cartilage ligaments and vascular walls. Collagen in bone is synthesize by osteoblast. The collagen superfamily has at least 27 collagen types and more than 15 additional proteins that have collagen-like domains. The superfamily can be divided into several classes based on their supramolecular structures or other features. Collagens have an important role in wound healing and fracture (Ichioka *et al.*, 2005). The inhibition of collagen synthesis will delay healing, however the excessive collagen formation can lead to fibrosis which impairing the normal organ function.

Collagen type I is the most abundant protein in bone extracellular matrix and is essential for bone strength. It is composed of two α1 chain and one α2 chain [$\alpha1(I)_2$ - α2(I)] coiled around each other in triple helix structures but a few collagen type I can be form by three [$\alpha1(I)_3$]. α1 and α2 chain consist of a long helical domain followed by a short N-terminal peptide and followed by a short C-terminal peptide. In bone, hydroxyapatite crystal lies in the gaps between collagen molecules.

Differentiating osteoblast are known to synthesize collagen type I, alkaline phosphatase (ALP) and other non-collagenous extracellular bone matrix protein such as osteonectin, osteocalcin osteopontin and bone sialoprotein. This bone matrix protein is useful to characterize the stages of osteoblast differentiation. Collagen type I is expressed during the initial period of proliferation and extracellular matrix synthesis, whereas ALP is expressed during the post-proliferative period, whereas the expression of osteonectin, osteopontin, osteocalcin and bone sialoprotein were occur later during third period of extracellular matrix maturation. (Kokubo, 2008)

2.8 Fibronectin

Fibronectin is a glycoprotein that is non-collagenous that involved in cellular attachment and calcification of bone matrix (Lee *et al.*, 2006). Fibronectin is synthesized by many connective tissue cells and is major component of serum. Fibronectin have ~400 kDa protein with two identical subunits of ~200kDa each, composed of type I, II and III repeats. Osteoblasts express fibronectin during early osteogenesis process by mediating cell attachment and spreading (Lacouture *et al.*, 2002).

Bone matrix could contain fibronectin that derived by exogenous and endogenous sources. Fibronectin is produced during early stages of bone formation and is highly upregulated in the osteoblastic cell (Stephansson *et al.*, 2002). Major glycoprotein in bone matrix usually contains the amino acid sequence Arg-Gly-Asp (RGD), which have the ability to bind to the integrin class of cell surface receptors (Silva *et al*., 2004).

2.9 Biomaterials classification

Once the biomaterials implants in the body, it will interact depends on material types. Biomaterials can be classified based on their reaction toward sites of implants. These are bioinert, bioactive and biodegradable. (Dee *et al*., 2002)

2.9.1 Bioinert

Biologically inert or bioinert materials is any material that retains its structure once implanted in the human body, has minimal interaction and do not initiate any response with its surrounding tissue or induce any immunologic host reactions (Tschernitschek *et al.*, 2006). Bioinert materials such as Al_2O_3 and ZrO are used as femoral heads in artificial hip joints.(Nicholson, 2002)

2.9.2 Bioactive

Bioactive materials are any materials once placed inside the human body, it will react with surrounding tissue and form bonds with living tissue (Sul *et al.*, 2005). Bioactive materials such as hydroyapatite and bioglass are used in various dental and orthopaedic devices. It becomes fixed in place by chemical bonding between the materials and implants sites. Study done by (Fathi and Doostmohammadi, 2009)

shows that bioactive materials exhibits the improvement of corrosion resistance and bone bonding when applied as a human body implant. Bioactive materials also can be used as coating materials for metallic orthopaedic implants to improve interfacial reactions (Lopez-Esteban *et al.*, 2003).

2.9.3 Biodegradable

Biodegradable materials are referred to any materials once placed inside the body it will degrade by hydrolytic breakdown and eventually being replaced by regenerating of natural tissue (Park and Bronzino, 2003). The chemical by-products of degrading materials are absorbed and released via metabolic processes of the body. The degradation rate is crucial to ensure the complete healing process and function properly (Wong *et al.*, 2010).These biodegradable materials have been used as drug delivery devices, bone filler and for repairing maxillofacial and dental defects (Mangual *et al.*, 2010, Park and Bronzino, 2003).

2.10 Synthetic materials

Synthetic materials that widely used for medical and dental applications include metals, polymers, and ceramics. Due to the differences in structures of these materials, they have different properties and therefore being applied differently in the body.

2.10.1 Metal

Metals have been used extensively as load-bearing implants. The high tensile and fatigue strength of metals, compared with ceramics and polymers, make metals as the materials of choice for implants that carry high mechanical loads. However, the selection of metals and its alloys as a medical device must be biocompatible, corrosion resistance, acceptable cost and have appropriate mechanical properties.

There are three major classes of metals includes stainless steel, cobalt-chromium and titanium. These material are used in pure form or alloys (metal containing two or more elements) to improve the material properties. The properties of metallic biomaterial are shown in the Table 2.1.

Table 2.1 : The properties of metallic biomaterial (Dee *et al*., 2002)

Material	**Young's Modulus, E (GPa)**	**Yield Strength, σ_y (MPa)**	**Tensile Strength, σ_{UTS} (MPa)**	**Fatigue Limit, σ_{end} (MPa)**
Stainless steel	190	221-1213	586-1351	241-820
Cobalt-chromium alloys	210-253	448-1606	655-1896	207-950
Titanium	110	485	760	300
Ti-6Al-4V	116	896-1034	965-1103	620
Corticol bone	15-30	30-70	70-150	

The immersion of metals in human body surrounding that is 37° C aqueous solutions, at pH7.3 with dissolved gases, electrolytes, cells and proteins can lead to corrosion by metal ions release that may reduce the biocompatibility and interrupt the material performance.

The oxidized layer on metallic surfaces can reduced and prevented the corrosion. So, the overall corrosion resistance depends on different oxides film from different metals. Cobalt-chromium, titanium and its alloys have more corrosion resistance than stainless steel which make them suitable for long term implantation. However titanium and its alloys have more stable oxides film which gives higher corrosion resistance and becoming osteointegrated *in vivo*. (Dee *et al*., 2002)

2.10.2 Ceramic

Ceramics are materials composed of metallic and non-metallic elements bind together by ionic and/or covalent bonds. Ceramics can be either bioinert, bioactive or biodegradable as shown in Table 2.2.

Table 2.2: Ceramics classification (Dee *et al*., 2002)

Ceramic	**Chemical Formula**	**Reaction**
Alumina	Al_2O_3	Bioinert
Zirconia	ZrO_2	Bioinert
Bioglass	$Na_2OCaP_2O_3$-SiO	Bioactive
Hydroxyapatite (sintered at high temperature)	$Ca_{10}(PO_4)_6(OH)_2$	Bioactive
Hydroxyapatite (sintered at low temperature)	$Ca_{10}(PO_4)_6(OH)_2$	Biodegradable
Tricalcium phosphate	$Ca_3(PO_4)_2$	Biodegradable

When exposed to the physiological environment, some ceramics are stimulated to degradation. It may undergo rapid or slow dissolution and resorbed by osteoclast due to its similar composition to natural bone. The mechanical properties of ceramic materials are shown in the Table 2.3.

Table 2.3 : The mechanical properties of ceramic materials (Dee *et al*., 2002)

Ceramic	Young's Modulus, E (GPa)	Compressive Strength, σ_{UCS} (MPa)	Tensile Strength, σ_{UTS} (MPa)
Alumina	380	4500	350
Bioglass-ceramics	22	500	56-83
Calcium phosphates	40-117	510-896	69-193
Pyrolic carbon	18-28	517	280-560

Ceramics that have lower mechanical properties are more suitable to be used as bone filler, and coating materials for metallic implants to promote bone fixation.

2.10.3 Polymer

Polymers are organic materials consists of large macromolecules composed of many repeating units called monomers. Polymers are used in medical field as vascular grafts, hearts valves, artificial hearts, breast implants, intraocular lenses and many more. The properties of polymers depend on the composition, structure and arrangement of their macromolecules. The mechanical properties of polymer are shown in Table 2.4 .

Table 2.4 : The mechanical properties of polymer (Dee *et al*., 2002)

Polymer	Tensile Strength, σ_{UTS} (MPa)	Young's Modulus, E (GPa)	% Elongation
Poly(methyl methacrylate)(PMMA)	30	2.2	1.4
Nylon 6/6	76	2.8	90
Poly(ethylene terephthalate)	53	2.14	300
Poly(lactic acid)	28-50	1.2-3	2-6
Polypropylene (PP)	28-36	1.1-1.55	400-900
Polytetrafluoroethylene	17-28	0.5	120-350
Silicon rubber	2.8	Up to 10	160

Ultra-high-molecular-weight polyethylene (UHMWPE)	>35	4-12	>300

Advantages of polymer compared to metal and ceramic materials is their easy manufacturability to produce various shapes, acceptable cost and its availability with desired mechanical and physical properties. Various physical forms being used in medical application include fibers, textiles, membranes, films, foams, solid rods, powder and many more. (Dee *et al*., 2002)

2.11 Biocompatibility

Biocompatibility is usually defined as the ability of a material to perform with an appropriate host response in specific application. The biomaterials performance includes attachment, proliferation, differentiation, protein adsorption and mild inflammatory response (Essen *et al*., 2016, Catto *et al*., 2015). The successful of biomaterials also closely related to synthesis and characterisation of physical and mechanical properties which include porosity, protein adhesion, contact angle, elastic modulus, tensile strength, durability and in vivo stability (Ostdiek *et al*., 2015, Derkus *et al*., 2015, Kopova *et al*., 2016). Ideally, the properties of the biomaterials must match and modified with the sites of application. For example, a hip prosthesis must be strong, stable and non-degradable in vivo, but a bone filler should be porous, osteoconductive and biodegradable correlated with healing period.

Osteoconduction is defined as the apparent growth of bone tissue along the surface of implants (Yu *et al*., 2011). It is important for stable long term orthopaedic and dental implants due to its three important features of osteoconductivity of biomaterials which are surface chemistry, surface topography and architectural geometry (Kokubo, 2008). Regarding the surface chemistry, bioactive materials possess a high

conductivity compared to bioinert materials while the surface topography is closely related to contact guidance of osteoblast such as the biomaterial smoothness and grooves. Architectural geometry is a porous implant structure which is important due to its size, extent and interconnectivity allow for bone ingrowths and the invasion of blood vessel. (Kokubo, 2008)

Osteoinductivity is a biological process that induces local mesenchymal cells to differentiate into bone-producing cells. Osteoinduction properties can be induced by the presence of adding cells or growth factors such as bone morphogenetic proteins (BMPs) or by synthetic biomaterials (Takahashi *et al.*, 2005). Most synthetic biomaterials shown to be osteoinductive contain calcium phosphate, which suggest the importance of calcium and phosphate ion in the process of osteoinduction by biomaterials. There are three differences in osteoinduction by BMPs and biomaterials which are the bone induced by biomaterials is always intramembranous while BMP-induced bone can be Fin formed via both osteochondral and intramemranous osteogenesis (Kokubo, 2008). The osteoinductivity of biomaterials can be confirmed by upregulation of osteoblast marker genes such as *Runx*2, collagen type I, osteopontin, and osteocalcin (Sun *et al.*, 2008)

The successful of biomaterials is depends on the ability of cells to adhere to material followed by differentiation which leading to the extracellular matrix production and organization. Cell adhesion is a series of events that involved initial cell attachment followed by cell spreading, actin cytoskeleton organization and focal adhesion formation. The attachment of cell to biomaterials is controlled by various families of adhesion receptors including integrins, selectins, cadherins and immunoglobulin.The

integrin receptor and ligand binding is often mediated through an Arg-Gly-Asp (RGD) amino acid sequence that serve as main cell attachment sites (Hersel *et al.*, 2003). Thus protein containing RGD sequence such as fibronectin, vitronectin and laminin are believed to play an important role in cellular morphology, migration, and also trigger signal for cellular proliferation, metabolism, function and differentiation in anchorage dependent cells such as osteoblasts.

2.12 Tests for biocompatibility

Biomaterials that are intended to be used as human medical devices need to be evaluated for biocompatibility. The methods for measuring biocompatibility are various and evolve rapidly. Biocompatibility test is important to ensure the material is biologically acceptable with minimal adverse effect response. The biocompatibility test include in vitro, in vivo and usage test. Materials need to be completely evaluated by each and various test before it can consider biocompatible and safe to human (Williams, 1989).

In-vitro test for biocompatibility evaluation is done outside the living organism (Donato *et al.*, 2009). The tested materials will incorporated with cell, enzyme or other isolated biological system. In vitro test can be used to measure cytotoxicity, cell growth, metabolic or other cell function and also to measure the genotoxicity potential. In vitro test offer advantages than other test because it is quick to perform, low cost and can be controlled and standardize. This in-vitro test is only can be used to screening the materials and expects the outcome during in vivo and other test but it cannot predict the overall biocompatibility of a material as medical devices.

In-vivo test is a biological test that used living organism to evaluate the biocompatibility of the materials (Browne, 1994). *In-vivo* test can be done using mice, rats, guinea pigs or others species. The use of animal allows many complex interactions and complete biological system to occur. The disadvantages of in vivo test are that they are difficult to control, time consuming, expensive and have ethical issues that need to be followed. The validity of the results may affected by species, tissue, gender and other factors.

Pre-clinical is a biological usage test that is done in animals or in human which is the most important stage to evaluate the biocompatibility because its mimic the clinical use of the material to be used inside human body. However, the materials need to pass in vitro and in vivo test before it can be implemented in human after several approved effective assessments. Pre-clinical is extremely expensive, difficult to control, very time consuming, difficult to interpret and involve major legal and ethical issues.

The entire test that design for biocompatibility evaluation must follow the standard and regulations that have been made by government agencies such as Food and Drug Administration (FDA) and ISO 10993 as guidelines.

2.13 Alamar blue assay

Alamar blue is a non toxic and cell permeable compound that can be used to analyse cell proliferation and cytotoxicity (Nakayama *et al.*, 1997). Alamar blue used as indicator for cell health by using resazurin. Resazurin is a non-fluorescent blue color dye which is converted to resorufin via reduction reaction of metabolic active cells that produced very bright red fluorescence as shown in (Figure 2.3). The amount of fluorescence generated is proportionally to the number of living cells and can be measured using spectrophotometer.

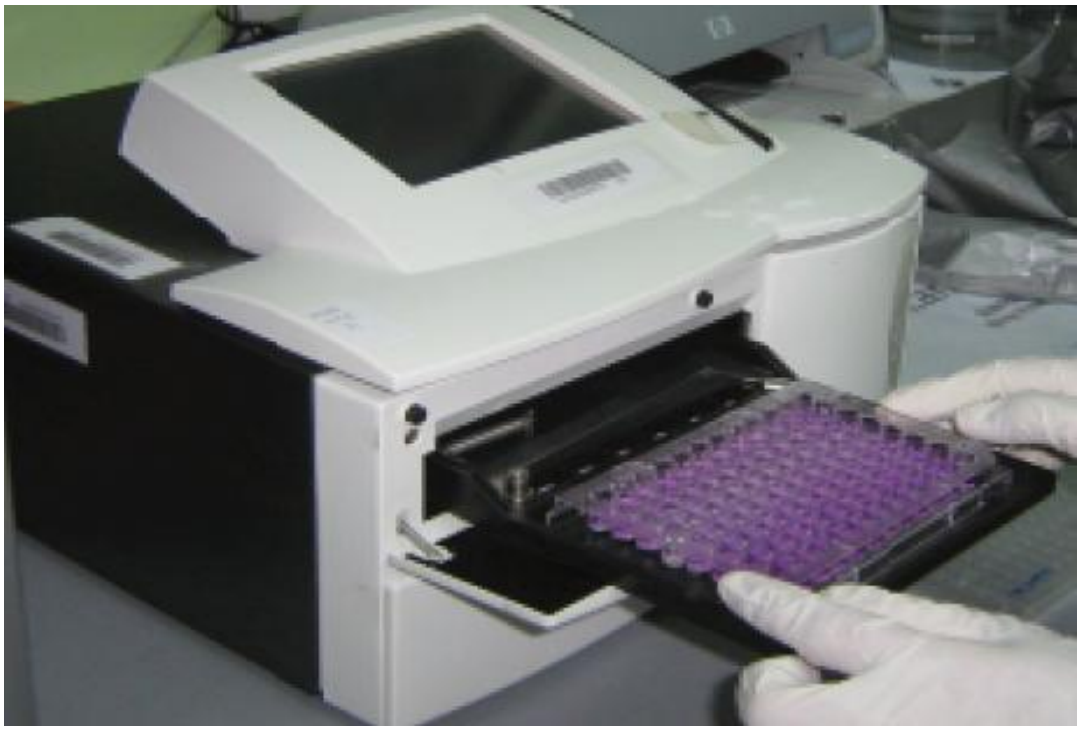

Figure 2.3 Conversion of resazurin to resorufin measured using spectrophotometer.

Alamar blue is non toxic and permits a long term exposure to the dye without any negative effects to the cell viability and health.

2.14 MTS assay

MTS contain a novel tetrazolium compound [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt; MTS]. MTS is chemically reduced by healthy cells into formazan, which is soluble in tissue culture medium. The assay measures dehydrogenase enzyme activity which found in metabolic active cells. The increased number of viable cells leads to the increase activity of mitochondrial dehydrogenase in the sample which correlates to the amount of formazan dye formed. The quantity of formazan product can be measured using spectrophotometer at absorbance 490nm.

The MTS assay offer advantages than MTT assay because it does not require solvent to solubilise the formazan product. MTT reduced to insoluble purple formazon by mitochondria of active cells. Formazan from MTT need to be solubilised by organic solvent such as DMSO. Thus, the used of MTS reagent can save time and cost with other advantages such as rapid indication of toxicity with acceptable sensitivity and specificity (Malich *et al.*, 1997). Nowadays the MTS assay have been used extensively by others researcher to measure the cytotoxicity and proliferation of the cells when incubated with drug and others compound.