SURFACE FUNCTIONALIZATION AND CHARACTERIZATION OF ANTIMICROBIAL POROUS P(3HB-*co*-4HB) SCAFFOLD WITH COLLAGEN PEPTIDE FOR BIOMEDICAL APPLICATIONS

TANA POORANI A/P GURUSAMY

UNIVERSITI SAINS MALAYSIA

2020

SURFACE FUNCTIONALIZATION AND CHARACTERIZATION OF ANTIMICROBIAL POROUS P(3HB-*co*-4HB) SCAFFOLD WITH COLLAGEN PEPTIDE FOR BIOMEDICAL APPLICATIONS

by

TANA POORANI A/P GURUSAMY

Thesis submitted in fulfilment of the requirements
for the degree of
Master of Science

May 2020

ACKNOWLEDGEMENT

I would like to express our gratitude to supervisor Professor Dr. Amirul Al-Ashraf Abdullah for giving us opportunity and help us endlessly in finishing my research. His valuable advice, comments and motivation have guided me in completing my research successfully. I am also indebted to my co-supervisor Dr. Vikeneswari Sevakumaran from UTM for her guidance in aminolysis study and cell culture and also scientifice views which are very much appreciated.

I am fortunate to be a part Lab 318, School of Biological Science and I am very grateful to Mr. Huong Kai Hee, Ms.Aida, Mrs. Izzaty, Mrs. Azura, Ms.Wani, Ms. Azuraini for their guidance, assistance and support which have benefited me in coutless ways. I would also like to thank Mr.Zul, Mr.Afiq of cell culture Research Facility, IPharm a nd all other staffs of School of Biological Science for their assistance and guidance. Thank you for the tremendous positive feedbacks given along with the continous support received.

Last but not least I would thank to My god and my family – father, Mr. Gurusamy, mother, Mrs. Logambal and my husband, Mr. Jeshigan Jothivel who are being with me and had gave all time encouragemnet and support in accomplishment my research study.

TABLE OF CONTENTS

LIST OF TABLES

Page

LIST OF FIGURES

Page

LIST OF SYMBOLS

ºC	Degree celcius
º	Degree
®	Registered
%	Percentage
µL	Microliter
™	Trade mark
β	beta
α	alpha
γ	gamma
ω	Omega (lowercase)
nm	nanometer
M_W	Weight average molecular weight
M_n	Number-average molecular weight
mg	miligram
M	Molar
mol%	mole Percent
L	Liter
kg	kilogram
g	gram
kDa	kilodalton
γ	gamma
ω	Omega (lowercase)
nm	nanometer
M_W	Weight average molecular weight
rpm	Revolution per minute

v/v	Volume per volume
vvm	Volume per volume per min
wt%	Weight percent
U	One unit of enzyme activity
V	Volt
w/v	Weight per volume
w/w	Weight per weight
kPa	kilopascal
M_w/M_n	Polydispersity index
h	hour
MPa	Megapascals
MS	Mean Square
Da	Dalton
df	Degrees of freedom
cm	Centimeter
min	minute
mM	Millimolar
µg/mL	Miligram/Mililiter

LIST OF ABBREVIATIONS

CMP	calcium metaphosphate
CHL	Chinese hamster lung cell
CME	Caprylic methyl ester
C/N	Carbon to nitrogen ratio
CoA	Coenzyme-A
DMSO	Dimethyl sulfoxide
DMF	dimethylformamide
ECM	Extracellular matrix
ED	Ethylene diamine
E-spun	Electrospun
FBS	Feral bovine serum
FDA	Food and Drug Administration
FT-IR	Fourier transform mid-infrared spectrometry
G	gauge
GA	Glutaraldehyde
GC	Gas chromatography
GPC	gel permeation chromatography
H	Hydrogen atom
HA	Hydroxyalkanoates
HAEC	Human aortic endothelial cells
HDI	Hexamethylene diisocyanate
HFIP	1,1,3,3,3-hexafluro-2-propanol
H_2SO_4	Sulphuric acid
MTS	(3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium)
MTT	3-(4,5- dimethylthiazol-2-yl)-2,5-diphenyltetrazolium
OD	Optical density
P(3HB)	Ply(3-hydroxybutyrate)
P(3HO)	Poly(3-hydroxyoctanoate)
P(4HB)	Poly(4-hydroxybutyrate)
P(3HB-*co*-3HV)	Poly(3-hydroxybutyrate-*co*-3-hydroxyvalerate)
P(3HB-*co*-3HHx)	Poly(3-hyroxybutyrate-*co*-3-hydroxyhexanoate)

P(3HB-*co*-4HB)	Poly(3-hydroxybutyrate-co-4-hydroxybutyrate)
PBS	Phosphate buffer solution
PCL	Poly(Ɛ-caprolactone)
PEG	Poly(ethylene glycol)
PVA	Polyvinyl alcohol
PGA	Poly(glycolic acid)
PHA	Polyhydroxyalkanoate
P(GA-*co*-LA)	Poly(glycolic-*co*-lactic acid)
PhaC	PHA synthase
PLLA	Poly(l-lactic acid)
PMS	Phenazine methosulfate
PU	polyurethane
Ltd.	Limited
MCL	Medium chain length
MEM	Minimal Essential Medium
MM	Mineral medium
RGD	L-arginine-glycine-L-aspartic acid
SCL	Short chain length
SEM	Scanning electron microscope
SSD	Silver sulfadiazine
TGF-1	Transforming growth factor-1
UV	Ultraviolet

LIST OF APPENDICES

PEMFUNGSIAN PERMUKAAN DAN PENCIRIAN PERANCAH ANTIMIROBIAL BERLIANG P(3HB-*KO*-4HB) DENGAN PEPTIDA KOLAGEN UNTUK BIDANG BIO PERUBATAN

ABSTRAK

Poli(3-hidroksibutirat-*ko*-4-hidroksibutirat) [P(3HB-*ko*-4HB)] merupakan polimer biodegradasi bakteria dengan kadar kebioserasian yang tinggi. Walaubagaimanapun, permukaan perancah P(3HB-*ko*-4HB) adalah hidrofobik dan tidak mempunyai bahagian bioaktif bagi tujuan pertumbuhan sel. Ini menyebabkan perancah ini kurang mendapat sambutan dalam bidang regenerasi tisu dan perubatan. Maka, kajian ini dijalankan bertujuan untuk menghasilkan P(3HB-*ko*-4HB) dengan menambahbaik permukaan perancah dengan peptida kolagen untuk meningkatkan ciri penyerapan air dan juga percambahan sel untuk bidang bioperubatan. *Cupriavidus malaysienisis* sp.USMAA1020 transformator merupakan bakteria yang berupaya menghasilkan polihidroksialkaoat (PHA) dengan menggunakan prekursor 1,4-butanediol dan 1,6-heksanadiol. Perancah berliang P(3HB-*ko*-4HB) dihasilkan melalui kaedah pengeringan beku dan penyucian garam. Ini diikuti dengan pergabunggan pelbagai kepekatan peptida kolagen (2.5 wt%- 12.5 wt%) dengan perancah P(3HB-*ko*-4HB) melalui dua jenis teknik iaitu aminolisis dan penyalutan peptida kolagen atas permukaan perancah. Terdapat tiga jenis perancah P(3HB-*ko*-4HB) dihasilkan iaitu salutan sulfadiazina perak (SSD)/ salutan peptida kolagen P(3HB-*ko*-4HB), campuran sulfadiazina perak /salutan peptida kolagen P(3HB-*ko*-4HB) dan campuran sulfadiazina perak/aminolisis P(3HB-*ko*-4HB). Sebagai tambahan, kaedah penghubungan silang peptida kolagen telah digunakan dengan 1 wt% glutaraldehid (GA). Secara hasilnya, ciri hidrofilik dan ciri penyerapan air

kesemua ketiga-tiga perancah didapati semakin meningkat dengan peningkatan kepekatan peptida kolagan daripada 2.5 wt% kepada 12.5 wt%, tetapi permukaan berliang didapati semakin menurun. Analisis antimikrobial menunjukkan kadar perencatan percambahan mikroorganisma mencapai pada tahap 100%. Namun, tempoh perencetan mikroorganisma dipengaruhi oleh dua teknik pengabungan bahan antimikrobial dimana sulfadiazina perak disalutkan atas permukaan perancah P(3HB-*ko*-4HB) manakala satu lagi teknik dimana sulfadiazina perak digabungkan dengan polimer. Salutan sulfadiazina perak atas permukaan perancah P(3HB-*ko*-4HB) hanya mengambil masa perencetan dalam 24 jam manakala pengabungan sulfadiazina perak di dalam polimer mengambil masa 48 jam. Kajian *in-vitro* menggunakan MTS analisis menunjukkan percambahan sel fibroblas tikus (L929) dengan baik pada permukaan P(3HB-*ko*-4HB) yang dihasilkan. Dalam kajian ini, perancah SSD salutan/10 wt% peptida kolagen salutan P(3HB-*ko*-4HB) and perancah SSD campuran/10 wt% peptida kolagen salutan P(3HB-*ko*-4HB) menunjukkan sel percambahan tinggi manakala untuk aminolisis perancah 12.5 wt% peptida kolagen P(3HB-*ko*-4HB) merekodkan percambahan sel yang tinggi. Secara keseluruhannya, ketiga-tiga perancah boleh dikembangkan sebagai produk biomedikal terutamanya untuk pembalutan luka dan kejuruteraan implantasi tisu tulang di mana ia menyerupai ciri kejuruteraan tisu semulajadi.

SURFACE FUNCTIONALIZATION AND CHARACTERIZATION OF ANTIMICROBIAL POROUS P(3HB-*co*-4HB) SCAFFOLD WITH COLLAGEN PEPTIDE FOR BIOMEDICAL APPLICATIONS

ABSTRACT

P(3-hydroxybutyrate-*co*-4-hydroxybutyrate), [P(3HB-*co*-4HB)] is a bacterial derived biopolymer known for its high levels of biocompatability. However, surface of P(3HB-*co*-4HB) is found to be hydrophobic with minimal recognition sites for cell attachment. This makes it less desirable candidate to be tailored as scaffolds for tissue engineering and biomedical applications. Due to this, this research was undertaken to fabricate P(3HB-*co*-4HB) with collagen peptides scaffolds using physiochemical modifications to further enhance their surface wettabilty as well as to support cell growth for biomedical applications. In this case, a bacterial strain, *Cupriavidus malaysienesis* sp. USMAA 1020 transformant, was found to synthesize P(3HB-*co*-4HB) from 4-hydroxybutyrate (4HB) precursor substrates, namely 1,4-butanediol and 1,6-hexanediol, respectatively. Firstly, a porous P(3HB-*co*-4HB) was fabricated through salt leaching and freeze drying techniques. This was then followed by the incorporation of collagen peptides at various concentrations (2.5 wt% - 12.5 wt%) to P(3HB-*co*-4HB) using two different modifications technique, amiolysis and collagen-coating. As a result, three types of P(3HB-*co*-4HB) scaffolds was fabricated including silver sulfadiazine(SSD) coated/collagen peptide-coated P(3HB-*co*-4HB) scaffolds, silver sulfadiazine blend/collagen peptide-coated P(3HB-*co*-4HB) scaffold and silver sulfadiazine blend/aminolysed P(3HB-*co*-4HB). In addition, cross-linking was employed on P(3HB-*co*-4HB) scaffolds with 1 wt% glutaraldehyde (GA). Consequently, the wettability and water uptake properties of all

the three scaffolds increased along with the increasing concentrations of collagen peptides, from 2.5 wt% to 12.5 wt%, however their porosity was found to be decline. On the other hand, antimicrobial analysis of the fabricated scaffolds exhibited 100% inhibition towards various pathogenic microorganims. On contrary, the time inhibition profile was influenced by two different techniques of silver sulfadiaziane incorportion, namely polymer coating and polymer impregnation. Results clearly revealed 24 h and 48 h of inhibition in coated SSD scaffold and impregnated SSD scaffold, respectively. In terms of the cyototoxic results, MTS assay demonstrated the L929 fibroblast cells grow well to the fabricated scaffolds. In this study, SSD coated/10 wt% collagen peptides coated P(3HB-*co*-4HB) and SSD blend/10 wt% collagen peptides coated P(3HB-*co*-4HB) displayed highest cell proliferation rate; whereas for aminolysed scaffolds, highest proliferation rate recorded at 12.5 wt% collagen peptides scaffolds. On the whole, all the three types of scaffolds have potential to be developed as a potent medical product especially for wound dressing and also as implants for bone tissue engineering mimicking the natural tissue engineering.

CHAPTER 1

INTRODUCTION

Biomaterials based scaffolds are well acknowledged in providing three-dimensional structure and extracellular matrix (ECM) platform for tissue regeneration. They induced cells to proliferate, synthesize tissue and other functional molecules. According to Williams and Martin. (2005), biopolymers apparently served as an alternate ECM for regeneration of cells. This is because they have the tendency to represent the key characteristics of natural ECM. As such, biodegradable scaffolds are sought for adequate cell-cell material interactions and aggregation. Biodegradable polymer is an innovative approach for recovering the function of damaged or diseased tissue (Chee et al., 2010). The key of success in designing potential scaffolds for tissue engineering depends on its capability in emitting highly porous and well interconnected configuration (Hollister, 2005; Loh and Choong, 2013; Mukheem et al., 2018). This will enhance and facilitates cell infiltration, proliferation, differentiation, and finally leading to the generation of new bone tissue. There are various fabrication techniques developed such as phase separation freeze-drying, foaming, particle leaching, electrospinning and sintering (Chen et al., 2002). Polyhydroxyalkonates (PHA) is one of the desirable biodegradable polymers that has been widely used due to its biocompatibility and biodegradability properties. Polyhydroxyalkanoate is an insoluble granule synthesized and stored in the cell cytoplasm as carbon and energy storage compounds under excessive carbon source and limiting nutrient conditions (Anderson and Dawes, 1990; Loo and Sudesh, 2007). These polyesters mainly synthesized by many microorganisms with various monomers. All PHA have similar properties which is hydrophobicity (insoluble in water), non-toxic and high degree

of polymerization (10^5-10^7 Da). Also interestingly exhibited as non-toxic, non–carcinogen, non-genotoxic biopolymers and are extensively studied for application in tissue engineering (Chen, 2009).

PHAs are widely applied as the conventional plastics (Sudesh et al., 2000). Thus, PHAs have gained great approach and interest as advantageous biomaterial as it can be produced from a variety of renewable resources and exhibited biodegradable and biocompatible thermoplastic material. Among the variety of biopolymers, poly(3–hydroxybutyrate-*co*-4-hydroxybutyrate) P(3HB-*co*-4HB) are widely applied as biomaterial in biomedical industry due to its biocompatibility and biodegradability (Martin and Williams, 2003). This copolymer has efficiently promoted cell viability as 4HB monomer is commonly found in human metabolites (Doi et al., 1990; Chanprateep et al., 2010). According to Wu et al., (2009) and Sudesh et al., (2016), P(3HB-*co*-4HB) is a potential medium for drug delivery. Recent studies involving commercially available PHAs such as P(3HB-*co*-4HV), P(4HB) and P(3HB-*co*-4HB) were developed as these polymers are capable of degrading slowly. Since PHAs are biocompatible, it does not cause any immune response in the organism. Due to this property, PHAs are widely used in surgery, bone defects treatment and as drug delivery device (Lim et al., 2017). Besides, studies have shown that, P(4HB) is one of the prominent monomers in medical industry as this is due to the self- healing property of P(4HB) which helps in preventing leakage of blood (Williams and Martin, 2005).

However, P(3HB-*co*-4HB) has a drawback with its hydrophobicity characteristics with minimal recognition sites for cell attachment and aggreration. This makes it less desirable candidate to be designed as scaffolds for tissue engineering and regenerative medicine. Therefore biomodification of this copolymer with natural polymers such as collagen, gelatine and chitosan has enhanced its properties for tissue engineering. Among the entire natural polymer, collagen is well known because it is key component of ECM. Collagen is the ECM element thus enhancing cell proliferation and differentiation. This ECM offers structure and mechanical integrity to tissues. Furthermore, it also prompts dynamic communications with the cellular components that facilitate and regulate daily cellular processes and wound healing. Additionally, it is undergoing persistent remodelling for an appropriate biological functions (Barnes et al., 2007). Surface architecture copolymer with collagen peptides is one of the most effective methods for providing scaffolds with improved cell adhesion, bioactivity, and degradation rate for tissue engineering applications.

Besides that biomaterial derived polymer source are susceptible to microbial infection which leads to nosocomial infections. The polymeric materials with resistance to vast microbial infections are highly recommended. The root of hospital acquired infections arises from medical devices although vast improvements have innovated in materials and technique (Huang et al., 2016). Moreover, antimicrobial polymers extensively gain priority as substitute to existing biocides and in some cases even to antibiotics (Timofeeva and Kleshcheva, 2011). As such, an attempt was made to develop biopolymer with antimicrobial that improvise the resistance towards pathogenic microorganism. There have been number of studies reported in the literature about the coating and impregnation of different antimicrobials agents.

In this study silver sulfadiazine (SSD) was impregnated to enhance the antimicrobial properties of biomaterial. The released silver ions could act as an inhibitor agent against organism growth and spreading.

In this study, surface functionalization of P(3HB-*co*-4HB) was enhanced by conjugating collagen peptides with impregnation or coated with antimicrobial agents (SSD) was carried out in order to produce a novel biomaterial. Three different scaffolds were fabricated which called as SDD/collagen peptides coated P(3HB-*co*-4HB) scaffold, SSD blend/collagen peptides-coated P(3HB-*co*-4HB) scaffold and SSD blend/ aminolysis P(3HB-*co*-4HB) scaffold by combination salt leaching and freeze drying technique.

In order to evaluate of the antimicrobial scaffolds, minimal inhibitory concentration and colonization test were conducted. The modified P(3HB-*co*-4HB) scaffolds were subjected to cell viability assay using fibroblast cell L929. The hydrophilicity of the fabricated scaffolds were also evaluated to enhance cell proliferation.

1.1 Problem Statements:

P(3HB-*co*-4HB) has caused limitation in used for biomedical applications. This is because the surface of P(3HB-*co*-4HB) is hydrophobic with minimal recognition sites for cell attachment and non sufficient biocontact properties of the polymer. Besides that, various types of polymers are currently used for indirect implantation for biomedical and tissue engineering applications. However, most of these lack inherent antimicrobial property and are vulnerable to bacteria attack due to the emergence of multidrug resistant microorganims. Antibiotics and biocides used to develop antimicrobial polymer but they are toxic to human health and ecosystem.

As such, modification of P(3HB-*co*-4HB) polymer to hydrophilicity properties and usage of silver ions are considered as the most promising antimicrobial scaffolds for biomedical applications.

1.2 Objectives of this study:

1. To develop surface functionalized antimicrobial porous P(3HB-*co*-4HB) scaffolds by salt leaching and freeze drying technique followed by collagen-coated and aminolysed technique.

2. To evaluate the antimicrobial and physical characteristics of the surface functionalized P(3HB-*co*-4HB) scaffolds.

3. To evaluate the biocompatibility and cell proliferation of the fabricated scaffolds.

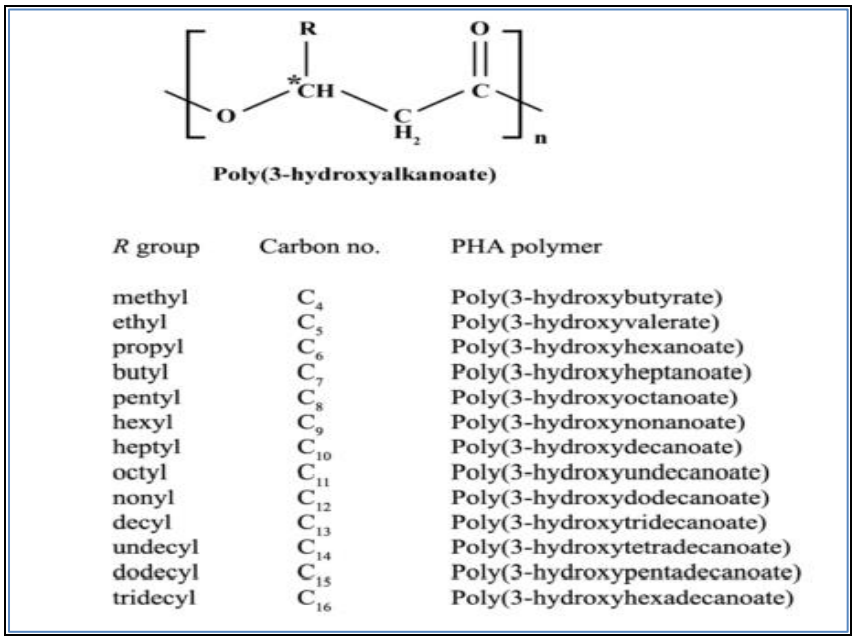
CHAPTER 2

LITERATURE REVIEW

2.1 Polyhydroxyalkonates (PHA)

Polyhydroxyakonates belongs to optically active biological polyesters family with (R)-3HA monomer units. Polyhydroxyakonates has gained biggest attraction for commercial and research interests due to its biocompatibility, chemical-diversity, biodegradability and its production from renewable carbon resources (Shah et al., 2008). PHA is synthesized by numerous microorganisms with carbon source material for circumstances with limiting nutrients with extreme carbon source (Anderson and Dawes, 1990). PHA was widely distributed in Gram-negative bacteria. PHA monomer known as P(3HB), was first identified by Lemoigne in 1926 and undergone extensive research there after (Macrae and Wilkinson, 1958). P(3HB) received broad attention as bacterial storage material which was equivalent to starch and glycogen. The molecular weight of these compounds ranges from 2×10^5 to 3×10^6 Da which was influenced by growth conditions and type of microorganisms synthesizing it (Byrom.,1992). Besides, PHA is assembled as granules particularly in cell cytoplasm (Pötter and Steinbüchel, 2005).

PHA is produced by bacteria which utilized carbon sources as complex plant oils or simple sugars (Loo and Sudesh, 2007; Tsuge, 2002). The hydroxyalkonates (HA) monomer units of these microbial polymers were found in configuration (-3D) due to the stereospecificity of biosynthetic enzymes (Anderson and Dawes, 1990). The distribution of the accumulated PHA varies in numbers and sizes per cell among different species (Byrom, 1992). In terms of phase contrast light observation, PHA granules exist as light–refracting granules. Figure 2.1 demonstrate the PHA chemical structure.



R group	Carbon no.	PHA polymer
methyl	C_4	Poly(3-hydroxybutyrate)
ethyl	C_5	Poly(3-hydroxyvalerate)
propyl	C_6	Poly(3-hydroxyhexanoate)
butyl	C_7	Poly(3-hydroxyheptanoate)
pentyl	C_8	Poly(3-hydroxyoctanoate)
hexyl	C_9	Poly(3-hydroxynonanoate)
heptyl	C_{10}	Poly(3-hydroxydecanoate)
octyl	C_{11}	Poly(3-hydroxyundecanoate)
nonyl	C_{12}	Poly(3-hydroxydodecanoate)
decyl	C_{13}	Poly(3-hydroxytridecanoate)
undecyl	C_{14}	Poly(3-hydroxytetradecanoate)
dodecyl	C_{15}	Poly(3-hydroxypentadecanoate)
tridecyl	C_{16}	Poly(3-hydroxyhexadecanoate)

Figure 2.1 Polyhydroxyalkanoates (PHA) chemical structure. The number and nomenclature of carbon for PHA compounds is determined by the functional alkyl group. Asterisk represents chiral centre for PHA-building block (Loo and Sudesh, 2007)

In general, there were about 150 distinct monomers units of PHAs being identified in several bacteria and each monomer contains specific functional groups (Pötter and Steinbüchel, 2005). By some means, some polymers were highlighted in several applications with appropriate quantities. Example of these polymers are homopolymers of P(3HB), poly(4-hydroxybutyrate) P(4HB) and poly(3hydroxyoctanoate) P(3HO); and copolymers of poly(hdroxybutyrate-*co*-3-hydroxyhexanoate) [P(3HB-*co*-3HHx)], poly(3-hydroxybutyrate-*co*-3-hydroxyvalerate) [P(3HB-*co*-3HV], as well as poly(3-hydroxybutyrate-*co*-4-hydroxybutyrate) [P(3HB-*co*-4HB)].

On the other hand, PHA synthase (PhaC) is the main element in PHA biosynthesis (Sudesh et al., 2000). PHA also can be categorized as short-chain (scl-PHA; 3 to 5 carbon atoms) or medium-chain length PHA (mcl-PHA; 6 to 14 carbon atoms) based on their total carbon atoms in a PHA monomer (Khana and Sristava, 2005). Whereas, mcl-PHA comprised several functional groups namely branched alkyls, cyano, halogens, olefins and aromatics (Hazer and Steinbuchel, 2007). Since PHA can be synthesized according to their respective functional groups, this has been advantageous in designing and producing related biopolymers consisting physical characteristics ranging from brittle to elastomeric polymers (Anderson and Dawes, 1990). Also, PHA was treated as pharmaceutically- active compound which currently being investigated as potential anti-HIV drugs, anti-cancer drugs and antibiotics. However, the type of monomer constituents determine the PHA applications. In terms of PHA production, the process differs by the type of bacteria involving one-stage and two-stage cultivations. One-stage cultivation mode involves growth associated PHA producers whereby PHA accumulates together with the cells growth.

Whilst, the two-stage cultivation involves non-growth associated PHA producers. Here, the cell growth phase is carried out under isolated nutrient enriched medium which then transferred into a nitrogen-free mineral salts medium for the PHA build-up. Alternatively, fed-batch cultivation method is usually performed for larger scale production systems as well as industrial scale (Chen et al., 2001; Kahar et al., 2004). PHA accumulation in bacteria can be controlled by manipulating the ratio of carbon to nitrogen (C/N). The ideal C/N ratio is around 20-50 for optimum PHA accumulation (Amirul et al., 2008; Lee et al., 2008). Some PHAs are known for their biocompatibility, which resulting in an appropriate and good biomaterial

(Williams and Martin, 2005; Zinn et al., 2001, Bhubalan et al., 2011). Several *In vivo* studies on the biodegradation and biocompatibility of PHA has been carried out (William and Martin, 2005; Zinn et al., 2001). The key breakdown products of PHA are 3-hydroxyacids which can be found naturally in animals. Adam et al. (1987) and Wiggam et al. (1997) reported that 3-hydroxybutyric acid and 4-hydroxybutyric acid are common constituents of human blood. In addition, PHA polymer containing 4HB monomer was found to exhibit relatively higher *in vivo* degradation rate compared to other PHAs (Saito et al., 1996). The rate of degradation can be controlled by changing the 4HB monomer composition. PHA polymers of P(3HB), P(4HB), poly(3-hyroxybutyrate-*co*-4-hydroxybutyrate) [P(3HB-*co*-4HB), poly(3-hydroxybutyrate-*co*-3-hyroxyvalerate) [P(3HB-*co*-3HV)], poly(3-hyroxybutyrate--*co*-hyroxyhexanoate) [P(3HB-*co*-3HHx)] and poly(3-hydroxyoctanoate) P(3HO) are of specific interest in tissue engineering applications such as surgical sutures, implants, bone plates, gauzes, osteosynthetic materials and also as matrix material assisting in slow release of drugs, vaccines and hormones (Freier, 2006; Chen and Wu, 2005; Williams and Martin, 2002; Zinn et al., 2001).

Further, introducing secondary monomers is another strategy to produce polymers in higher quality and quantity for processing. According to Matsusaki et al. (2002), the integration of other monomers into 3HB polymer chain significantly improved its thermal and physical proper. It was proposed SCL_{PHA} resembles conventional plastics, whereas MCL_{PHA} is identified as elastomers and rubbers. Apart from that, mixtures of PHA polymers with other biodegradable materials have also been investigated (Suriyamongkol et al., 2007).

In this case, PHA was combined with components such as poly (ethylene glycols) (Foster et al., 2001), poly(vinyl alcohol) (Yoshie et al., 1995b). Poly(lactic acid) (Blümm and Owen ,1995; Koyama and Doi, 1997) and few other natural products such as rubber (Bhatt et al., 2008) or bamboo fibers (Singh et al., 2008). Few instances of commercially available PHA are Biomer®, Mirel^{TM}, Biogreen®, Biocylce® and Biopol® (Sudesh and Iwata., 2008).

2.2 Poly(3-hydroxybutyrate-*co*-4-hydroxybutyrate) [P(3HB-*co*-4HB)]

In general, Poly(3-hydroxybutyrate-*co*-4-hydroxybutyrate) [P(3HB-*co*-4HB)] is a copolymer that can be converted into different composition of monomer using two various carbon sources (Lee et al., 2004). This type of copolymer attains exceptional features for medical and pharmaceutical fields (Loo and Sudesh, 2007). This is due to the ability of this copolymer which can be hydrolysed into natural metabolites by eukaryotic lipase that mimics those that exist in the human system (Martin and Williams, 2003). Additionally, some of the microorganisms possessed ability to integrate 4-hydroxybutyrate (4HB) monomers to form P(3HB-*co*-4HB) copolyme and these properties were specific to microorganisms that have a SCL PHA synthase (Loo and Sudesh, 2007). While, the synthesis of the copolymers 3HB and 4HB, respectively were carried out using 4-hydroxybutyric acid as the main carbon source (Doi et al, 1995).

Besides, recent findings revealed that copolymer can be even synthesized through the incorporation of 4-chlorobutyric, γ-butyrolactone, and 1,4-butanediol (Kunioka et al., 1989). Along the line, the combination of 4HB monomers resulted in copolymers consisting different physical features ranged from highly crystalline to elastomeric (Lee et al., 1999). Instead, modification of P(3HB-*co*-4HB) can be

executed to decrease the short comings in their physical properties such as lack of bioactivity and hydrophilicity (Keshavarz and Roy, 2010).

On top of it, biosynthesis of P(3HB-*co*-4HB) can be implemented using few techniques such as shake flasks, batch and fed-batch systems in bioreactor In the shake-flasks cultivations via two-stage cultivation is usually preferred due to its potency in producing higher 4HB molar fraction (Amirul et al., 2008a; Lee et al., 2004; Sudesh et al., 1999). Sugars (glucose, fructose and sucrose), butyric acid together with acetic acid are normally used for the generation of 3HB monomer (Lee et al., 2004). On the contrary, 4HB is generated using 4HB precursors. Several factors that may affect this copolymer biosynthesis inclusive of feeding of single or mixtures of carbon sources,carbon to nitrogen (C/N) ratio, aeration, inoculum concentration and the pH rate (Valentin and Dennis,1997; Li et al., 2010; Amirul et al., 2008a; Lee at al., 2004).

Besides, γ-butyrolactone is cleaved to 4-hydroxybutyric acid by the reaction of esterases or lactonases. ω-alkanediols such as 1,4–butanediol, 1,6-hexanediol, and 1,8-octanediol whereby these substrates are oxidized via enzymatic reactions including β-oxidation to form 4-hyroxybutyric acid before being converted to 4-hydroxybutyryl-CoA. Catabolism of the 4-hydroxybutyric acid also results in development of 3HB intermediate, 3-hyroxybutyryl-CoA. Generation of 3HB monomer leads to build-up of P(3HB-*co*-4HB).

2.3 Biomaterials

Biomaterial is ‘material intended to interface with biological systems to evaluate, augment, treat, or replace any tissue, organ or function of the body’. In detail, biomaterials influence biological processes by interacting with body in order to meet tissue regeneration (Lutolf and Hubell, 2005). In line with this, scaffolds made of biomaterials shall complement certain requirements, cells and growth-stimulating signals, which are familiar as the triad of tissue engineering. The scaffolds made from polymeric biomaterials, must be biodegradable and biocompatible upon implantation. The usage of porous 3D scaffolds is eminent to provide a suitable and an ideal environment for the organ and tissues regeneration.

Basically, these scaffolds act as a template for tissue formation and typically seeded with cells and occasionally growth factors. Also, they can be subjected to biophysical stimuli in the form of a bioreactor or as a device or system which applies different type of mechanical or chemical stimuli to cells. These cell-seeded scaffolds are either induced *in vivo* by direct implantation into the injured site, via body’s own systems to regenerate the tissues or organs or; cultured *in vitro* to synthesize tissues which can then be implanted into an injured site (Hutmacher, 2006). This combination of cells, scaffold and signals is frequently denoted as a tissue engineering triad (Fig 2.2).

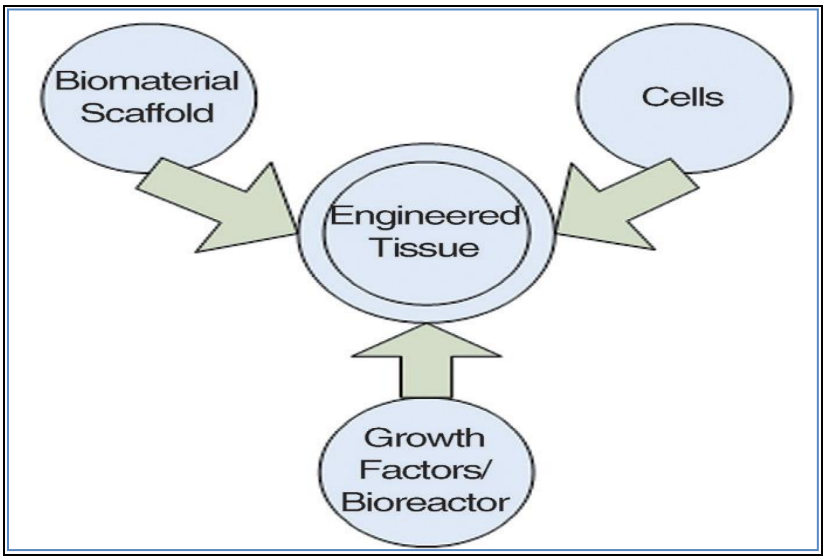


Figure 2.2 Tissue enginnering triad with the combination of cells, scaffolds and signals (Langer and Vacanti,1993).

The term 'tissue engineered construct' is used to distinguish scaffolds which have endured extensive *in vitro* culture prior to implantation.In depth, scaffold refers to the 3D biomaterial before cells were added with rate equal to the new matrix production by the developing tissue. Besides, they should be porous with interconnected pores of adequate size and absorbable by the cells to allow cell proliferation and cell adhesion for subsequent tissues development. Further, the matrix must also possess the ability to bind and couple up to neighbouring the tissues and be clinically practicable (Langer and Vacanti, 1993; Chang and Wang, 2011).

Selection of scaffolds for tissue engineering can be varied with plentiful options. There are two categories of biomaterials used for making porous based scaffolds natural and synthetic (Meyer et al., 2009). The natural biomaterials normally have remarkable biocompatibility to allow cells to attach, adhere and grow with excellent viability. In this sense, the scaffolds can be obtained from different animal species, for instances, bovine, pigs and horses, using intestine, pericardium, skin, bone, tendon and demineralized bone (Carletti et al., 2014; Chen et al., 2011).

Biomaterial also comprises of various type synthetic and natural materials, such as polymers or their protein, polysaccharides and composites.

In the body, a biomaterial is degraded or water solubilised to disappear from the implanted sites. In this case, the material is enzymatically digestedor hydrolysed into smaller fragments and finally disappears. Natural polymers undergo enzymatic degradation while synthetic polymers are through simple hydrolysis (Takahashi et al., 2005). Usually, the synthetic polymers can be modified to change their molecular weight and chemical composition. Generally, the synthetic polymers are mechanically strong and hydrophobic compared to the natural ones with slower degradation rates because of its properties itself. Exceptionally, in tissue engineering or medical application, the retention of biomaterials implanted in the human or animal body causes physical impairment of tissue regeneration (Takehara et al., 2008). Further, the absolute mechanical strength of materials is predicted through numerous applications. Material combination and design can assist in balancing two opposite properties of a material (Fernyhough et al., 2008).

Collagen is another type of protein classified under natural polymers together with gelatin, fibrinogen, actin, elastin, silk, keratin and others (Ratner et al., 2004). This type of protein is widely distributed in human body. This investigation revealed that half of the total collagen in the body built up in the skin and 70% of skin comprises collagen other than water (Lee et al., 2001). Collagen marked as the most studied molecules of the extracellular matrix due to its presence in all the connective tissues and cells (Parenteau-Bareil et al., 2010). Basically, the collagen family can be further divided into several groups based on the polymeric structures formed and related structural characteristics. For instance, collagen that form fibrils are

made up of Type I, II, III, V and XI (Prockop and Kivirikko, 1979; Gaspar et al., 2011).

The molecules of the collagen are made up of three α chain grouped together Each of α chain is made up of more than few thousand of amino acids following the sequence of –Gly-X-Y (Parenteau-Bareil et al., 2010). Apart from that, collagen is the major insoluble fibrous protein found in the connective tissue and ECM. Collagen has been extensively utilized in tissue engineering applications due to its abundance in the ECM, non-imunogenicity and various sources of existing isolation methods (Glowacki and Mizuno, 2008). Likewise, the collagen fibers also have some unique structural properties which plays an important role in providing a biological signals to stimulate and adjacent cells that regulate functional response and tissue engineering (Kolacna et al., 2007). Moreover, collagen is packed with good cell compatibility, resorbable, low antegenicity with high water affinity, as well as ability to promote tissue regeneration and growth. Nevertheless, collagen scaffolds possess some limitations in their structural and biological properties (Barnes et al., 2002). As such, blending collagen with other polymer especially nature polymer may enhance the properties resulting in more desirable ones for medical applications. Also, this can yield ideal biopolymers for tissue engineering applications.

2.4 Biocompatibility of P(3HB-*co*-4HB)

P(4HB) and P(3HB-*co*-4HB) exhibited high tolerance *in vivo* therefore it highly gained attention as biomaterial in biomedical and tissue engineering applications (Martin and Williams, 2003). Several previous studies showed positive cell proliferation and adhesion after seeding L929 mouse fibroblast and ovine vascular cellsonto P(4HB) films (Sodian et al., 2000). The results clearly showed P(3HB-*co*-4HB) promote cell attachment, migration, proliferation and differentiation with good surface characteristics. In addition, P(3HB-*co*-4HB) with 4HB monomer (11 to 45 mol%) were found to support proliferation on the scaffolds tested as well as the L929 cell growth (Chee et al., 2008).

Chanparteep et al. (2010) investigated the effects of P(3HB-*co*-4HB) with 4HB molar fractions (5, 24 and 38 mol%) for dermal and orthopaedic properties using L929 and human dermal fibroblasts. The results summarized L929 and human dermal fibroblasts cell densities grown on P(3HB-*co*-4HB) were higher compared to the positive control, poly(lactic-*co*-glycolic acid) [P(LA-*co*-GA)] films. Thus, the study concluded the number of human dermal fibroblasts increased proportionally with 4HB monomer composition. Similarly, Ee et al. (2009) demonstrated the cytotoxicity of P(3HB-*co*-4HB) scaffolds with fibroblast cell L929 and Chinese hamster lung fibroblast cells V79 cells using MTT assay [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium] and alkaline assay, respectively. On the contrary, another study demonstrated no genotoxic effects through the alkaline comet assay. However, positive results were obtained for P(3HB-*co*-4HB) drug loading level and composition ratio of *Mitragyna speciosa* crude extracts on the biocompatibility of P(3HB-*co*-4HB) (Azizi et al., 2010).

As matter of fact, different cells prefer different surfaces for attachment The capacity and morphology of a cell for proliferation and differentiation are influenced by the quality of cellular adhesion on materials. Also, P(3HB-*co*-4HB) films tend to possess different surface morphologies depending on their 4HB contents (Chee et al., 2008). On top of that, surface modification of P(3HB-*co*-4HB) films were executed to promote cell growth and improve cell adhesion. For this, various techniques such as lipase treatment, alkaline hydrolysis and ammonia plasma treatment were applied. Apart from that, immobilization of biomolecules such as collagen also plays an important role in cell proliferation. This was further proved by Rao et al. (2011), where depyrogenated of P(3HB-*co*-4HB) were blended with vitamin E or collagen for *in vivo* investigation. Hypothetically, it was presumed that the addition of either vitamin E or collagen will improve the biological performance of P(3HB-*co*-4HB). This type of blends enhance the cell L929 growth to agree with tissue engineering.

2.5 Surface modifications of PHA

To a point, proliferation and adhesion of different types of cells on polymeric materials depend on surface characteristics such as wettability (hydrophilicity/hydrophobicity or surface free energy), charge, chemistry, roughness and rigidity. Extensive research on the interactions between different types of cultured cells and various polymers with distinct wettability were carried out to study the correlation between surface wettability and cell interaction (Shen et al., 2008). In general, surfaces act as crucial platform in biology and medicine fields since most of the biological reactions occur at the surface and interfaces. The cell surface

comprises variety of receptors that able to bind with other cells or proteins in order to compose the surrounding of the cells (Kirchhof and Groth, 2008).

In fact, the development of biomaterials require detailed understanding on the interaction among the material surface and ECM to promote ideal tissue response. There are chances for numerous reactions to occur between the biomaterial surface of the biological system once biomaterials are exposed to living organisms (Kingshott et al., 2011). Those reactions may vary based on the surface roughness, topography and chemical composition of the surface which meets the biological system. The reactions involve water absorption which attracts biomolecule adsorption followed by cell attachment to the biomaterial surface (Vladkova, 2010). On the other hand, the surface properties of the biomaterials can affect the reactions between ECM and the biomaterial which act as determinant factor for the success or failure of a biomaterial in the end of result outcomes (Katsikogianni and Missirlis, 2004).

An ideal material surface is necessary to stimulate a constructive cell response for wound reparation and tissue engineering, while unfavourable surface structure may indicate the material are to be removed (Wilson et al., 2005). Biomaterial implanted in living organisms are able to trigger chronic and an acute inflammatory responses. Besides, the effect of surface topography and chemistry on cellular response becomes the central importance, especially when living systems encounter device surfaces in medical implants, tissue engineering and cell-based sensors. To comprehend these biological processes on the surfaces, there is an extensive interest in tailored surface-active materials produced by surface chemistry with advanced patterning processes (Senaratne et al., 2006).

Surface modifications are an effective approach in designing scaffolds in order to achieve biocompatability properties in tissue engineering applications (Chen et al., 2002). Surface modifications involve changes only at the outermost surface orientation and composition of biomaterial, without affecting its bulk properties (Yim and Leong, 2005). The quality of scaffolds governs the biological performance of the scaffold and cell interaction (Ratner and Bryant, 2004). The interactions of the scaffold and cell is also influenced by various structure of the biomaterial surface such as the presence of pores, pore distribution pore size, orientation and surface roughness, surface chemical properties or characteristics such as hydrophilicity, ionic interaction and surface charge (Dalby et al., 2002). Extensive research has been conducted to prepare various specific surfaces using several methods of surface modifications. This is performed to study the correlation between cell proliferations and cell anchorages on the structure of scaffold to be applied in biomedical field (Falconnet et al., 2006). There are few types of surface modifications being employed in fabricating surface modified biomaterials such as mechanical, physiochemical and biological modifications.

2.5.1 Biological Modifications

Surface biological modification is achieved by adsorption or chemical bonding of biomolecules to the polymer surface to stimulate a specific cell response. Numerous studies have reported immobilization of collagen, gelatine, chitosan and RGD peptide (L-arginine, glycine and L-aspartic acid) onto the polymer surface to improve the properties (Joddar and Ito, 2011). However, the attachment of biomolecules requires the activation of functional groups on both polymer surface and biomolecules (Marwa et al., 2015).

Among many modification, physical adsorption is among the simplest methods to biofunctionalized biomaterials, which is obtained by incubating the scaffold in solutions comprising biomolecules. The biomolecules attach to the material surface owing to surface interactions and attachment, such as hydrogen bonds, hydrophobic interactions, electrostatic forces, intramolecular forces and Van der Waal forces (Kingshott et al., 2011). The physical adsorption efficiency can be intensified by treating the biomaterial with air plasma to increase its hydrophilicity (Domingos et al., 2013). Usually, hydrophilic surfaces inclined to enhance and improve biocompatibility, adhesion strength and other pertinent properties (Hlady and Buijs, 1996; Vogler, 2012). Furthermore, surface functionalization through physical adsorption is a simple and mild procedure, which ensures limited damage to fragile biomolecules and structure; however biomolecule binding to scaffold surfaces is relatively weak. Meanwhile, non-covalent immobilization is based on electrostatic interactions. For example, ionic complex of gelatin and transforming growth factor-1 (TGF-1) can be attained when gelatin microparticles loaded with TGF-1 are encapsulated in oligo [poly(ethylene glycol) fumarate] hydrogels at pH 7.4. The interactions between gelatin and TGF-1 occur due to the negatively charged chemical groups on the gelatin surface and positive charge on TGF-1 (Madry et al., 2014). Typical adsorption of a TGF-1 onto a polymer is charge interaction between the polymer surface and TGF-1. This an outcome can also be obtained through an indirect interaction using an intermediate biomolecule (Sohier et al., 2007).

One alternative simple method would be the coating of a polymer surface with biomacromolecules. Biomacromolecules are proteins such as fibronectin, collagen and vitronectin provide an ideal adhesive effects between biomaterial surfaces and cells (Geißler et al., 2000). In order to fabricate blood compatible biomaterial, heparin was covalently coated to a polycarbonate urethane. Surface bound heparin polymer exhibited significant direct thrombin inhibitory activity. This is due to the anticoagulant nature of heparin, which able to support modified surfaces of the polycarbonate urethane into blood compatible. Thus, heparin coated polycarbonate urethane is a potential candidate in coating cardiovascular devices for long term compatibility (Lu et al., 2012). Alternatively, the sol-gel process is a wet-chemical technique which was employed to produce bioactive coatings onto the material surface. This altered the biological behaviour of cells and proteins to the implants.In the process, the sol (or solution) gradually forms a gel-like network comprising both liquid and solid phase on the surface of the material. There are plentiful applications on the use of the sol-gel process for production of biomaterials (Podbielska and Jarza, 2005).

2.5.1 Mechanical Modifications

Mechanical modifications comprised surface topographical structures such as grooves, rigid, micro and nano-structures on cell responses (Oakley et al., 1997). The interaction of cell adhesion and surface topography were investigated by Wan et al. (2005) which involved the preparation of PLLA scaffolds with patterned surfaces which were created using polystyrene hemispherical pit as template. The cell adhesion strength of the OCT-1 osteoblast-like cells was improved due to the roughness or porous structure of the scaffold surface as compared to the

smooth surface of the control. In this case, the cellular response of biomaterials can be enhanced by mimicking the surface roughness or porosity of the structural ECM components in the natural tissue (Dhandayuthapani et al., 2011). In order to create the porous or micro-rough surface on scaffolds, freeze drying technique was incorporated. This freeze-drying method involved freezing the polymer solution against various concentrations. Following this, the ice/polymer scaffold which was freeze-dried resulted in porous structures on the surface of the biomaterial (Lv and Feng, 2006). Additionally, a biocompatible (3D) porous polysaccharide scaffolds was fabricated through freeze-drying. Correspondingly, cross-linking process using biomacromolecules such as pullulan was applied to fabricate scaffolds with desired pore shapes and sizes. The biocompatibility of the scaffolds was proven through the enhanced proliferation of mesenchymal stem cells (Autissier et al., 2010).

Similarly, Ma et al. (2003) fabricated collagen/chitosan porous scaffold with enhanced stability for skin tissue engineering by engaging the freeze-drying technique. The *in vitro* culture of human fibroblast cells and *in vivo* testing using animal model further verified the good cytocompatibility of the scaffold with accelerated cell infiltration and proliferation. Interestingly, biocomposite scaffolds with porous surface synthesized P(3HB-*co*-4HB) was fabricated using the freeze-drying method. This scaffold exhibited accelerated cell proliferation with Chinese hamster lung (CHL) fibroblast cells. This scaffold which enhanced degradation rate has been targeted for wound dressing or tissue engineering based applications (Zhijiang et al., 2012).

Salt leaching is another widely used method to fabricate porous scaffolds Salts, gelatine or sodium bicarbonate ($NaHCO_3$) are used as porogens to create the pores. The porogen are grinded into small particles and cast together with the polymer solution. The porogen is leached out by rinsing the scaffolds with water leaving behind pores created by porogen on the scaffolds (Subia et al., 2010). Apparently, highly porous PLLA scaffolds with pore size ranging from 280-450 μm was achieved using gelatine particles as the porogen. The scaffold demonstrated efficient biocompatibility when evaluated by *in vivo* implantation and *in vitro* chondrocyte culture (Gong et al., 2008). Ansari and Amirul (2013) has reported the fabrication of PHA macroporous scaffold by salt leaching and enzyme degradation technique resulted in an increased water uptake capability of P(3HB-*co*-70mol% 4HB) scaffold.

2.5.3 Physiochemical Modifications

Physiochemical modifications is one of modification on surface biomaterial implicate the treatment with aminolysis, vapor, active gases, radiation or plasma treatment (Yoshida et al., 2013). Plasma treatment is broadly applied in surface modifications which involves the use of electrons, radicals, ions, neutral molecules and gasses to modify the surface of materials (Oehr et al., 1999). As such, a biocompatible of P(3HB-*co*-3HV) films was fabricated by plasma treatment especially for tissue engineering (Wang et al., 2006). In the respective study, the oxygen content of the surface was increased through oxygen plasma treatment; whereas nitrogen plasma treatment improved the surface with nitrogen atoms. Besides, new bonds such as COOH from oxygen plasma, and C-N, C═N, amide bonds from nitrogen plasma were created after the plasma irradiation.

Additionally, the cell adhesion and hydrophilicity of stromal cells seemed to increase with the plasma treated P(3HB-*co*-3HV) scaffolds (Wang et al., 2006).

On the flip side, chemical grafting is a technique of immobilizing biomacromolecules such as RGD peptide and proteins through photochemical method or plasma graft (Yang et al., 2001). P(3HB-*co*-3HV) films were activated by ammonia plasma treatment on the surface, followed by chemical grafting of RGD-containing peptides where the RGD contains peptides were covalently grafted onto the P(3HB-*co*-3HV) films. This clearly showed that the modified P(3HB-*co*-3HV) films grafted with RGD demonstrated significant improvement in cellular compatibility (Wang et al., 2011). Meanwhile, another study reported that the chitosan was covalently immobilized onto a P(LA-*co*-GA) surface by means of chemical grafting. The modified P(LA-*co*-GA) surface revealed an increase in the hydrophilicity of the modified film as well as enhanced cell compatibility with hepatocyte cell culture (Wang et al., 2003). Wang et al. (2009) also established P(3HB-*co*-3HV) collagen film using covalent immobilization onto polymer surface to increase its cell compatibility. Amide groups were photo grafted on P(3HB-*co*-3HV) films and collagen was then chemically bonded to amine groups to form the collagen-modified P(3HB-*co*-3HV). The hydrophilicity of the modified films was successfully improved. Along with that, chondrocytes cultured on the modified film demonstrated a decent cell growth as well (Wang et al., 2009).