RADICAL GRAFTING OF MEDIUM CHAIN-LENGTH POLY-3-HYDROXYALKANOATES WITH GLYCEROL 1,3-DIGLYCEROLATE DIACRYLATE TO FORM AMPHIPHILIC GELS: MECHANISM AND COPOLYMER CHARACTERIZATION

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ABSTRACT

Radical grafting of medium chain length poly-3-hydroxyalkanoates (mcl-PHA) with glycerol 1,3-diglycerolate diacrylate (GDD) in acetone was successfully carried out using benzoyl peroxide (BPO) as sole micro-initiator. A detail mechanism scheme provides significant improvement to previous literature. Radical-mediated grafting generated α-β carbon inter-linking of mcl-PHA and GDD resulted in macromolecular structure with gel properties. Thermal characterization distinguished the thermal properties of the copolymer for different graft yields as a function of initiator concentration, GDD monomer concentration, incubation period and temperature. The water absorption and porosity of the gel were significantly improved relative to neat mcl-PHA.

Keywords: Polyhydroxyalkanoates, radical grafting, amphiphilic, biogel, biomaterial

CANTUMAN SECARA RADIKAL MELIBATKAN POLI-3-HIDROKSIALKANOAT BERANTAIAN SEDERHANA PANJANG (MCL-PHA) DENGAN GLISEROL 1,3-DIGLISEROLAT DIAKRILAT (GDD) BAGI MEMBENTUK GEL AMFIFILIK: MEKANIME DAN PENCIRIAN KOPOLIMER

ABSTRAK

Proses cantuman secara radikal melibatkan polimer poli-3-hidroksialkanoat berantaian sederhana panjang (mcl-PHA) dengan gliserol 1,3-digliserolat diakrilat (GDD) telah berjaya dijalankan dengan bantuan benzoil peroksida (BPO) sebagai satu-satunya bahan pemula mikro dalam larutan aseton. Mekanisme secara terperinci telah dikemukakan sebagai kemas kini berdasarkan kajian-kajian lepas. Proses cantuman berasaskan radikal telah menghasilkan rangkaian karbon α-β antara mcl-PHA dan GDD, menghasilkan struktur makromolekul yang mempunyai ciri gel. Pencirian terma telah menunjukkan ciri terma produk berdasarkan hasil cantuman mengikut kepekatan bahan pemula, kepekatan monomer GDD, tempoh pemeraman dan suhu. Daya serapan air dan keporosan gel yang terhasil juga dengan ketaranya telah dipertingkatkan, dibandingkan dengan mcl-PHA mentah.

Kata kunci: Polihidroksialkanoate, cantuman radikal, ampifilik, biogel, biobahan

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–OH	:	Hydroxyl group
°C	:	Degree Celsius
Å	:	Armstrong
cm	:	centimeter
Da	:	Dalton
E	:	Young's modulus
eV	:	Electronvolt
g	:	Gram
g/L	:	Gram/liter
GPa	:	Gigapascal
L	:	Liter
M	:	Molar
mg	:	Milligram
min^{-1}	:	Per minute
mJ	:	Millijoule
ml	:	Mililiter
mM	:	Millimolar
MPa	:	Megapascal
mW	:	Milliwatt
s^{-1}	:	Per second
T_d	:	Degradation temperature
T_g	:	Glass transition temperature
T_m	:	Melting temperature
T_{max}	:	Maximum degradation temperature

v/v	:	Volume/volume
X_{cr}	:	Crystallinity degree
ε	:	Epsilon (Elongation at break)
θ	:	Theta
σ	:	Sigma (Tensile strength)
$(NH_4)_2SO_4$	:	Ammonium sulphate
AIBN	:	2,2'-azobisisobutyronitrile
BPO	:	Benzoyl peroxide
C	:	Carbon
$CaCl_2 \cdot 2H_2O$	:	Calcium chloride dihydrate
$CaCl_2 \cdot 6H_2O$	:	Calcium chloride hexahydrate
$CuCl_2 \cdot 2H_2O$	:	Copper (II) chloride dihydrate
DSC	:	Differential Scanning Calorimetry
$FeSO_4 \cdot 7H_2O$	:	Iron (II) sulphate heptahydrate
FTIR-ATR	:	Fourier Transform Infrared - Attenuated Total Reflectance
GC	:	Gas Chromatography
GDD	:	Glycerol 1,3-diglycerolate diacrylate
HD	:	Hydroxydecanoate
HDD	:	Hydroxydodecanoate
HHx	:	Hydroxyhexanoate
HO	:	Hydroxyoctanoate
HPLC	:	High-Performance Liquid Chromatography
K_2HPO_4	:	*di*-Potassium hydrogen phosphate
KH_2PO_4	:	Potassium dihydrogen phosphate
lcl-PHA	:	Long-chain-length polyhydroxyalkanoates
mcl-3HA-CoA	:	Medium-chain-length-3-hydroxyalkanoic Coenzyme A

mcl-PHA	:	Medium-chain-length polyhydroxyalkanoates
$MgSO_4 \cdot 7H_2O$	:	Magnesium sulphate heptahydrate
$MnCl_2 \cdot 4H_2O$	:	Manganese (II) chloride tetrahydrate
N_2	:	Nitrogen gas
$NaNH_4HPO_4 \cdot 4H_2O$	:	Ammonium sodium phosphate dibasic tetrahydrate
NMR	:	Nuclear Magnetic Resonance
OTR	:	Oxygen transmission rate
P(3HB-*co*-3HHx)	:	Poly-3-hydroxybutyrate-*co*-3-hydroxyhexanoate
P(3HB-*co*-3HV)	:	Poly-3-hydroxybutyrate-*co*-3-hydroxyvalerate
P(3HHx-*co*-3HO-*co*-3HD-*co*-3HDD)	:	Poly-3-hydroxyhexanoate-*co*-3-hydroxyloctanoate-*co*-3-hydroxydecanoate-*co*-3-hydroxydodecanoate
P(3HHx-*co*-3HO)	:	Poly-3-hydroxyhexanoate-*co*-3-hydroxyoctanoate
P(3HV) or PHV	:	Polyhydroxyvalerate
PHA	:	Polyhydroxyalkanoates
PHA-*g*-GDD	:	Polyhydroxyalkanoate-*grafted*-glycerol 1,3-diglycerolate diacrylate
PhaC	:	PHA polymerase
PhaP	:	Phasin
PhaZ	:	PHA depolymerase
PHB or P(3HB)	:	Polyhydroxybutyrate
scl-PHA	:	Short-chain-length polyhydroxyalkanoates
STA	:	Simultaneous Thermal Analysis
WAXS	:	Wide Angle X-ray Scattering
WVTR	:	Water vapor transmission rate
XRD	:	X-ray Diffraction Analysis
$ZnSO_4 \cdot 7H_2O$	:	Zinc sulphate heptahydrate

LIST OF APPENDICES

CHAPTER 1: INTRODUCTION

1.1 Introduction

Polyhydroxyalkanoates (PHA) is a well-known biopolymer class with attractive biocompatibility. PHA is accumulated within certain bacterial species in the form of granules when the microorganisms experience imbalanced growth conditions *viz.* simultaneous excess carbon source and limitation of nutrients such as nitrogen and phosphorus (Hazer & Steinbuchel, 2007; Keshavarz & Roy, 2010; Rai et al., 2011; Leong et al., 2014). Two categories of PHA can be differentiated i.e. short chain-length polyhydroxyalkanoates or scl-PHA, comprising monomers with carbon atoms of four and five, and medium chain-length polyhydroxyalkanoates or mcl-PHA, made up of monomers with carbon atoms of 6 to 14 (Keshavarz & Roy, 2010). Modification and functionalization of PHA, intended for fine-tuning the features, are important for certain applications. Functionalization of PHA on the side chain, for example, can alter the polymer interaction behavior by introducing elements of hydrophilicity (Stigers & Tew, 2003).

One of the functionalization techniques is grafting. Graft copolymerization of PHA forms a modified segmented copolymer with interesting properties, particularly in terms of wettability and thermo-mechanical strength. The grafting processes can be carried out in several ways, including chemical, radiation, and plasma discharge methods (Nguyen, 2008; Gumel et al., 2015a). The current grafting methods for many polymers are equally applicable in the case of PHA functionalization. For example, the benzoyl peroxide-initiated graft polymerization of 2-hydroxyethylmethacrylate (HEMA) onto poly-(3-hydroxybutyrate-*co*-3-hydroxyvalerate) (PHB-*co*-HV) enhances the crystallinity and wettability of the biopolymer (Lao et al., 2007b; Nguyen, 2008).

Grafting applies radical intermediates during the reaction, which mediate the polymerization of a vinyl monomer as grafted branches on the main polymer chain through 'grafting onto', 'grafting through' and 'grafting from' techniques (Nguyen, 2008; Roy et al., 2009). Free radical grafting *via* radical initiators is widely used for the modification of polymers. Benzoyl peroxide is one of the initiators extensively used to functionalize bacterial polyesters (Lee & Lee, 1997; Ilter et al., 2001; Lao et al., 2007b; Wang et al., 2007; Kim et al., 2008; Nguyen, 2008; Ansari & Annuar, 2018). It is a simple and robust method. Benzoyl peroxide has been reported to be more efficient compared to other common thermal initiators, such as azo-*bis*-isobutyronitrile (AIBN) (Bhattacharya & Misra, 2004) and other peroxyl initiators (Wilson et al., 2010). If the radical termination rate is balanced by the propagating radical concentration during the reaction, it is possible to yield high number of average molecular weight polymer as well as low dispersity index. Equilibrium constant across active and dormant species is determined by the catalyst and eventually determine the rate of polymerization (Barner-Kowollik et al., 2003; Coessens & Matyjaszewski, 2010).

The mechanism of radical-mediated copolymerization of mcl-PHA with GDD was not clearly elucidated by previous studies. The stochastic nature of the radicalized reaction made it difficult to delineate concise structure for the final grafted product. Hence, in this study, medium-chain-length poly-3-hydroxyalkanoates (mcl-PHA), obtained from bacterial fermentation, was graft copolymerized with glycerol 1,3-diglycerolate diacrylate (GDD) *via* free radical polymerization. PHA-*g*-GDD, the product of the grafting was prepared through thermal incubation process with all reaction components mixed together in a selected organic solvent. The effects of different initiator concentrations, in this case benzoyl peroxide (BPO), were investigated alongside incubation temperature and time, and initial GDD concentration. The PHA-*g*-

GDD copolymer was characterized and mechanism of the grafting reaction was proposed.

1.2 Objectives

The objectives of the study are:

- To investigate mcl-PHA graft copolymerization with GDD using benzoyl peroxide as micro-initiator;
- To authenticate and characterise the grafted product, PHA-*g*-GDD;
- To elucidate the reaction mechanism, and established chemical structure of PHA-*g*-GDD.

CHAPTER 2: LITERATURE REVIEW

2.1 Polyhydroxyalkanoates (PHA)

Polyhydroxyalkanoates was first discovered in 1926, in Gram-positive bacterium *Bacillus megaterium* (Doi & Steinbuchel, 2002; Kim et al., 2007). A French microbiologist, Maurice Lemoigne chanced upon the PHA in the form of poly(3-hydroxybutyrate) or P(3HB) made from linear polyester of D (-)-3-hydroxybutyric acid (Keshavarz & Roy, 2010; Bugnicourt et al., 2014). The analytical method developed by Lemoigne at that time involved the usage of hot alcohol for extraction, and chloroform and diethyl ether for purification hence the polymer was saponified (Lemoigne, 1926; Salmiati et al., 2009).

Subsequent to its discovery, poly-*β*-hydroxybutyrate became the type of PHA to be utilized widely and analytically for further investigations. Most of the literatures described better and improved analytical methods with time. In 1950s, physiological studies were carried out to obtain information regarding the function of PHA as intercellular carbon and energy reservoir. These involved the conversion of PHA sample into crotonic acid prior to analysis (Lemoigne, 1926; Slepecky & Law, 1960; Law & Slepecky, 1961; Kichise et al., 2002). Early methods were lacking in the ability to distinguish different monomer in PHA, which include spectrophotometric, infrared methods, and the sensitive enzymatic method, but nonetheless are useful for fast screening techniques (Rijk et al., 2002).

The chronological order of the PHA analytical studies since 1926 is shown in Table 2.1.

Table 2.1: Chronology of PHA analytical studies.

Year	Progress	Reference
1926	Maurice Lemoigne discovered PHA in the form of P(3HB). Hot alcohol for extraction, and chloroform and diethyl ether were used for purification.	(Lemoigne, 1926)
1958	Faster analytical spectrophotometric method developed. Treating *Bacillus* cells with sodium hypochlorite for a turbid solution to be measured spectrophotometrically.	(Williamson & Wilkinson, 1958)
1960 -1961	P(3HB) was treated with concentrated sulphuric acid to be converted to crotonic acid. Quantitation achieved by ultraviolet spectrophotometric measurement.	(Slepecky & Law, 1960; Law & Slepecky, 1961)
1973	Development of a disk assay method.	(Ward & Dawes, 1973)
1974	Identification of 3-hydroxyvalerate and 3-hydroxyhexanoate, types of 3-hydroxyalkanoates besides 3-hydroxybutyrate in chloroform extract of activated sludge from an alkaline hydrolysis method. PHA was separated using hot ethanol (95 %) and analyzed by nuclear magnetic resonance (NMR). Discovered the presence of longer fatty acids due to a methylene group adjacent to a methyl group.	(Wallen & Rohwedder, 1974)
1978	Development of analytical method based directly on whole cell biomass sample by having simultaneous extraction, hydrolysis and methylation in a test tube. Mild acid hydrolysis using 3 % sulphuric acid in methanol allows for identification and quantitation of methyl ester of a specific monomer present in PHA sample. The use of internal standard in GC improves quantitation.	(Braunegg et al., 1978)

Table 2.1, continued.

Year	Progress	Reference
1983	Application of ion-exclusive high-pressure liquid chromatography and UV-detection in PHA recognition and measurement. Discovery of mcl-PHA in *P. oleovorans* grown on 50 % octane that showed flexible property during freeze-fracture electron microscopy preparation procedure.	(Karr et al., 1983) (Desmet et al., 1983)
1986	Utilization of NMR for analysis of PHA.	(Doi et al., 1987)
1999	Improvement of HPLC method in analyzing PHA sample.	(Hesselmann et al., 1999)

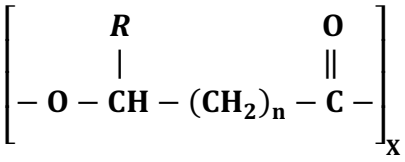
2.1.1 Types of PHA

Generally, PHA are biopolyesters consisting *R*-3-hydroxy fatty acids, with various types of side chain. The pendant group *R* determines PHA monomer identity, of which more than 150 kinds of hydroxycarboxylic acid have been identified, varying from C_1 to C_{13}, containing substituents and whether saturated or unsaturated (Berezina & Martelli, 2015). There are also PHA of 4, 5, and 6-hydroxyalkanoic acids, synthesized by a wide variety of bacteria *viz*. approximately 300 different Gram-positive and Gram-negative species by fermentation utilizing different types of carbon sources (Martinez-Abad et al., 2016).

There are three types of PHA according to their chain length, short-chain-length (scl-PHA), medium-chain-length (mcl-PHA), and long-chain-length (lcl-PHA). Scl-PHA contains 3 to 5 carbon atoms in each monomer, such as poly-3-hydroxybutyrate (P3HB) and poly-4-hydroxybutyrate (P4HB), meanwhile, mcl-PHA is PHA with 6 to 14 carbon atoms, for example poly-3-hydroxyhexanoate (P3HHx) and poly-3-hydroxyoctanoate (P3HO). Long chain fatty acids that contain more than 14 carbon atoms will produce

long-chain-length (lcl)-PHA (Rai et al., 2011; Berezina & Martelli, 2015). Furthermore, a PHA could be either homopolymer that consists only one type of monomer unit or heteropolymer with more than one type of hydroxyalkanoates monomer. Poly-3-hydroxybutyrate-*co*-3-hydroxyvalerate (P(3HB-*co*-3HV)), poly-3-hydroxyhexanoate-*co*-3-hydroxyoctanoate (P(3HHx-*co*-3HO)), and poly-3-hydroxybutyrate-*co*-3-hydroxyhexanoate (P(3HB-*co*-3HHx)) are some example of heteropolymers in PHA. These distinctive structures and the resulting material properties of PHA are due to the low substrate specificity of the synthases, influenced by the bacteria in line with the carbon source utilized during the accumulation process (Berezina & Martelli, 2015).

The chemical identity of *R* group determines the type of PHA (Figure 2.1). *R*-group that consists of a hydrogen atom, methyl and ethyl are considered as scl-PHA, while mcl-PHA composed of propyl, butyl, pentyl, hexyl or heptyl. *R*-groups with more than 14 carbons per repeating unit are lcl-PHA (Berezina & Martelli, 2015), examples are in Figure 2.1.

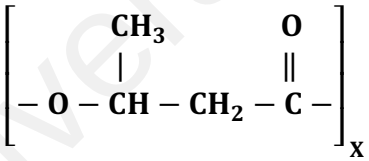


X = 100 – 30,000

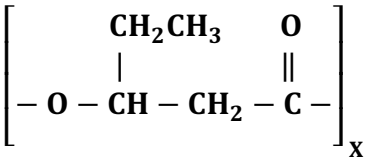
For *n* = 1	*R* = H	Poly-3-hydroxypropionate	(P3HP)
	R = methyl	Poly-3-hydroxybutyrate	(P3HB)
	R = ethyl	Poly-3-hydroxyvalerate	(P3HV)
	R = propyl	Poly-3-hydroxyhexanoate	(P3HHx)
	R = pentyl	Poly-3-hydroxyoctanoate	(P3HO)
	R = nonyl	Poly-3-hydroxydodecanoate	(P3HDD)
For *n* = 2	*R* = H	Poly-4-hydroxybutyrate	(P4HB)
	R = methyl	Poly-3-hydroxyvalerate	(P4HV)
For *n* = 3	*R* = H	Poly-5-hydroxyvalerate	(P5HV)
	R = methyl	Poly-5-hydroxyhexanoate	(P5HHx)
For *n* = 4	*R* = hexyl	Poly-6-hydroxyodecanoate	(P6HDD)

Figure 2.1: General chemical structure of a monomer in PHA (Reddy et al., 2003; Castilho et al., 2009), permission obtained from Elsevier

(a)



(b)



(c)

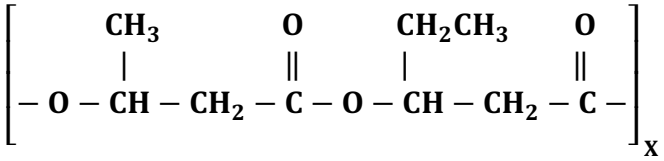


Figure 2.2: Examples of common chemical structure of a monomer in PHA, where *X* is an integer for the repeating units (a) P(3HB), (b) P(3HV), and (c) P(3HB-*co*-3HV)

The most abundant type of PHA found is P(3HB), a homopolymer consisting methyl group for its *R*-group. PHA with 3-hydroxy acid are optically active due to the PHA synthase, a polymerizing enzyme that having stereospecificity property (Sudesh et al., 2000). They also exhibit isotactic properties due to stereochemical specificity in the repeating monomer units for their well-known high degree of polymerization (Reddy et al., 2003). Besides that, PHA are famous for their exceptionally high molar mass polyester, in between 100-1000 kDa, depending on the microbial species, and culture condition especially the carbon source (Braunegg et al., 1998). The copolymers of PHA are typical in bacterial production as well, for example for mcl-PHA are poly-3-hydroxyhexanoate-*co*-3-hydroxyoctanoate-*co*-3-hydroxydecanoate and poly-3-hydroxyhexanoate-*co*-3-hydroxyloctanoate-*co*-3-hydroxydecanoate-*co*-3-hydroxydodecanoate as represented in Figure 2.3 (Kubota et al., 1988; Chen, 2010).

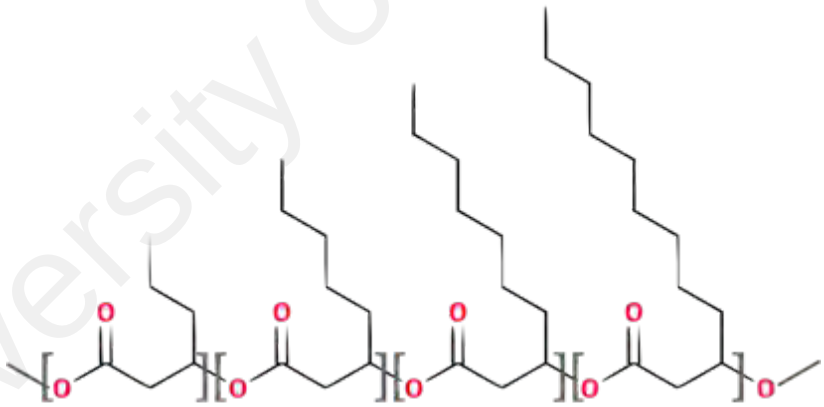


Figure 2.3: Basic chemical structure of mcl-PHA

On the mcl-PHA itself, there are several classifications to be considered to characterize them based on to the chemistry of the compound present (Table 2.2). These classifications distinguish the way PHA differs in terms of composition, and evidently drawing attention among researchers to tailor made desired mechanical properties, especially for the biocompatibility purposes in medical applications. The presence of additional functional groups such as halogen, hydroxyl, carboxyl epoxy, phenoxy,

cynophenoxy, and nitrophenoxy helps to customize the hydrophobic properties (Rai et al., 2011).

Table 2.2: Classification of mcl-PHA (Rai et al., 2011).

	Classification	Examples	Reference
1.	Saturated aliphatic mcl-PHA without any functional moieties	P(3HO), P(3HHx), P(3HN)	(Rai, 2010)
2.	Unsaturated mcl-PHA containing carbon-carbon double bonds (C=C) and carbon-carbon triple bonds (C≡C) in their terminal reactive groups	poly(3-hydroxy-4-hexenoic) P(3HN(≡)) (poly-3-hydroxy-8-nonynoate), P(3HUD(≡)) (poly-3-hydroxy-10-undecynoate)	(Kim et al., 1998; Rai et al., 2011)
3.	mcl-PHA with halogenated reactive groups like bromine, chlorine, and fluorine	poly(3-hydroxyalkanoate-co-3-hydroxy-ω-chloroalkanoate)	(Doi & Abe, 1990)
4.	mcl-PHA containing bulky aromatic side chains	poly(3-hydroxy-5-phenyl-valerate)	(Fritzsche et al., 1990b)
5.	mcl-PHA containing branched monomers	poly(3-hydroxy-7-methyloctanoate)	(Fritzsche et al., 1990a)

2.1.2 Biosynthetic Pathway of Polyhydroxyalkanoates

Polyesters of 3, 4, and 5-hydroxyalkanoate units were discovered in many bacteria and the genes coding for the enzymes involved in PHA biosynthesis are cloned in *Ralstonia eutropha* in the 1980s (Doi & Steinbuchel, 2002). In fact, more than 150 different PHAs could be produced by choosing the right microorganism and carbon substrate.

Typical biosynthesis in bacteria from sugar begins with the glycolysis pathway, forming pyruvate and eventually acetyl-coenzyme A to be condensed by β-ketothiolase, an enzyme encoded by *pha*A gene followed by the production of acetoacetyl-coenzyme A from 2 acetyl-coenzyme A molecules, to be reduced into *R*-3-hydroxyacyl-coenzyme A by acetoacetyl-coenzyme A reductase enzyme, encoded by the *pha*B gene. *R*-3-hydroxyacyl-coenzyme A is now a monomer or building block for PHA polymer. PHA synthase, encoded by *pha*C gene is responsible for polymerizing the monomers, to form the PHA polymer (Madison & Huisman, 1999; Reddy et al., 2003; Gumel et al., 2012a). The key component of acetyl-coenzyme A supplies different length of monomer for PHA synthases and polymerization hence the chain length of the PHA polymer (Figure 2.4) (Verlinden et al., 2007; Chen, 2010).

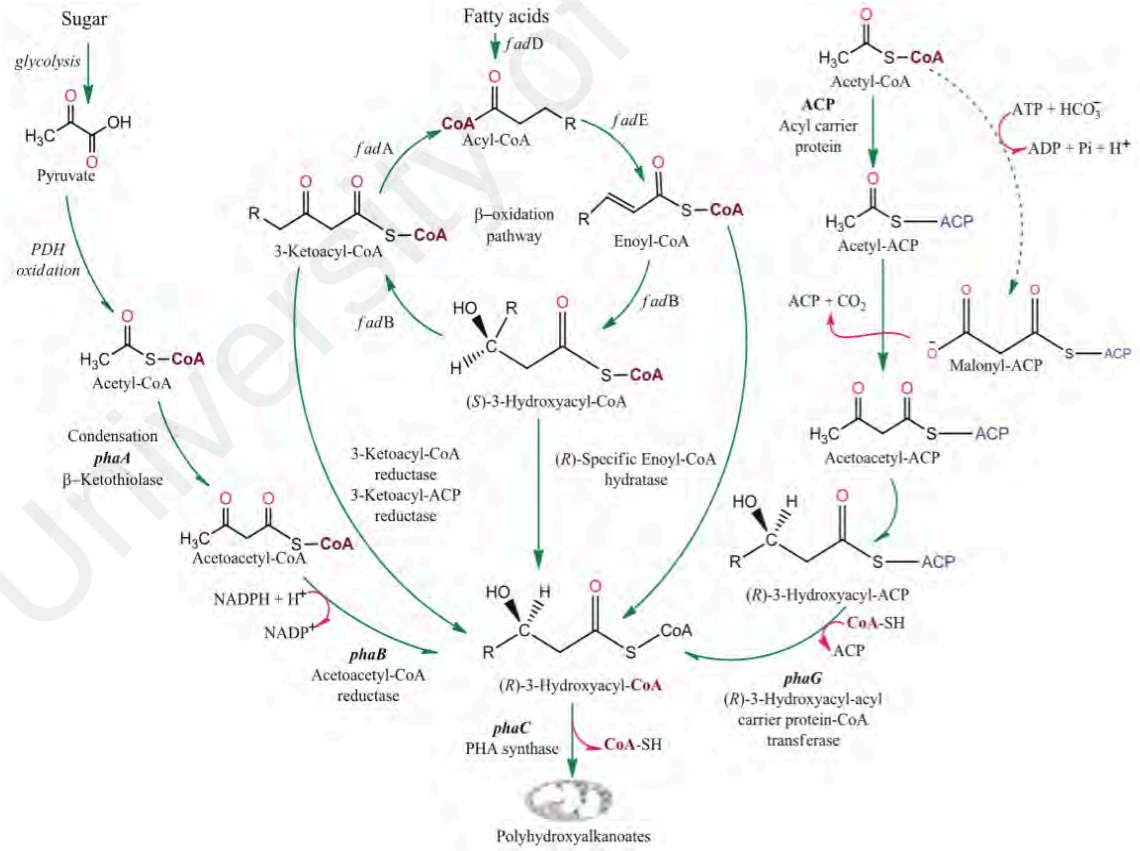


Figure 2.4: PHA biosynthesis general scheme by sugar catabolism, fatty acid β-oxidation and intermediary pathways (Gumel et al., 2012a), permission obtained from Elsevier

The polymerization of a specific type of PHA is regulated by several built-ins genetic information within the microbe. It depends on the subunit composition and substrate specificity of the enzyme PHA synthase which can be classified into four group, class I, II, III and IV and influenced by the *pha*C gene for polymerization process. Class I PHA synthases favorably utilized coenzyme A thioester for various kind of $3HA_{scl}$ monomers. On the other hand, PHA synthases of class II show specificity for $3HA_{mcl}$. Both class I and II synthases are regulated by *pha*C genes to form the subunit. Class III PHA synthases subunits are transcribed by *pha*C and *pha*E genes, and specifically for the production of scl-PHA but capable of accommodating mcl-PHA. Two subunits from *pha*C and *pha*R are observed in class IV PHA synthases for the biosynthesis of PHB (Rehm, 2007; Jendrossek, 2009; Park et al., 2012; Berezina & Martelli, 2015).

2.1.3 Biosynthesis of Medium Chain-Length Polyhydroxyalkanoates

The imbalance in nutrient provisions, such as oxygen, nitrogen, phosphorus, sulphur and magnesium forced the bacteria to excess carbon intake by polymerization into PHA within the cells. PHA accumulation as carbon assimilation route acts as energy reservoir during the times when common carbon source is deficient (Bugnicourt et al., 2014). The carbon source that is fed into the bacterial culture may include alkanes, alkenes, alcohols, and carbohydrates instead of fatty acids, and they affect the polymer structure, quantity and quality (Gumel et al., 2014; Bassas-Galia et al., 2015).

Most common cultivation methods used are fermentation by parameter-regulated bioreactor and shake-flask fermentation (Annuar et al., 2008a). Bioreactor setups include fed-batch fermentation, continuously stirred tank reactor, sequencing batch reactor and partition reactor (Madison & Huisman, 1999). The range of bacteria utilized as reported in numerous literatures are diverse including *Alcaligenes* sp., *Pseudomonas*

sp., *Enterobacter* sp., *Necator* sp., *Rhodobacter* sp., *Ralstonia* sp., and *Cupriavidus* sp. (Annuar et al., 2007; Gumel et al., 2012a).

PHA fermentation can be performed in two stages. The idea is to proliferate and grow the bacterial culture in sufficient density during the first stage without nutrient limitation. In the second stage of fermentation, the culture is dedicated for the production of PHA in nutrient-limited condition. The cells in the second stage are almost constant in number but increased in terms of cellular size due to accumulation and storage of intracellular PHA (Chee et al., 2010; Keshavarz & Roy, 2010; Gumel et al., 2012b). An example of the two-stage fermentation by shake-flask method is the application of nutrient-rich medium for the first stage, and minimal medium for the latter stage (Gumel et al., 2014).

Many types of bacterial species were prospected and isolated in search of diversity in PHA accumulation and identities. Production of mcl-PHA, for instance, is very challenging because of low accumulation level, and the limitation in substrate utilization across species (Gumel et al., 2012b).

However, the production of mcl-PHA among *Pseudomonas* sp. are generally known to biosynthesize intracellular PHA under nutrient-limited condition, and in the presence of abundant carbon source, typically fatty acids (Annuar et al., 2008b). Notwithstanding, *Pseudomonas* species that accumulate mcl-PHA as growth-related phenomenon have also been reported (Gumel et al., 2014). They are active in preferably producing C_8 – C_{10} monomer, and made several passes through β-oxidation pathway a necessity when long chain fatty acids are metabolized for PHA accumulation (Madison & Huisman, 1999). Wild-type *Pseudomonas* sp. is widely utilized as mcl-PHA production model organism, for instance *P. putida* Bet001 (Gumel et al., 2014). The consumption of specific types of fatty acids will determine whether the cells

produce even or odd-numbered monomer, and assemble saturated and/or unsaturated PHA monomers (Gumel et al., 2014).

2.1.4 Polyhydroxyalkanoates Granules

PHA granules formed intercellular i.e. enclosed within the cell wall of producing microbes. The granules formed within the bacterial cells are designated as carbonosome, in the analogy of carboxysomes and magnetosomes. Native PHA is composed of pure PHA polymer with small amounts of phospholipid layer and other proteins (Jendrossek, 2009). Studies have reported that the coating encasing the polymer is made up of phospholipid monolayer (Zinn et al., 2001). Amorphous structure of PHA is established as partially crystalline in terms of T_g of the amorphous phase and T_m of the crystalline phase (Marschessault et al., 1990; Preusting et al., 1990; Sudesh et al., 2000; Rai et al., 2011).

PHA granules-associated proteins have their own roles. Apart from being compositional with phospholipid monolayer, they have a prominent contribution in accumulation process. They can be classified into four types: (i) the PHA polymerases or *PhaC* that are only activated when localized on the surface of granule for functioning, (ii) PHA depolymerases or *PhaZ* for PHA degradation in the instance of starvation and mobilization, (iii) phasins or *PhaP* as stabilizing protein, and (iv) some other proteins that are yet to be ascertained their functionalities (Zinn et al., 2001). Granule-associated protein, *PhaP*, is attracting attention for its major responsibility to determine the surface-to-volume ratio of PHA granules. It also avoids the affiliation of the hydrophobic granules within the cells to the hydrophilic cytosol of cytoplasm by the formation of the protein boundary (Rehm, 2003; Grage et al., 2009; Jendrossek, 2009).

2.1.5 Polyhydroxyalkanoates Recovery and Purification by Solvent Extraction

Biosynthesized PHA polymer eventually needs to be recovered in its pure form for further utilization. However, this step has a pertinent impact on the production cost industrially. Therefore, development of high purity recovery method along with least degradation of PHA polymer along the way is crucial for a large-scale production. A significant number of techniques have been established for recovery and purification of PHA from microbial cultures.

The most common approach in PHA extraction is the non-mechanical methods of utilizing diverse kind of organic solvents. Types of solvents used differ accordingly to the type of PHA to be extracted and bacterial strain. The typical solvents applied are chloroform and acetone. Chloroform is known to purify PHA sample without the risk of degradation (Kunasundari & Sudesh, 2011; Gumel et al., 2012a). The dry cell biomass is refluxed in the solvent at specific temperature for a specified period, and filtered to obtain the PHA solution filtrate. Then, the filtrate is concentrated using solvent evaporator (Gumel et al., 2012b). Non-solvent such as methanol and ethanol is commonly used to precipitate the PHA out of the concentrated filtrate (Kunasundari & Sudesh, 2011).

One of the advantages of solvent extraction is the removal of cellular endotoxin of Gram-negative bacteria hence made it practical for medical applications. PHA extracted also has high purity level with high molecular weight, alongside with insignificant degree of degradation of the polymer. On the other hand, the solvent can be recycled many times for the same purpose (Kunasundari & Sudesh, 2011; Gumel et al., 2012a; Mohammadi & Ghaffari-Moghaddam, 2015).

However, there is some drawback of this method. It is known to be environmentally hazardous. In addition, the consumption of a large amount of solvent that is toxic and

volatile leads to high operational cost. The process is also tedious, consisting many steps and consume time. Due to the usage of the solvent in modifying the permeability of the cell, disarrangement of the native order of PHA chains is made possible (Kunasundari & Sudesh, 2011).

2.1.6 Physical and Mechanical Properties of PHA

Generally, the physical and material properties of PHA are determined by the chemical structure and composition of the homo- or copolyester of hydroxyl fatty acid (Bugnicourt et al., 2014) which is the monomer composition, namely the pendant group that stretch out from the polymer backbone, as well as the chemical nature of the particular pendant group and the distance between the ester linkages of the polymer (Visakh, 2015). In addition, selective behavior of the producer microbes to the substrate resulted in only hydrophobic polymer being produced during PHA biosynthesis (Hazer et al., 2012).

The structures of mcl-PHA are much more diverse compared to scl-PHA, hence flexibility in customizing their physical and mechanical properties that may fit desirable attributes according to highly specific applications. Their molecular weights ranging from 60,000 to 412,000 Da, which are relatively low compared to scl-PHA, for both saturated and unsaturated pendant group (Rai et al., 2011). The studies on PHA were mostly conducted on PHB, a basic PHA in the form of short length chain with 4 carbon atoms as it is the best characterized and common in the PHA family (Gumel et al., 2013; Bugnicourt et al., 2014). Some properties of PHA are represented in Table 2.3 and Table 2.4.

Table 2.3: Range of typical properties of PHA (Bugnicourt et al., 2014)

*Property (Units)	Values
T_g [°C]	2
T_m [°C]	160-175
X_{cr} [%]	40-60
E [GPa]	1-2
σ [MPa]	15-40
ε [%]	1-15
WVTR [g·mm/m^2·day]	2.36
OTR [cc·mm/m^2·day]	55.12

*T_g: Glass transition temperature; T_m: Melting temperature; X_{cr}: Crystallinity degree; E: Young's Modulus; σ: Tensile strength; ε: Elongation at break; WVTR: Water vapor transmission rate; OTR: Oxygen transmission rate

Table 2.4: Comparison for properties of scl-PHA (P(3HB-*co*-3HV)) and mcl-PHA (P(3HHx-*co*-3HO-*co*-3HD-*co*-3HDD)) (Rehm, 2007)

**Property (Units)	scl-PHA	mcl-PHA
T_g [°C]	2	-36
T_m [°C]	177	61
X_{cr} [%]	70	30
ε [%]	5	300

**T_g : Glass transition temperature; T_m: Melting temperature; X_{cr}: Crystallinity degree; ε: Elongation at break

Among mcl-PHA species, differences in terms of crystallinity from distinct pendant group and their irregularity impair the ability for close packing array (Rai et al., 2011). Another possibility is the isotactic and syndiotactic nature of polymers contributing to the crystalline structure since the axial geometry of a chain is a major influence in forming crystallite and being optically active (Preusting et al., 1990; Reddy et al., 2003). Different crystallinity properties are observed in several different kinds of PHA ranging from scl-PHA to mcl-PHA with declining crystallinity in PHA consisting longer pendant group (Madison & Huisman, 1999). The melting temperature T_m of a particular PHA is obtained for determination of crystalline phase, and glass transition temperature T_g is for the amorphous phase determination (Rai et al., 2011).

On the other hand, durability attributes are highly considered in PHA physical studies. Mechanical properties such as tensile strength (σ) for toughness, Young's modulus (E) for stiffness and elongation to break (ε) for flexibility percentage were introduced for sturdiness determination purpose (Rai et al., 2011).

2.1.7 Biodegradability

The prominent attribute that attracts attentions is the biodegradability of PHA. The confidence in replacing conventional plastic with PHA is extensively expressed through diverse publications and literature. Since the potential application of PHA is very substantial, several degradation factors are taken into consideration focusing on the environmental factors and enzymatic degradation. Degradation process is directly correlated with the chemical structure especially the side chains, orientation and morphological properties (Mochizuki & Hirami, 1997). The degree of degradation is reduced with higher order structures, determined by crystallinity analysis (Rai et al., 2011). Usually, the process will ultimately form water-soluble monomer and oligomer as byproducts (Sudesh et al., 2000). Further degradation by microbes in aerobic condition produce carbon dioxide and water, and anaerobic condition liberate carbon dioxide and methane from PHA (Reddy et al., 2003).

Abiotic factors for example temperature, moisture level, pH and nutrient supply, also play important roles in the breakdown of the biopolymer. However, the extent of degradation is directly related to PHA material properties typically represented by the composition, crystallinity, additives, and surface area (Sudesh et al., 2000). Generally, higher crystallinity contributes to higher T_m hence decline in degradability.

Enzymatic degradation is another factor that assists in hydrolysis of biodegradable compound. The role of microbes is pertinent here, to introduce enzymes such as PHA depolymerases and PHA hydrolases (Madison & Huisman, 1999). The product will eventually be utilized as the carbon source for the microbe (Reddy et al., 2003). Different compositional elements are expected to exert different degradability for different PHA species due to dissimilarity in physicochemical properties. Among most significant factors are stereoregularity of PHA that only allow enzymatic hydrolysis on monomer in *R*-configuration with ester linkages, perfection of crystalline phase of PHA thus having reduced degradability, relative strenuousness for higher molecular mass PHA to break down compared to PHA with lower molecular mass, and the monomeric composition of homopolymer compared to copolymer (Jendrossek et al., 1996; Braunegg et al., 1998; Sudesh et al., 2000; Castilho et al., 2009; Rai, 2010).

2.1.8 Biocompatibility

Another important feature of PHA is biocompatibility. The wide potential of PHA in medical area demands the development of their practicality. The candidates for biocompatible polymers need to be non-toxic, mostly not producing any toxic and harmful compound upon degradation (Reddy et al., 2003; Lao et al., 2007a). In tissue engineering, for instance, the effects of cellular behavior towards the degradation product(s) of PHA after implantation are a great concern for biocompatibility evaluation (Rai et al., 2011).

The polymer utilization in medical area required more elastomeric polymers, which are prominently diverse in PHA copolymers and mcl-PHA species since scl-PHA species are known for their limitations such as the brittleness and stiffness properties (Braunegg et al., 1998; Rai et al., 2011; Bassas-Galia et al., 2015). For that, mcl-PHA is

typically more biocompatible than scl-PHA. Further modifications of PHA were taken to include varying the monomer constituent to form copolymer and surface modification to alter the properties for improved biocompatibility (Chen & Wu, 2005; Berezina & Martelli, 2015).

2.2 Functionalization of PHA

PHA is envisaged to replace conventional plastics. On the other hand, the roles of PHA in medical application are also extensive. However, scientists realized the shortcomings of raw PHA produced by bacteria and started to explore and eventually design methods to modify the raw material to obtain desirable characteristic, therefore their utilizations are made possible to the other levels. The processes in altering PHA molecular structure to produce specific characteristics for targeted usage are known as functionalization of PHA, typically related to some techniques including chemical modification, physical modification, and enzymes modification (Gumel et al., 2015b).

Chemical functionalization for instance, is one of the modification methods consisting several specific methods in the attempt to chemically alter PHA macromolecule. This approach offers a uniform and bulk production of the functionalized PHA (Mohammadi & Ghaffari-Moghaddam, 2015). Furthermore, it offers changes in terms of chemical group, functionality, hydrophilicity, wettability and surface charge (Lao et al., 2007a). There are a number of reports on the functionalization of PHA chemically through halogenation, carboxylation, hydroxylation, epoxidation and graft copolymerization (Gumel et al., 2015b).

2.3 Radical-mediated Reaction in Copolymerization

The objective of PHA modification is mostly for better interaction with aqueous environment, and altering its thermo-mechanical strength. The reaction could be carried out chemically, through radiation or plasma discharge methods (Gumel et al., 2015b). There are several established ways of grafting, focusing on PHA as the polymer of interest namely "grafting onto", "grafting from", and "grafting through" (Nguyen, 2008).

"Grafting onto" method involved covalent coupling of active sites along the polymer as well as the end of copolymer to be grafted. The examples of grafting onto processes include esterification of PHB and PHBV with acrylic acids by ozone treatment (Hu et al., 2003), amidation or condensation reaction between carboxylic group of PHO, PHBV and linoleic acid with amine group of chitosan (Arslan et al., 2007a), and also free-radical reaction of polystyrene consisting active peroxide group reaction with poly(β-hydroxynonaoate) (Hazer, 1994).

"Grafting from" method is another grafting mechanism of having active sites along the main polymer chain, with grafting monomer polymerized from these sites. An example is grafting reaction between chlorinated PHB and polymethymethacrylate in toluene solution, in order to allow copolymerization of monomer by using the active sites of the polymer as macroinitiator, for atom transfer radical polymerization (Arslan et al., 2007b).

"Grafting through" or macromonomer technique involves grafting mechanism of utilizing copolymerization of main macromonomer with lower molecular mass oligomer with at least one active site. This method offers more control on branching formation. Furthermore, choosing the size of macromonomer is possible post-polymerization, and the distribution along the main copolymer chain is based on the reactivity ratio of

comonomer. This mechanism can be observed in grafting PHB with methylmethacrylate, producing copolymer of polymethylmethacrylate grafted with isotactic PHB (Nguyen & Marchessault, 2005; Nguyen, 2008).

2.3.1 Types of Radical-mediated Functionalization Reaction involving Polyhydroxyalkanoates

2.3.1.1 Free Radical Polymerization (FRP)

Generally, FRP is a typical radical-mediated reaction involving functionalization of polymer. This reaction is utilized to create a brand-new macromolecule consisting more than one type of polymer within a similar backbone, assisted by a sole radical initator. Most widely used radical initiator are benzoic peroxyanhydride or benzoyl peroxide (BPO), 2,2′-azobis(2-methylpropionitrile) or azobisisobutyronitrile (AIBN), and potassium persulfate. The procedure is straightforward without the involvement of metal catalyst or ligand. Thermal decomposition of the radical component will provide active anionic radical with unpaired electron during the initiation steps. However, radical-mediated reaction utilizing cationic radical have also been reported, using triaryl sulfonium salts and induced by UV irradiation (Versace et al., 2012).

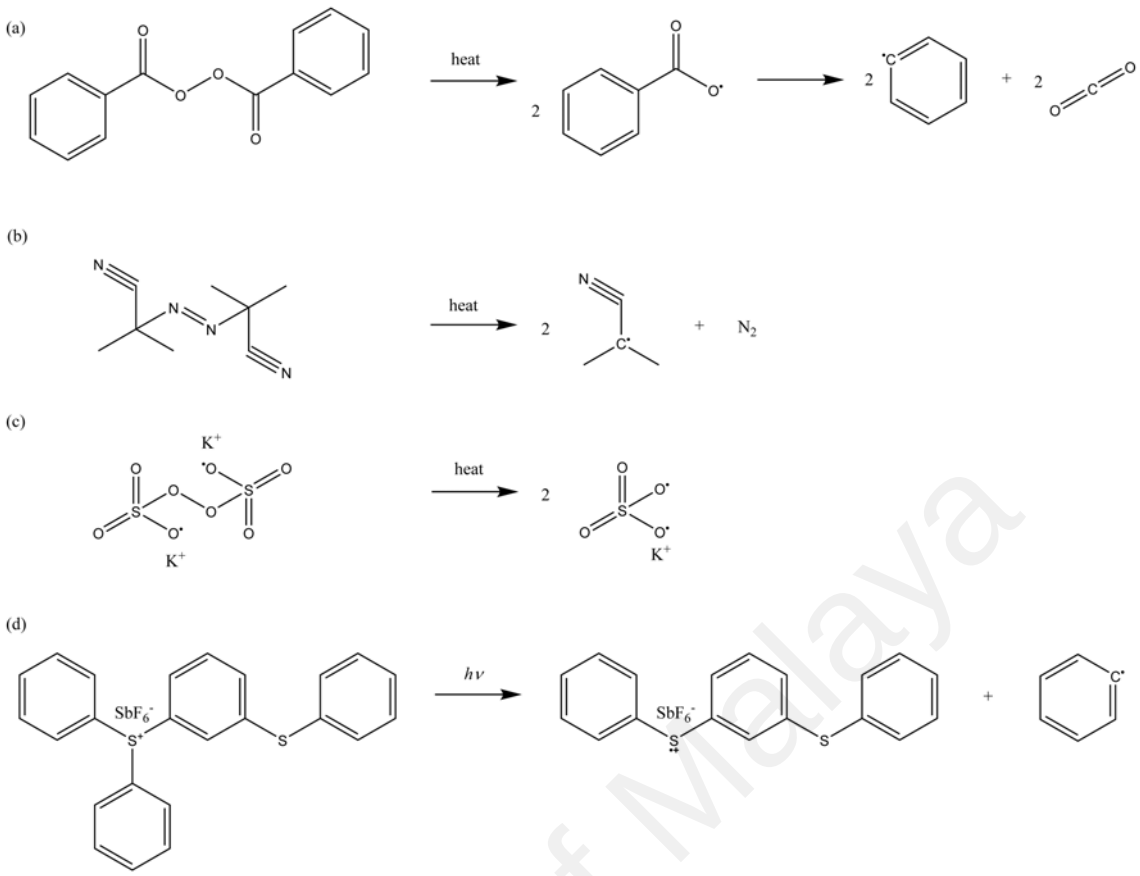


Figure 2.5: Thermal decompositions of (a) benzoyl peroxide, (b) AIBN, and (c) potassium persulfate, meanwhile irradiation-induced decomposition of (d) triaryl sulfonium salt

The mechanism for the graft copolymerization consists of three major steps, namely, initiation, propagation and termination. The mechanism can be described as an addition reaction of radicalized unit to the active site to form higher molecular mass polymer. As the reaction going, monomer concentration will decrease, as they are being linked to the polymer backbone (Stille, 1981; Jenkins & Hudson, 2001).

Initiation step begins with the radicalization of monomer and polymer substrate by the action of radical initiator, for instance, benzoyl peroxide and eventually generating radicals. Radicalized monomers later also play the role to undergo chain transfer to the backbone of the polymer. Therefore, radical formation is the process to enable the

monomer to be transferred to the main polymer backbone (Barner-Kowollik et al., 2003; Wang et al., 2007).

A particular polymer active site that was grafted with monomer will create propagation of radicalization, radicalizing other site of the backbone. This will allow another chain transfer reaction, forming successful graft copolymerization. However, radicalized monomers from the initiation step also have the ability to perform chain transfer among themselves to undergo unintended homo-polymerization process (Wang et al., 2007).

Termination step will come ultimately, resulting in graft copolymer and homopolymer as final products due to recombination process. The occurrence is influenced by several factors such as temperature, monomer concentration (Çelik, 2004), and especially by increasing radical initiator concentration which later would reduce the graft yield (Lee & Lee, 1997; Lao et al., 2007a; Wang et al., 2007). However, there are studies reporting that the radicals had no observable effect on polymer composition, for instance when micro-initiator such as AIBN is used (Jiao et al., 1985).

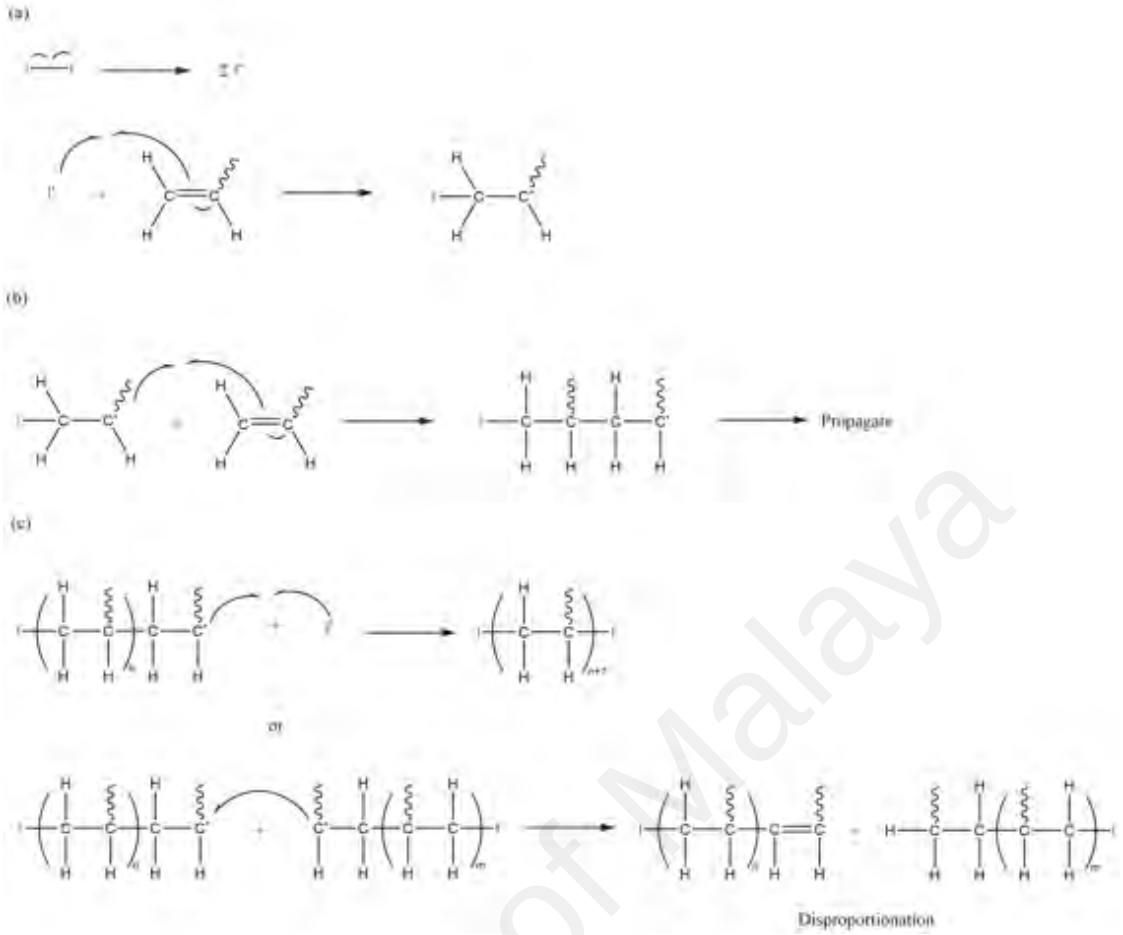


Figure 2.6: The mechanism for graft copolymerization involving radical initiator and polymer species with alkenyl end group, which consists of three major steps, namely, (a) initiation, (b) propagation and (c) termination

2.3.1.2 Atom Transfer Radical Polymerization (ATRP)

ATRP method or also known as atom transfer radical addition polymerization (ATRAP) is a reaction to create carbon-centered radical to form carbon-carbon bond. It is a controlled polymerization technique by reversible deactivation process. The process involves the abstraction of halogen species of an organic halide compound catalyzed by transition metal ligand. This reaction was first founded by Wang and Matyjaszewski (1995).

a) i) $R\text{-}X + M_t(n) \rightarrow R^{\bullet} + M_t(n+1)\text{-}X$

ii) $R^{\bullet} + M_1 \rightarrow R\text{-}M_1^{\bullet}$

iii) $R\text{-}M_1^{\bullet} + M_t(n+1)\text{-}X \rightarrow R\text{-}M_1\text{-}X + M_t(n)$

b) i) $R\text{-}M_1\text{-}X + M_t(n) \rightarrow R\text{-}M_1^{\bullet} + M_t(n+1)\text{-}X$

ii) $R\text{-}M_1^{\bullet} + M_2 \rightarrow R\text{-}M_1\text{-}M_2^{\bullet}$

iii) $R\text{-}M_1\text{-}M_2^{\bullet} \rightarrow$ Propagate

Figure 2.7: General mechanism of ATRP, (a) initiation, and (b) propagation. ***R*** = typically alkyl group; ***X*** = halogen; $\boldsymbol{M_t}$ = halogenated transition metal ligand; ***n*** = initial transition metal oxidation number; and ***M*** = monomer, adapted with permission from ACS Publications (Matyjaszewski & Xia, 2001)

The initial halogenated species, mostly with chlorine and bromine acting as initiator of the process. The transition metal catalyst, typically copper (I) salt is used to abstract halogen from the organic halide molecule to form carbon centered radical and oxidized metal-halide complex. The radicalized species will react with alkenyl group available as target monomer by addition to form carbon-carbon bonding. Meanwhile, the oxidized metal-halide complex will reduce the halogen to growing radical species, temporarily terminating the growth and reforming the original metal ligand that is able to undergo oxidation again. Then, the halogen atom on the growing species will later be abstracted again for next addition reaction. The fast reaction between carbon-centered radical and oxidized metal-halide complex avoid termination between growing macroradical, instead introducing halogen at the end (Wang & Matyjaszewski, 1995; Matyjaszewski, 1996; Matyjaszewski & Xia, 2001).

The application of ATRP in PHA functionalization was reported by Lao et al. (2011). Poly(3-hydroxybutyrate-*co*-3-hydroxyvalerate) film was investigated for its surface modification. The surface of the copolymer film first underwent aminolysis and

immobilization of ATRP initiator by using 2-bromoisobutyryl bromide. The pretreatment was carried out to produce halogenated film prior to undergoing ATRP process. The resulting product allowed for selective modification of polymer surface, which preserve the integrity of the film and its biodegradability (Lao et al., 2011).

2.3.1.3 Reversible Addition-Fragmentation Chain-Transfer Polymerization (RAFT)

RAFT is another "living" radical polymerization developed in order to have control on the chain growth during the reaction. The basis of the reaction utilizes typical free radical component such as AIBN. The presence of RAFT agent *viz.* thiocarbonylthio species such as thioesters, trithiocarbonates, xanthates, and dithiocarbamates, is key to RAFT polymerization reaction (Yeole, 2010). This method was developed by Chiefari et al. (1998) as another way of performing living free-radical polymerization to promote effectiveness and versatility in obtaining controlled copolymer molecular weight with low polydispersity index (Chiefari et al., 1998).

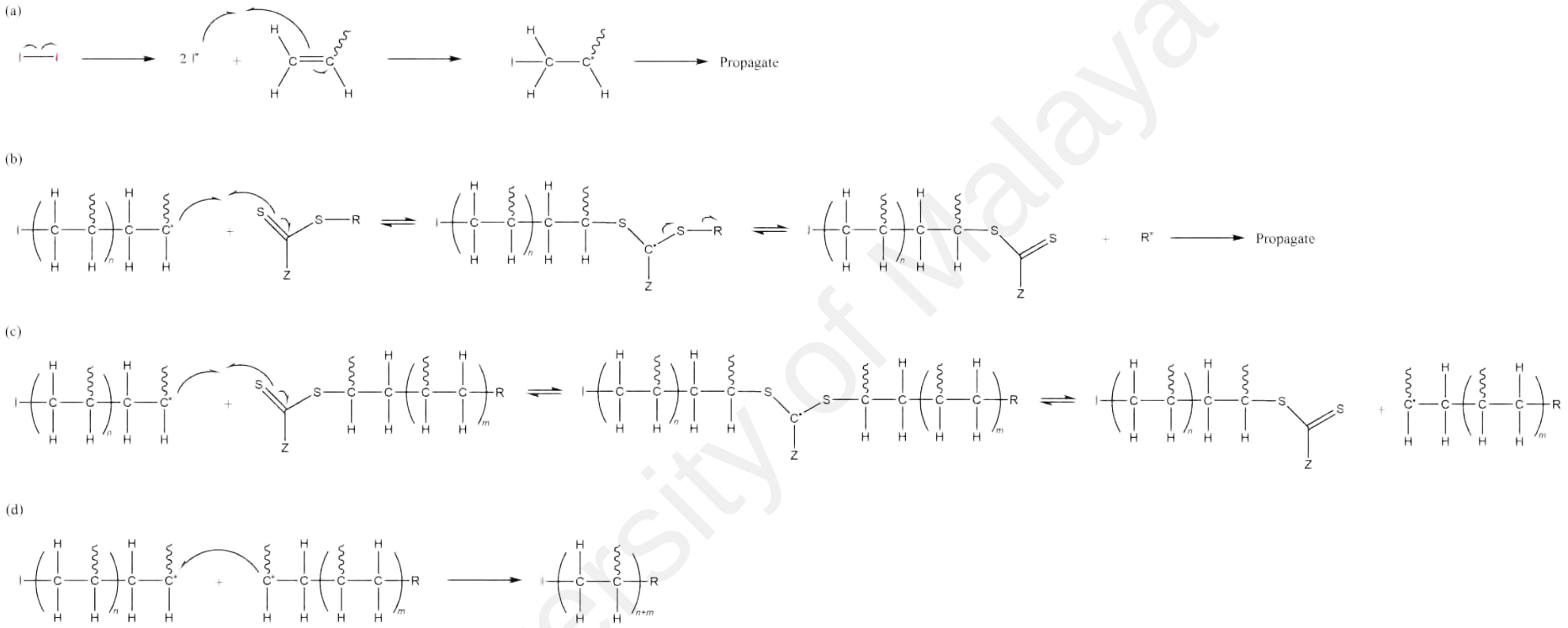


Figure 2.8: RAFT polymerization general scheme. (a) Initiation step involving radical, eventually promotes the polymer growth by propagation; (b) RAFT pre-equilibrium stage; (c) RAFT main equilibrium step; (d) termination by bimolecular reaction

The initiation step of the polymerization begins with free-radical reaction involving radical component whether azo- or peroxy- initiator introduced within the reaction milieu. The growth is then induced by conventional propagation reaction to form higher molecular mass polymer. The presence of RAFT molecule as transfer agent, a thiocarbonylthio component, employs reversible addition-fragmentation sequence in transferring the thiocarboylthio group between actively growing polymers. This particular behavior and characteristic preserve the "living" polymerization throughout the reaction period (Chiefari et al., 1998).

Application of this robust method was demonstrated in processes involving functionalization of PHA. RAFT polymerization was utilized in preparing component prior grafting with PHA samples. The reaction produces uniform oligomer of the graft component. Yao et al. (2016) reported the utilization of 4-cyano-4-(phenylcarbonothioylthio)pentanoic acid as the transfer agent in polymerizing 2-dimethylamino-ethylmethacrylate (DMAEMA) monomer by thirteen degrees of polymerization to yield oligomers with weight average molecular weight (M_w) of approximately 2,000 using AIBN as radical initiator. The oligomer is then grafted by thiol-ene click chemistry method to poly(3-hydroxydodecanoate-*co*-3-hydroxy-9-decanoate) synthesized by *Pseudomonas entomophila* LAC23 (Yao et al., 2016). On the other hand, a similar study was also conducted with 2-(dodecylthiocarbonothioylthio)-2-methylpropionic acid as chain transfer agent to produce poly(N-isopropylacrylamide) (PNIPAm) oligomer. This oligomer was later grafted to poly(3-hydroxydodecanoate-*co*-3-hydroxy-10-undecylenate), which contain unsaturated side chains, also synthesized by *Pseudomonas entomophila* LAC23 (Ma et al., 2016).

CHAPTER 3: METHODOLOGY

3.1 Two-stage Shake Flask Fermentation

Two-stage shake flask fermentation was used in cell cultivation and PHA production by *Pseudomonas putida* Bet001 (Gumel et al., 2014). Gravimetric measurement was made using Sartorius Analytical Balance ENTRIS224-1S. Medium components and chemicals for fermentation were weighed to the nearest hundredths. Pure cultures of *P. putida* Bet001 were maintained inside 1 ml of glycerol stock at -20 °C.

3.1.1 Nutrient Broth

Bacterial culture reconstitution step was carried out using the ratio of 8 g nutrient broth powder (Merck, Darmstadt, Germany) to 1 L of distilled water. Thirty milliliters in two replicates from each 125 ml conical flasks containing the broth were used. Mixing was carried out on Nuova® II magnetic hot plate stirrer (Barnstead Thermolyne, IA, USA). The media were autoclaved at 121°C for 45 minutes using autoclave SX-500 (TOMY Digital Biology Co. Ltd., Tokyo, Japan). After cooled to room temperature, each of the replicate was then added with the glycerol stock containing the pure culture. It was done in laminar flow chamber for aseptic condition, and then shaken on reciprocal shaking platform (Daihan Labtech Co. Ltd., Gyeonggi-do, Republic of Korea) at the rate of 200 rpm for 24 hours, 27 °C.

3.1.2 Nutrient Rich Medium

Bacteria growth medium or nutrient rich medium was prepared as follows: 10 g of yeast extract (Bacto, BD Bioscience, CA, USA), 15 g nutrient broth (Merck, Darmstadt, Germany), and 5 g ammonium sulphate ($(NH_4)_2SO_4$, CAS 7783-20-2) (SYSTERM, Selangor, Malaysia) were mixed in 1 L of distilled water. The mixture of 1.5 L was prepared, and evenly distributed into 15 pieces of 250 ml conical flasks containing 100 ml of the medium each, which were autoclaved.

The nutrient rich flasks were left cooled to room temperature after autoclaving. One milliliter of the activated culture prepared earlier was pipetted into each of the flasks. Culture inoculation step was carried out in laminar flow chamber for aseptic condition. The nutrient broth flasks containing the pure culture were shaken on reciprocal shaking platform model number LSI-1 (Daihan Labtech Co. Ltd., Gyeonggi-do, Republic of Korea) for 24 hours at 200 rpm and 27 °C. Later, proliferated bacteria were collected by centrifugation at 7656 × *g* for 3 minutes using centrifuge machine RC 5C Plus (Sorvall, Thermo Fisher Scientific, MA, USA). The bacterial pellet was washed and resuspended in 0.9 % (*w/v*) sterilized saline solution to maintain turgidity of the cells.

The cell concentrations were determined based on their optical densities. A cuvette containing 0.05 ml of the concentrated cells and 2.95 ml distilled water was prepared for 60 times dilution. Four cuvettes were prepared, one containing 3 ml of distilled water for blank, and triplicates containing 1 ml each of the diluted sample from the earlier cuvette and 2 ml of distilled water for 180 times dilution. Optical density measurement was carried out using spectrophotometer V-630 EHC-716 (JASCO Corp., MD, USA) at 600 nm wavelength. The mass of the cell was determined based on standard calibration of dry cell mass against absorbance.

3.1.3 Mineral Salt Medium

E2 medium or mineral salt medium was prepared to create a nutrient-limited condition for the bacteria to start accumulating intracellular mcl-PHA. The ingredients were mixed by mixing 1 L of distilled water with 3.5 g ammonium sodium phosphate dibasic tetrahydrate ($NaNH_4HPO_4 \cdot 4H_2O$, CAS 7783-13-3) (Merck, Darmstadt, Germany), 5.7 g dipotassium hydrogen phosphate (K_2HPO_4, CAS 7758-11-4) (SYSTERM, Selangor, Malaysia), and 3.7 g potassium dihydrogen phosphate (KH_2PO_4,

CAS 7778-77-0) (SYSTERM, Selangor, Malaysia). 2 L of the medium was prepared and distributed into ten pieces of 500 ml conical flasks, containing 200 ml of the solution each. Then, 1.12 g of lauric acid powder ($C_{12}H_{24}O_2$, CAS 143-07-7) (Merck, Darmstadt, Germany) was weighed and added into each of the conical flasks, as the sole carbon and energy source for the cell culture to produce medium chain-length poly-3-hydroxyalkanoates (mcl-PHA) (Gumel et al., 2012b, 2014). The flasks were autoclaved at 121 °C for 45 minutes by using autoclave SX-500 (TOMY Digital Biology Co. Ltd., Tokyo, Japan) and then left cooled to room temperature.

Other ingredients needed for the culture are magnesium sulphate heptahydrate ($MgSO_4 \cdot 7H_2O$, CAS 10034-99-8) (Merck, Darmstadt, Germany) and trace element solution. The preparation of magnesium sulphate heptahydrate was as 1.0 % (*v/v*) of 10 ml solution with distilled water. Meanwhile, trace element solution was prepared with 1.47 g of calcium chloride dihydrate ($CaCl_2 \cdot 2H_2O$, CAS 10035-04-8), 2.38 g calcium chloride hexahydrate ($CaCl_2 \cdot 6H_2O$, 7774-34-7), 0.17 g copper (II) chloride dihydrate ($CuCl_2 \cdot 2H_2O$, CAS 10125-13-0), 2.78 g iron (II) sulphate heptahydrate ($FeSO_4 \cdot 7H_2O$, CAS 7782-63-0), 1.98 g manganese (II) chloride tetrahydrate ($MnCl_2 \cdot 4H_2O$, CAS 13446-34-9), and 0.29 g zinc sulphate heptahydrate ($ZnSO_4 \cdot 7H_2O$, CAS 7446-20-0) in 100 ml of 1 M of hydrochloric acid solution.

Sterilized E2 medium flasks at room temperature were added with the following components, (i) the specified volume from the concentrated bacteria, (ii) 2 ml of 1.0 % (*v/v*) $MgSO_4 \cdot 7H_2O$, and (iii) 0.2 ml of the prepared trace elements solution. Then, the flasks were placed on orbital chaker 721 (Hotech, CA, USA) shaking platform, and left shaken at 200 rpm, 27 °C for 48 hours.

At the end of cultivation, cells from E2 medium were collected by centrifugation using centrifuge machine RC 5C Plus (Sorvall, Thermo Fisher Scientific, MA, USA)

(7656 × *g*, 3 minutes). The cell pellet was resuspended and washed twice using distilled water to remove residual carbon source and nutrients. Then, the pellets were left to dry inside heating drying oven (CONSTACE, London, UK) at 70 °C until constant dry cell mass was achieved (~ 3-5 days).

3.2 PHA Recovery

3.2.1 Solvent Reflux, Filtration and Distillation

The dried cells pellets were ground using mortar and pestle to obtain tiny particles. Two grams of ground solid were weighed and suspended in 200 ml acetone ($(CH_3)_2CO$, CAS 67-64-1) (Merck, Darmstadt, Germany) inside 250 ml round bottomed flask for reflux process. The solution was heated on stirring mantle MS-EAMS (Favorit, PLT Scientific Sdn. Bhd., Selangor, Malaysia) with temperature ranging from 70 °C to 90 °C to allow the mcl-PHA within the cells to dissolve into the acetone. The solution was later cooled to room temperature and filtered using No. 1 paper (Whatmann, GE Healthcare Life Sciences, PA, USA) to separate the cells debris from the refluxed solvent, which was collected in a 500 ml round-bottomed flask.

The solution was subjected to distillation step using water bath BM510 and rotary evaporator RE300 (Yamato Scientific Co. Ltd., Tokyo, Japan) to separate out acetone from the mixture. Concentrated yellow liquid of unpurified PHA remained at the flask bottom at the end of the distillation.

3.2.2 Purification and Casting

The concentrated polymer mass was further purified as it still contained cell residue and cultivation components carryover. The solution was pipetted using Pasteur pipette

into trident vials around 1 ml in volume. 1 ml acetone was added to the polymer, and then vortexed. Cold methanol (CH_3OH, CAS 67-56-1) (Merck, Darmstadt, Germany) at volume ratio of 3:1 to acetone was added to precipitate the solubilized polymer and left the other residues in suspension. Yellowish precipitate was observed, as the polymer is insoluble in cold methanol. Several vials were used to enable multiple sample purifications to be carried out simultaneously.

The vials containing polymer, acetone, and cold methanol were re-vortexed. In order to collect the polymer, the vials were centrifuged at 601 × *g* for 10 minutes using centrifuge machine EBA 20S (Hettich, MA, USA). The supernatant was discarded. The purification step was done repeatedly until visually clear supernatant solution was obtained.

Finally, the polymer mass was recovered from each vials through dissolution in 1 ml of acetone and then poured collectively into a glass petri dish. The purified polymer was left to dry at room temperature until constant weight was obtained (~ 3 to 4 days), and in the form of intact film.

3.3 Preparation of PHA-*g*-GDD Copolymers

P(3HHx-*co*-3HO-*co*-3HD-*co*-3-HDD) or mcl-PHA was mixed with benzoyl peroxide (BPO) ($C_4H_{10}O_4$, M_w 242.23, CAS 94-36-0), (with 25 % H_2O, used as received), (Merck Millipore, Darmstadt, Germany) and applied as a sole radical initiator for the grafting reaction. Glycerol 1,3-diglycerolate diacrylate (GDD) ($C_{15}H_{24}O_9$, CAS 60453-84-1) (Sigma-Aldrich, Saint Louis, USA). Acetone was used as reaction medium. Oxygen was purged out of the liquid acetone with nitrogen gas for ten minutes and the vial was subsequently capped to introduce airtight condition. It was incubated

within a heating block at 70 °C and 90 °C. The mixture was left to cool to ambient temperature (25 ± 1 °C) post-incubation before adding methanol to allow precipitation of the product and separate the non-grafted GDD and homo-polymerized GDD monomers at the same time. Successful grafting was indicated by the increase in the mass of precipitated product over the initial mass of mcl-PHA used (Ansari & Annuar, 2018).

Graft yield was presented in percentage (%), i.e. ratio of increased mass of PHA divided by the initial mass before reaction (Lao et al., 2007a; Kim et al., 2008) as shown by equation (3.1):

$$Graft\ yield\ (\%) = \frac{W_f - W_i}{W_i} \times 100 \qquad (3.1)$$

where W_f is the final mass of grafted PHA after reaction, and W_i is the initial mass of PHA before reaction.

3.4 Characterization of Polymer Samples

3.4.1 Gas Chromatography (GC) Analysis

The PHA content and monomer composition were determined using gas chromatography method. Four types of methyl 3-hydroxyalkanoates GC standards were used to determine the monomer concentration of mcl-PHA samples and retention time for each monomer peaks.

Table 3.1: 3-hydroxyalkanoic methyl ester standards and their retention time

Standard sample	Retention time (min)
Methyl 3-hydroxyhexanoate (C_6) (Sigma Aldrich, Germany)	4.76 ± 0.01
Methyl 3-hydroxyoctanoate (C_8) (Larodan, Sweden)	7.45 ± 0.01
Methyl 3-hydroxydecanoate (C_{10}) (Larodan, Sweden)	10.04 ± 0.00
Methyl 3-hydroxydodecanoate (C_{12}) (Larodan, Sweden)	12.39 ± 0.00

Standards were prepared in 1000 ppm as initial stock before being diluted in dichloromethane (CH_2Cl_2, CAS 75-09-2) (Merck, Darmstadt, Germany) to obtain 100, 200, 300, 400 and 500 ppm of concentrations. A standard calibration plot was constructed and a linear equation was derived for each monomer standard. Hence, the monomer concentration was calculated from the standard plot by using equation 3.2.

$$y = mx + c \tag{3.2}$$

where y is the standard monomer peak area, m is the slope of the plot, x is standard monomer concentration in ppm, and c is the y-intercept of the plot.

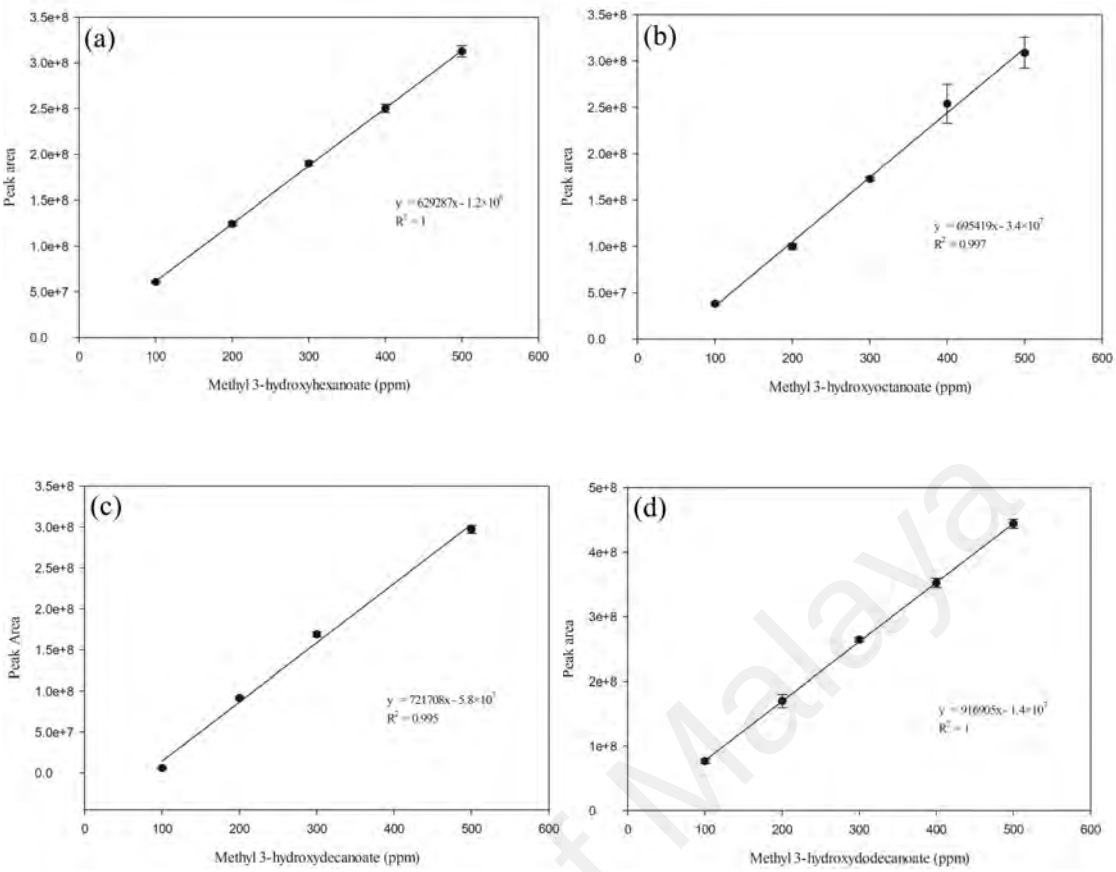


Figure 3.1: GC calibration plot for standard monomer concentration (a) Methyl 3-hydroxyhexanoate; (b) Methyl 3-hydroxyoctanoate; (c) Methyl 3-hydroxydecanoate; and (d) Methyl 3-hydroxydodecanoate

Approximately 15 to 20 mg of lyophilized cells was subjected to methanolysis. The sample was prepared in triplicate in thermal glass tubes. Two milliliters of dichloromethane (DCM) and 2 ml mixture of methanol and sulphuric acid (1:1 volume ratio) were added to the sample. The mixture was incubated at 100 °C for 2 hours and 20 minutes in the heating block HB48 (WiseTherm, Daihan Labtech Co. Ltd., Gyeonggi-do, Republic of Korea). After incubation, the sample was left to cool to room temperature. Distilled water was added and the mixture vortexed for about one minute before left overnight or at least four hours for phase separation. The organic layer at the bottom part containing the reaction products was transferred into gas chromatography

vial and mixed with sodium sulphate (Na_2SO_4, CAS 7757-82-6) (Merck, Darmstadt, Germany) to remove excess water.

The PHA concentration and monomer composition were determined using gas chromatography (GC) (Trace GC Ultra: Thermo Scientific, Rodano, Milan, Italy) equipped with flame ionization detector and a fused silica capillary column (30 m length × 0.32 mm internal diameter × 0.25 μm film) (Supelco SPBTM-1, Bellefonte, Pennsylvania, USA). During the process, helium was used as carrier gas at the rate of 48.3 ml min^{-1} and 0.41 bar pressure.

In order to calculate the mol percentage of the monomers, the concentration was obtained from equation 3.3 and used in:

$$Monomer\ molar\ concentration\ (M) = \frac{Concentration\ of\ monomers\ \ (\times 10^{-3}\ g\ L^{-1})}{Molecular\ mass\ of\ specific\ monomer\ (g\ mol^{-1})} \quad (3.3)$$

$$Mol\ percent\ (mol\%) = \frac{Specific\ monomer\ molar\ concentration}{Total\ of\ monomer\ molar\ concentration} \times 100 \quad (3.4)$$

The mcl-PHA sample was found consisting of four types of monomers, i.e. 3-hydroxyhexanoate (3HHx), 3-hydroxyoctanoate (3HO), 3-hydroxydecanoate (3HD), and 3-hydroxydodecanoate (3HDD), at 4 mol%, 37 mol%, 38 mol%, 21 mol%, respectively, hence, alternatively known as P(3HO-*co*-3HHx-*co*-3HD-*co*-3HDD).

3.4.2 Fourier Transform Infrared-Attenuated Total Reflectance (FTIR-ATR) Spectroscopy

FTIR-ATR was used to record the spectra on Spectrum 400 FT-IR and FT-NIR Spectrometer (Perkin-Elmer Inc., Wellesley, MA, USA), equipped with PIKE GladiATR hovering monolithic diamond ATR accessory (Pike Technologies Inc., USA) at room temperature. The samples were placed on the monolithic diamond ATR probe and fastened against the diamond crystal plate using force adaptor. Spectra were recorded between 4000 and 450 cm^{-1} using cuts of 0.5 cm × 0.5 cm films.

3.4.3 Simultaneous Thermal Analysis (STA) and Differential Scanning Calorimetry (DSC)

The applied thermal analysis was destructive Simultaneous Thermal Analysis (STA) of ASTM-E2550-11 thermal stability method. The machine used was STA 6000 (Perkin-Elmer Inc., Wellesley, MA, USA) running on tandem differential scanning calorimetry (DSC) and thermogravimetric analysis (TGA). The sample was prepared in the form of film. The analysis was programmed to initialize from 30 °C until 800 °C at a rate of 10 °C min^{-1} under a nitrogen gas stream of 10 ml min^{-1}.

3.4.4 Proton Nuclear Magnetic Resonance (^{1}H-NMR)

Sufficient quantity of mcl-PHA sample was dissolved in 2 mL of deuterated chloroform ($CDCl_3$, CAS 865-49-6) (Merck, Damstadt, Germany) and filtered into NMR tube using a borosilicate glass syringe equipped with 0.22 mm polytetrafluoroethylene (PTFE) disposable filter (Sartorius Stedim, Goettingen, Germany). The spectrum was acquired using a JEOL JNM-GSX 270 FT-NMR spectrometer (JOEL, Tokyo, Japan) at 400 MHz against tetramethylsilane (TMS) as

internal reference. Similar procedure was applied for the spectral measurement of grafted product.

3.4.5 Water Absorption and Porosity Studies

The samples were cut into small cubes with measured height, length and breadth, and then immersed in deionized water overnight. For porosity study, the cubes were immersed in 95 % ethanol solution for an hour before being left overnight immersed in deionized water (Kuo & Leou, 2006; Saadat et al., 2013; Ansari & Annuar, 2018). Gravimetric measurement was used to record the weight change for each of the samples in order to determine the degree of swelling from water absorption (Eq. 1). To calculate the porosity of the samples (Eq. 2), solvent replacement method was used. The calculations involved were as follows:

$$Degree\ of\ swelling\ (\%) = \frac{W_w - W_d}{W_d} \times 100 \tag{3.8}$$

$$Porosity\ (\%) = \frac{W_w - W_d}{\rho V} \times 100 \tag{3.9}$$

where W_d is the weight of the sample before immersing (dry), W_w is the weight of the sample after immersing (wet), V is the volume of the sample and ρ is the density of the solvent used, in this case, denatured 95 % ethanol, which is 0.79 g mL^{-1}.

CHAPTER 4: RESULT AND DISCUSSION

4.1 Characterization of PHA-*g*-GDD Copolymers

4.1.1 FTIR-ATR Spectroscopy

Copolymers were prepared from incubation of 50 g L^{-1} mcl-PHA and 0.14 M GDD in acetone at varying concentrations of BPO.

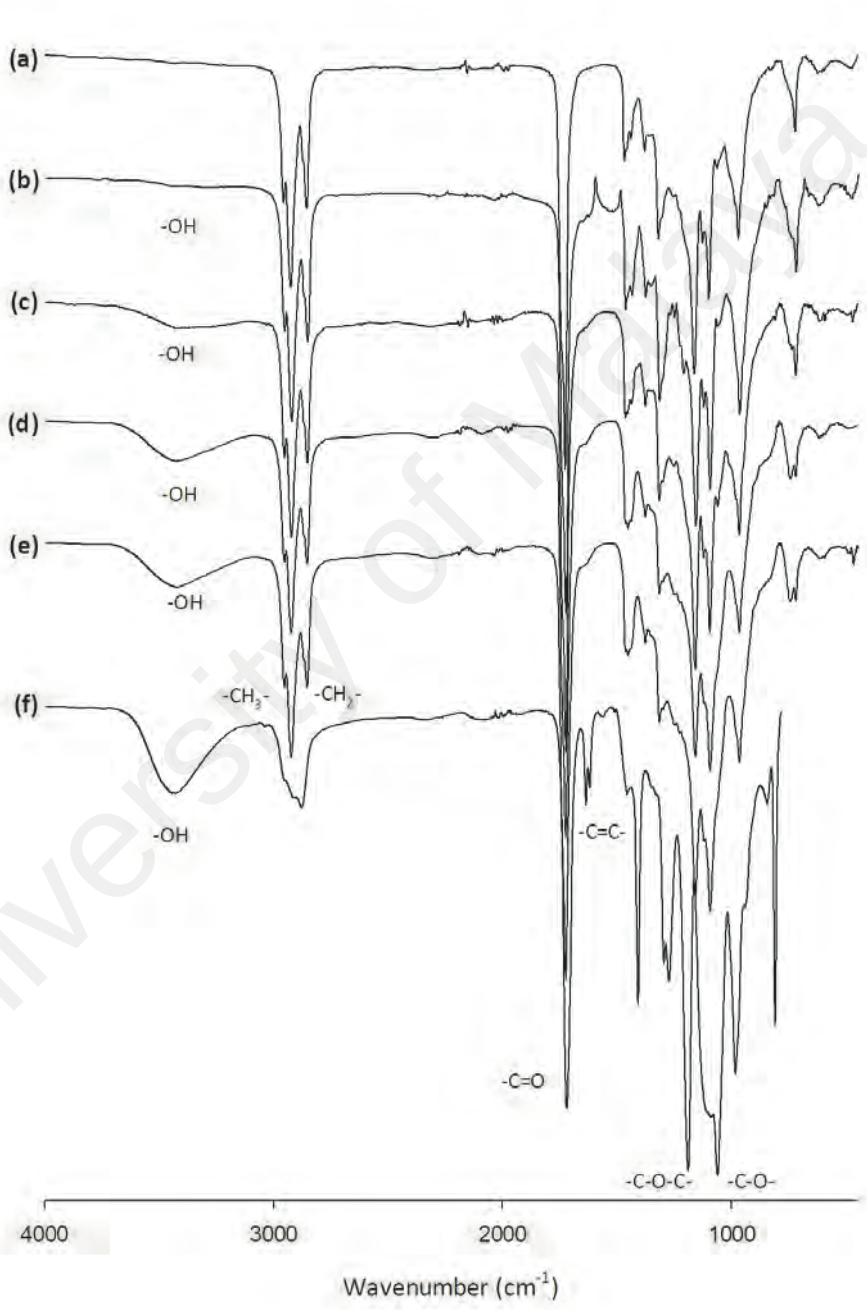


Figure 4.1: FTIR spectra of grafted materials obtained from reaction mixture at 70 °C. (a) mcl-PHA; (b) PHA-*g*-GDD with 3 mM BPO; (c) PHA-*g*-GDD with 5 mM BPO; (d) PHA-*g*-GDD with 10 mM BPO; (e) PHA-*g*-GDD with 15 mM BPO; and (f) GDD monomer

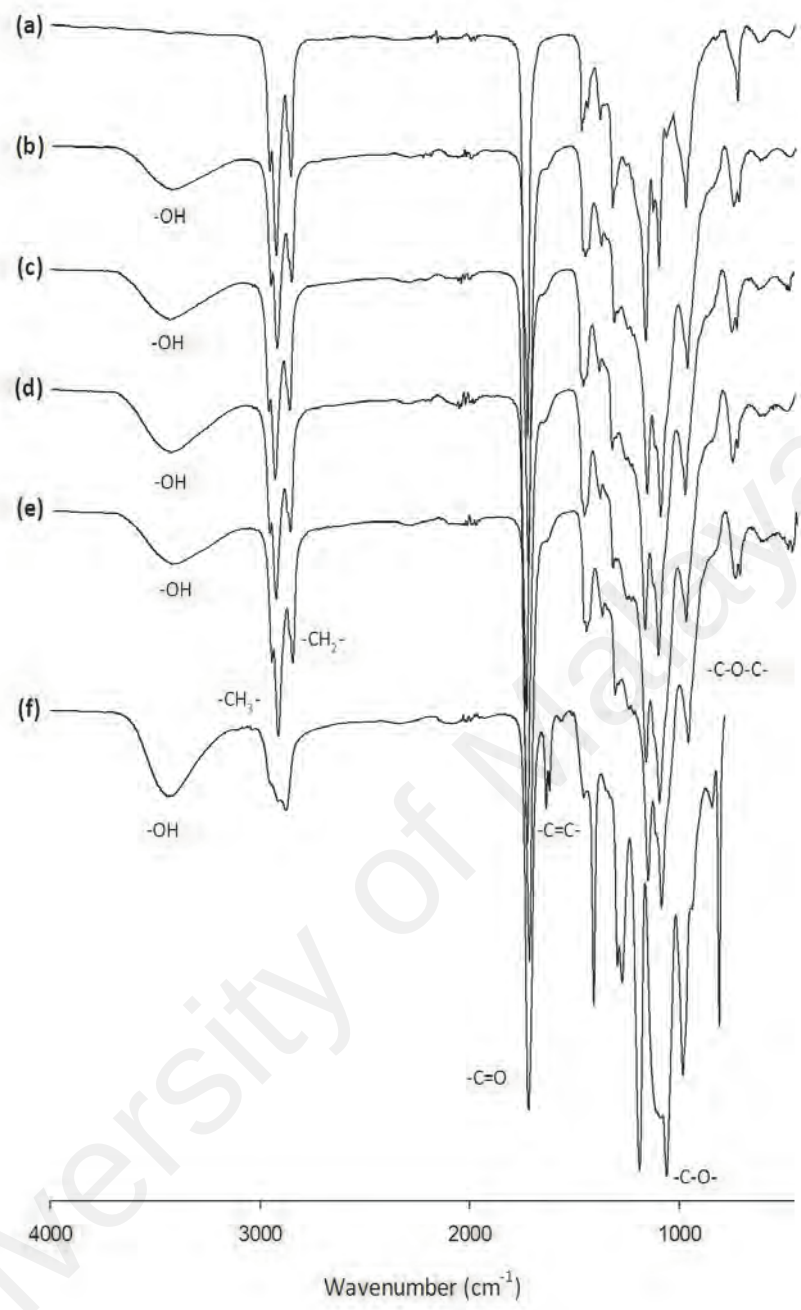


Figure 4.2: FTIR spectra of grafted materials obtained from reaction mixture at 90 °C. (a) mcl-PHA; (b) PHA-*g*-GDD with 3 mM BPO; (c) PHA-*g*-GDD with 5 mM BPO; (d) PHA-*g*-GDD with 10 mM BPO; (e) PHA-*g*-GDD with 15 mM BPO; and (f) GDD monomer

Ester vibrations were detected for both individual mcl-PHA and GDD samples. The carbonyl absorptions were observed at 1726 cm^{-1} and 1718 cm^{-1}, respectively, while the corresponding CO bond signals appeared at 1162 cm^{-1} and 1189 cm^{-1}. Meanwhile, the vibration signaling wavelength for symmetric -CH_2- of the samples were observed at

2858-2855 cm^{-1}, and 2926-2925 cm^{-1} for asymmetric -CH_3. No asymmetric -CH_3 signaling was detected in pure GDD samples (Fig. 4.1 & Fig. 4.2).

In grafted copolymer, signal from the presence of hydroxyl group introduced by the GDD monomers was evident as the copolymers exhibited new broad signals of -OH group shifted to 3432-3431 cm^{-1}. In addition, a strong shift at about 1151 cm^{-1} signifying ester bond signal, available in both mcl-PHA and GDD monomer, was present in abundant for the grafted product samples compared to the neat mcl-PHA samples. Ether bond presents exclusively in pure GDD samples was also evidenced with strong signal at about 1091 cm^{-1}. It can be concluded that grafting of mcl-PHA with GDD was successful.

4.1.2 ^{1}H NMR

The observed ^{1}H NMR spectrum for neat mcl-PHA, shown in Figure 4.3, matched previously published reports (de Rijk et al., 2005; Gumel et al., 2012b, 2014; Tan et al., 2014; Tan et al., 2015). The signal at 2.5 ppm was assigned to α-position methylene (-CH_2-) group bonded to carbonyl (-C=O) group, whereas the β-position methine (-CH) was found at 5.2 ppm (Figure 3). The remaining hydrogen atoms represent the side chain of the hydroxy fatty acid; the methylene protons were found at 1.3 ppm and the terminal methyl group (-CH_3) at 0.9 ppm (Figure 4.3).

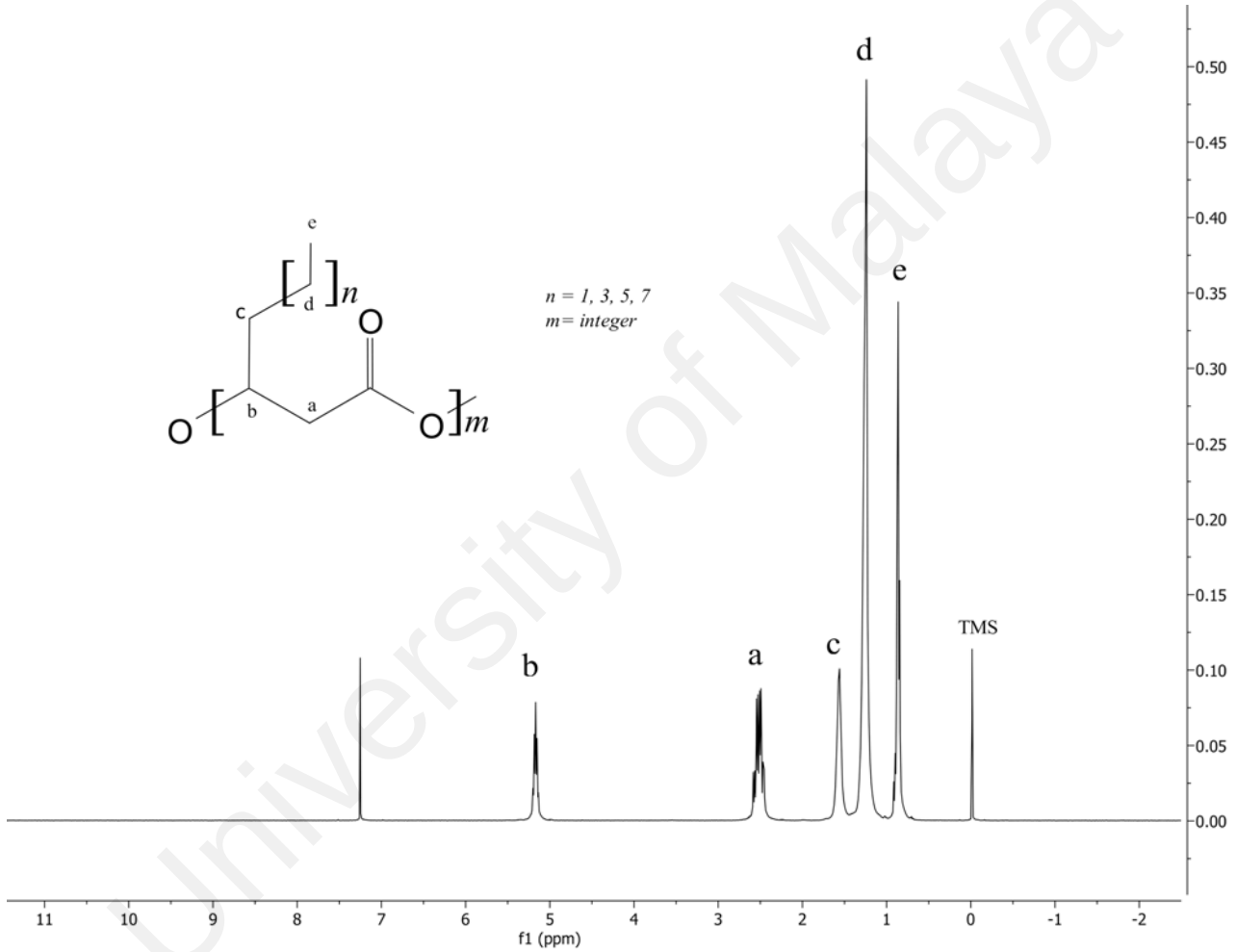


Figure 4.3: ^{1}H NMR spectrum for neat mcl-PHA

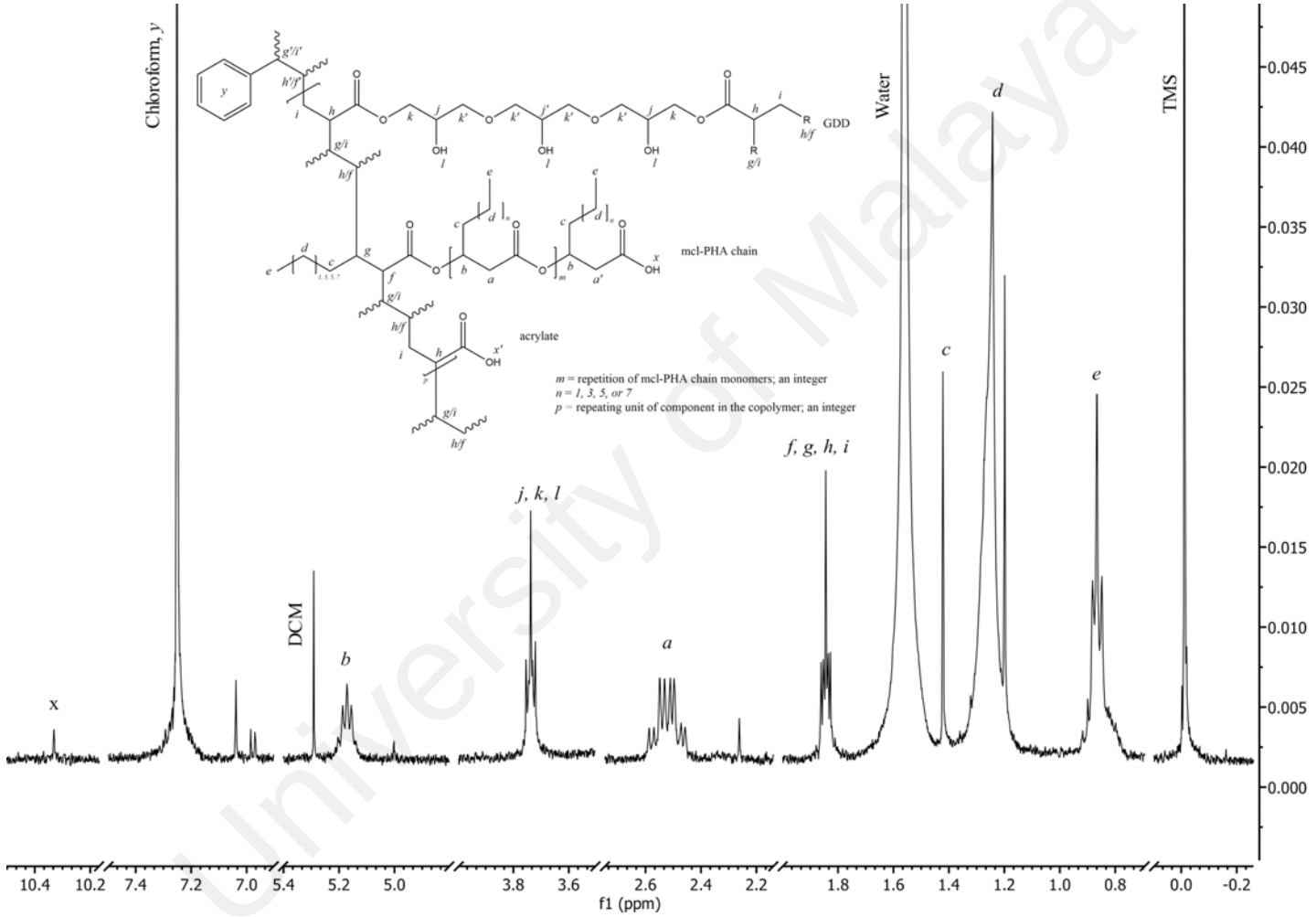


Figure 4.4: ^{1}H NMR spectrum for PHA-*g*-GDD

A representative ^{1}H NMR spectrum for the grafted products is shown in Figure 4.4. The spectrum shows all typical signals for neat PHA, comparable with those shown in Figure 4.3. The signal indicating a successful grafting process was found between 1.8 ppm to 1.9 ppm. The signal was assigned to overlapping signals of methine and methylene hydrogen atoms (-CH-) and (-CH_2-) on the backbone of polyacrylate, labelled as *h* and *i*. The peak also signifies the hydrogen atoms at the grafting position of PHA, reflecting α and β positions to the carbonyl of the terminal fatty acid of PHA, labelled as *f* and *g* in Figure 4.4, respectively. Additionally, another new signal of interest was evident between 3.7 ppm to 3.8 ppm. This was associated with hydrogens from methylene and methine groups of the triglycerol core of GDD, labelled as *j* and *k*.

Finally, a tiny signal at 10.3 ppm indicates hydrogen on a carboxyl group (-COOH), reflecting the terminal unit in PHA, which was not esterified. The position was labelled as *x* in Figure 4.4. A broad signal, overlapping with the chloroform peak at 7.3 ppm (labelled as *z*), probably indicates the presence of a benzene ring, originating from the phenylradical, which is generated by the initiator BPO and attaches to the very first monomer during the polymerization process.

4.2 Mechanism of mcl-PHA Grafting with GDD

From structural studies, a detail grafting reaction mechanism was proposed, which presented a significant improvement to previous literature (Ansari & Annuar, 2018). While the proposed reaction scheme still follows a typical three-step radical polymerization, which includes initiation, propagation and termination phases, it introduces a thorough revision of participating reactive components in the reaction.

Firstly, the grafting process involving mcl-PHA requires an unsaturated terminal monomer unit, referring to a double bond between the α- and β-carbon. This can originate from thermal degradation of mcl-PHA. There are several possibilities to

introduce the double bond *via* elimination processes, as corroborated by other similar studies (Aoyagi et al., 2002; Nguyen et al., 2002) and shown in Figure 4.5.

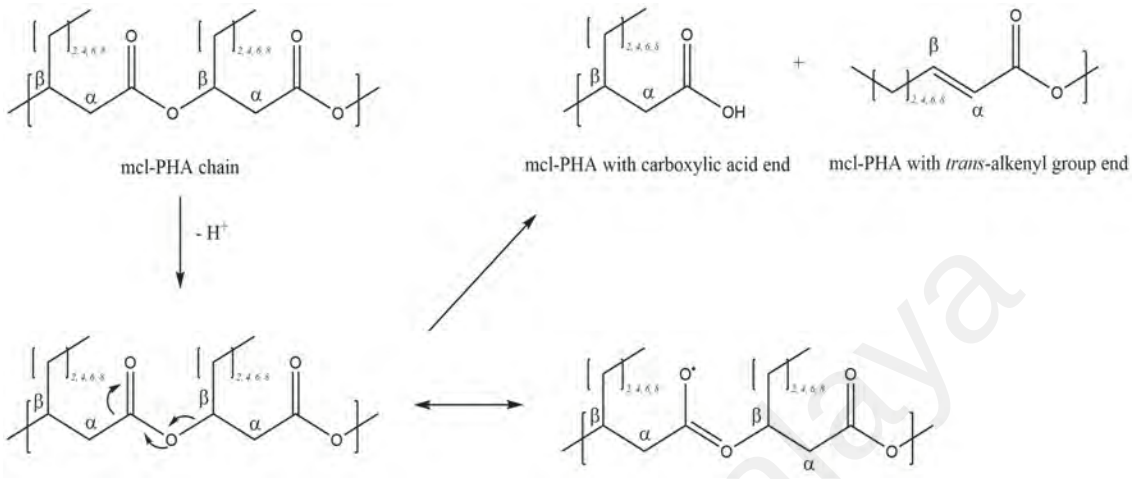


Figure 4.5: Possible routes of mcl-PHA degradation that contribute to alkenyl end group able to participate in grafting reaction *via* proton abstraction

Another side reaction that leads to additional acrylate monomers, thereby complicating the structure of the copolymer, is a (partial) hydrolysis of GDD, as shown in Figure 4.6.

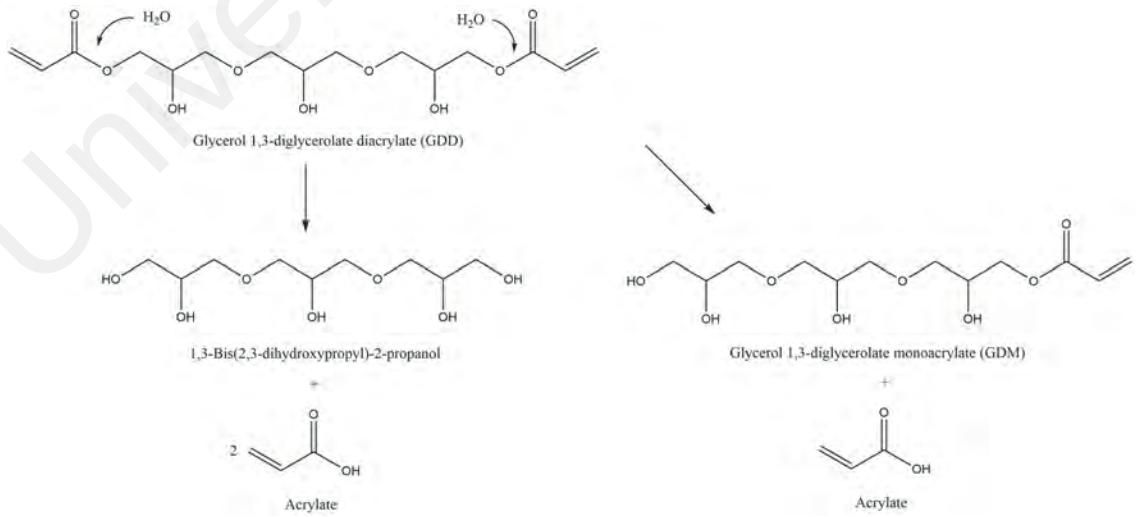


Figure 4.6: Thermal degradation of GDD to produce acrylate monomers *via* hydrolysis

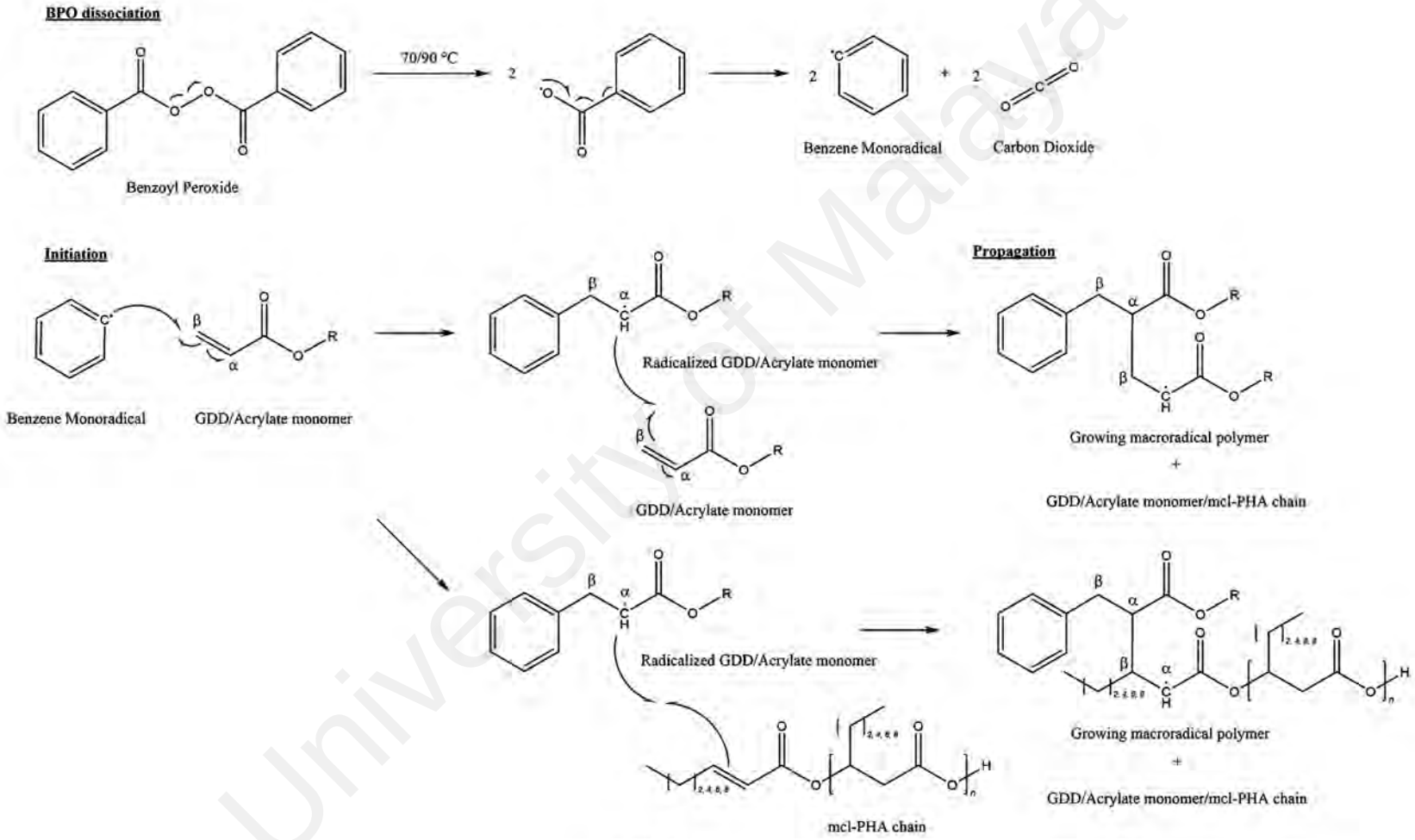


Figure 4.7: Proposed mechanism for graft copolymerization involving GDD and mcl-PHA

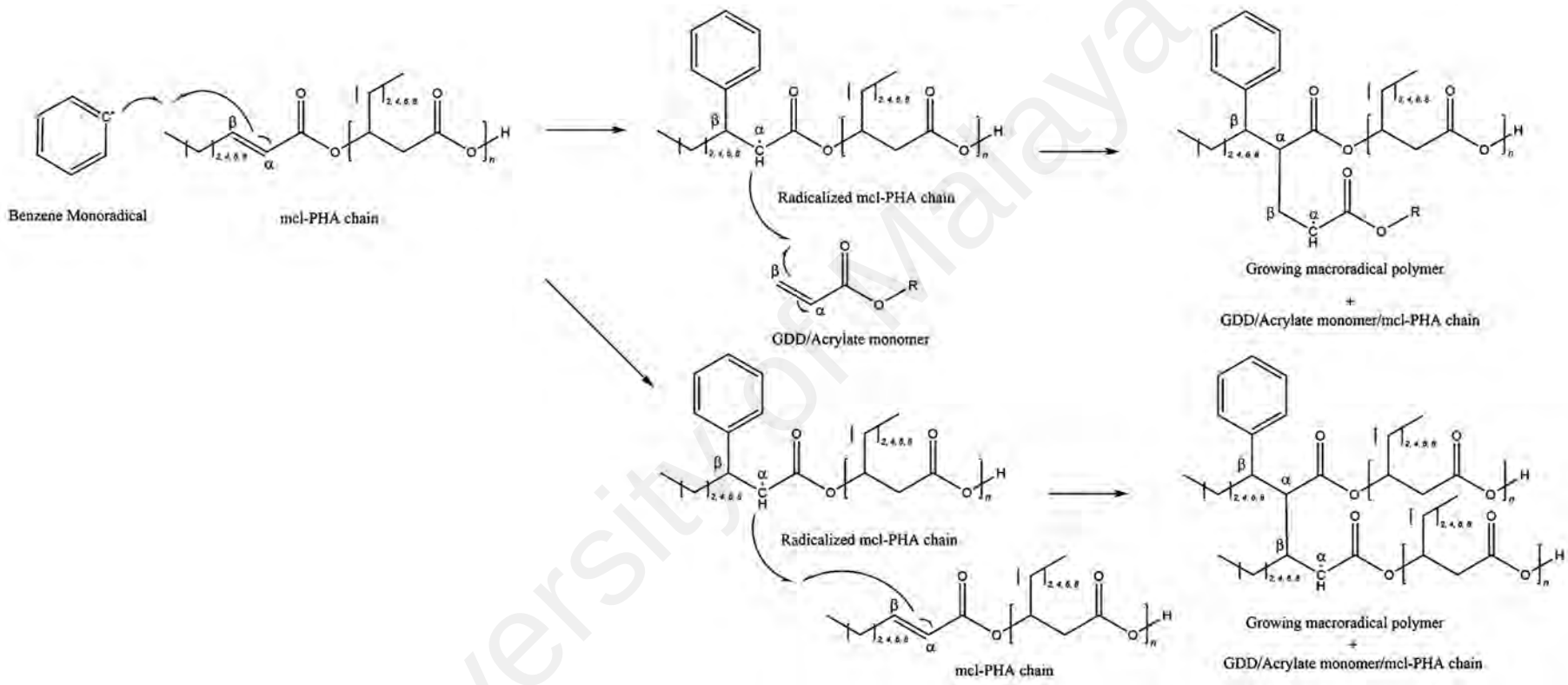


Figure 4.7, continued.

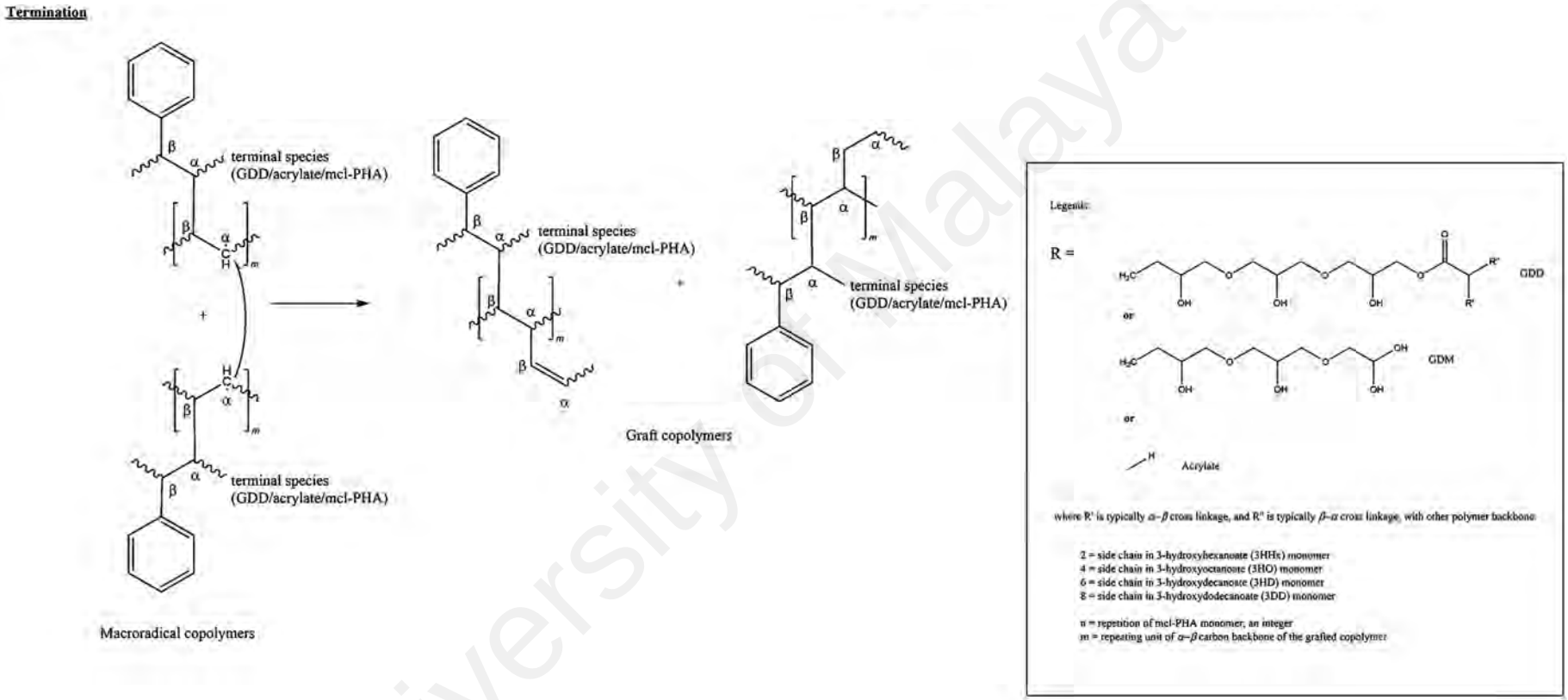


Figure 4.7, continued.

Grafting of mcl-PHA with GDD to obtain PHA-*g*-GDD copolymer starts with the dissociation of BPO into a benzene radical. As shown in Figure 4.7, it is proposed that the radicals start off the initiation step by attacking the β-carbon of the double bond to introduce a radical on the α-carbon. The reaction can happen at either end of GDD molecules. In the process, the benzene ring is covalently bonded to the monomer to become part of the terminal monomer unit of the copolymer. Although initiation could also happen at the unsaturated terminal fatty acid of mcl-PHA, the probability is small based on the low concentration density of mcl-PHA molecule compared to acrylate monomer. Nevertheless, grafting reaction with mcl-PHA does not hinder the propagation of the vinyl polymerization.

The terminal species with benzene ring plays a key role as radical to attack an alkenyl group of available GDD monomer, acrylate monomer, or mcl-PHA chain with alkenyl group. The β-carbon of the new species will be covalently bonded to the α-carbon of the next (Figure 4.8). When a radical attacks the β-carbon of an acrylic system vis-à-vis acrylates like GDD and its derivatives, and olefin-terminated PHA, the resulting radical is resonance-stabilized by the carbonyl group. The delocalization of the unpaired electron, as shown in Figure 4.8, reduces the energy of the intermediate and is responsible for the regioselective connection of α- to β-carbons (De Vleeschouwer et al., 2010). The chain process of the polymerization is continuously repeated to produce a growing macromolecule consisting of a mixture of GDD monomers, acrylate monomers and mcl-PHA with random connection pattern. The cascade of growing copolymer consistently assembles radicalized α-carbon to be bonded to β-carbon of monomers, thereby transferring the radical to the α-carbon of the newly attached monomer. Concurrently, the same process is presumably occurring at the other end of GDD monomers as well. The bivalent nature of GDD, thereby, is giving rise to a cross-

linked copolymer. This cross-linking affects the properties of the resulting gel, accounting for almost zero solubility in aqueous solution and a rigid shape.

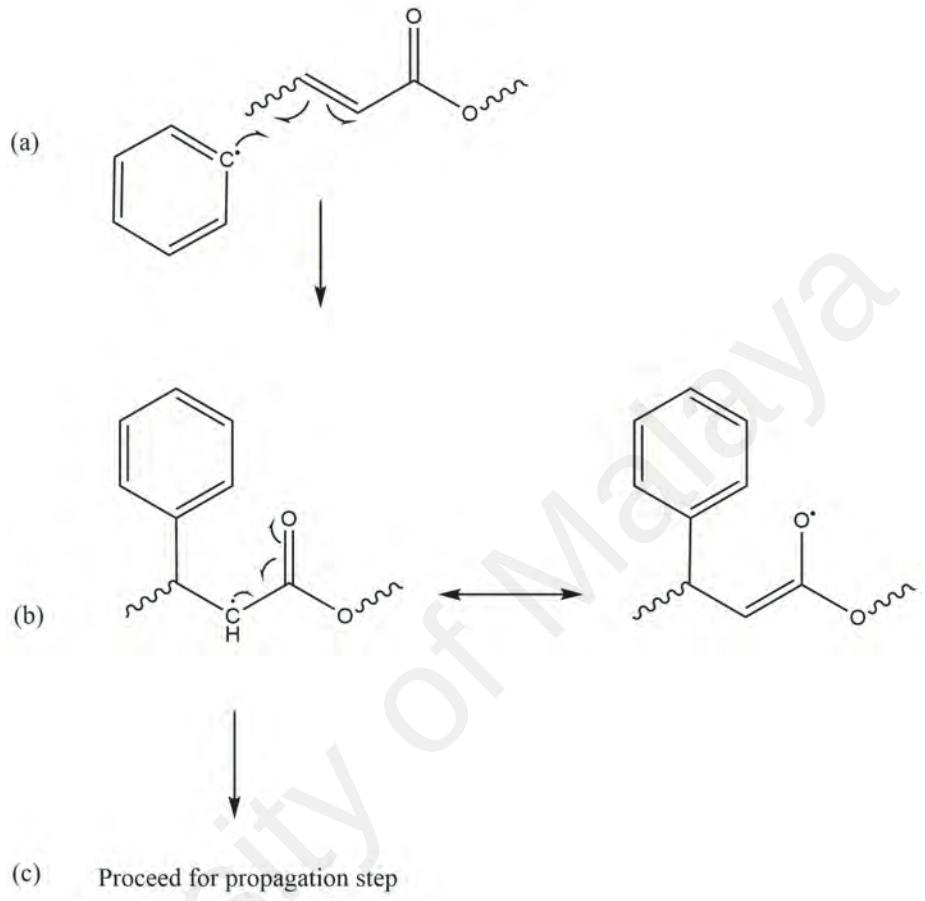


Figure 4.8: The allylic resonance involved after radical intervention on the reaction species, (a) benzene radical attacked the species with alkenyl group, (b) the allylic resonance shows the movement of the unpair electron along the bonds, and (c) entering propagation step

Termination step occurs when two different growing macroradical copolymers are reacting with each other, thereby loosing the radical character that is associated with unpaired electrons. Termination from disproportionation is more likely than combination of radicals.

During the grafting process, GDD monomers may also react with each other to form a densely cross-linked homopolymer gel, following the same mechanism that applies for

the grafting process. Owing to the divalent character of GDD and the nature of the vinyl polymerization, a high molecular weight is achieved. The crosslinking converts most of the monomers into a highly crosslinked gel, which consists of only a few interwoven polymer networks. A separation of these is practically impossible. However, GDD-polymers with low crosslinking, owing to incorporation of substantial contents of mono-valent acrylates, may be separated from grafted mcl-PHA based on its solubility in methanol, while highly cross-linked gels and polymers containing higher portions of mcl-PHA are expected to form a precipitate.

High frequency of crosslinking, due to the divalent structure of GDD, leads to a gel, which is insoluble in solvents applied for recovery and purification in this study. However, some of the grafted mcl-PHA copolymer could be dissolved and subsequently investigated for NMR studies, as reported earlier. This small portion of the copolymer is expected to contain a considerable amount of (partially) hydrolyzed GDD, which lowers the cross-linking, thereby providing a soluble copolymer of lower molecular weight.

4.3 Thermal Properties of PHA-*g*-GDD Copolymers

Thermal properties of PHA-*g*-GDD samples were determined using TGA and DSC analyses.

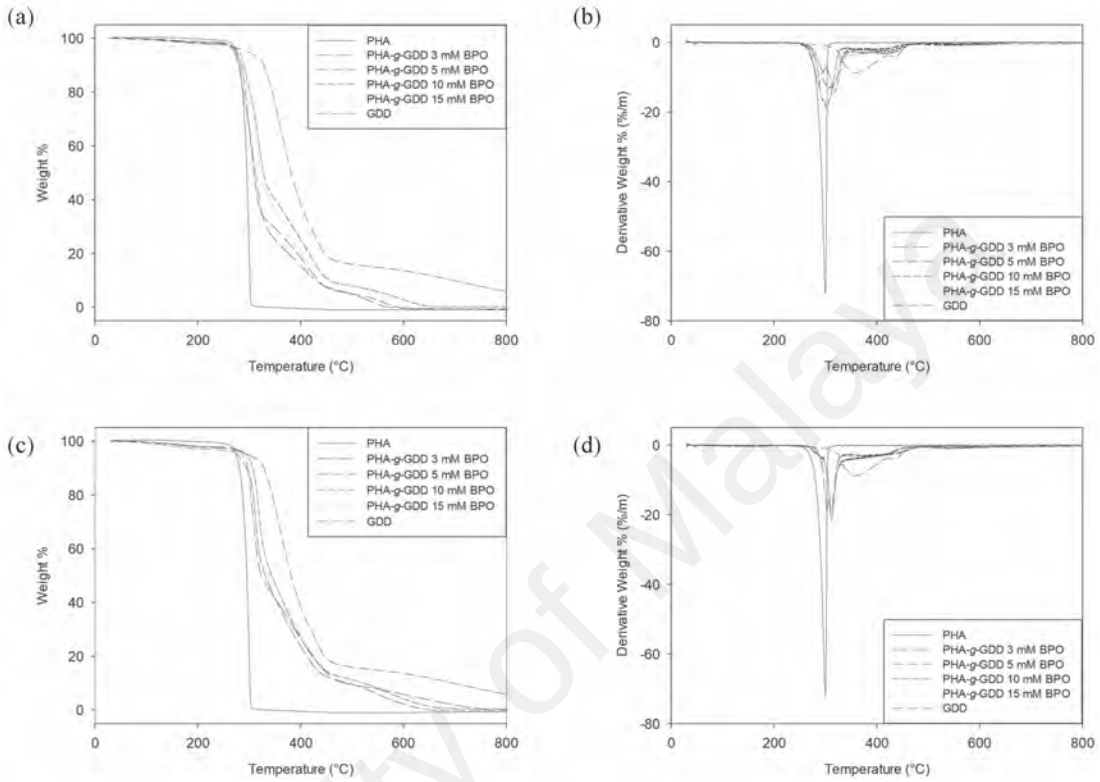


Figure 4.9: TGA analysis for PHA, PHA-*g*-GDD and GDD samples for different initiator concentrations, (a) & (c) weight percentage curves, and (b) & (d)) derivative weight percentage curves, while (a) and (b) represent samples incubated at 70 °C and (c) and (d) at 90 °C

From Figure 4.9, the grafted samples showed changes in terms of thermal degradation behavior. Neat PHA samples were degraded earlier around 260 °C compared to the rest of the samples (Figure 4.9 (a)-(d)). GDD sample was the most stable among all, with higher degradation temperature around 300 °C. The thermal curves of PHA-*g*-GDD samples from 90 °C incubation temperature were closer to the GDD curve (Figure 4.9 (c) & (d)), indicating that these samples were more stable compared to those incubated at 70 °C (Figure 4.9 (a) & (b)). The shapes of the curves were closely related due to molecular composition similarity. From DSC analysis, the

T_m of grafted samples was in the range of 53.0 °C – 55.5 °C, which was slightly higher compared to neat mcl-PHA at 52.7 °C, due to the presence of GDD monomer (Table 4.1).

Table 4.1: Thermal properties of PHA and PHA-*g*-GDD for different initiator concentrations and incubation time, with their graft yield percentages after two hours incubation, water uptake swelling and porosity percentages

Incubation temperature (°C)	Sample	Thermal Analysis		Graft yield (%)	Water uptake (swelling %)	Porosity (%)
		T_m (°C)	T_d (°C)			
	mcl-PHA	52.7	294.4	-	-	-
70	PHA-*g*-GDD					
	BPO (mM) 3	55.5	297.9	72 ± 5	2.4 ± 0.2	7 ± 1
	5	53.0	305.3	73 ± 11	4.6 ± 0.2	9 ± 1
	10	53.7	298.1	77 ± 2	8.2 ± 1.1	10 ± 1
	15	54.5	292.8	90 ± 9	6.6 ± 0.1	11 ± 1
90	PHA-*g*-GDD					
	BPO (mM) 3	53.5	295.6	73 ± 4	6.5 ± 0.9	12 ± 1
	5	54.7	295.0	86 ± 6	7.2 ± 1.0	10 ± 2
	10	53.8	291.6	88 ± 3	7.3 ± 0.4	10 ± 1
	15	53.9	298.7	85 ± 3	9.7 ± 0.5	8 ± 1

Generally, the thermograms were only slightly different from each other, since the grafting parameters *viz*. mcl-PHA concentration and GDD concentration were the same throughout the sample preparation reaction. On the other hand, the increase in initiator concentration contributed to the increment in graft yield (Table 4.1), hence resulting in more thermostable functionalized product with higher GDD concentration that were successfully grafted to the mcl-PHA backbone. The findings also agreed with the results from TGA analysis (Figure 4.9).

In terms of water absorption ability, the grafted samples exhibited increment in swelling percentage with higher initiator concentration used, although grafted samples from lower incubation temperature showed slightly less water absorption. Neat PHA material showed negligible water absorption due to its strong hydrophobicity. For samples obtained from grafting reaction at 70 °C and different initiator concentrations, approximately similar porosity percentages were determined except at the lowest concentration of BPO used (Table 4.1) attributed to lower grafting frequency. For grafted samples from 90 °C incubation, the increase in starting radical initiator concentration resulted in lower porosity percentages (Table 4.1). Neat PHA material showed no evidence of porosity. It is suggested that the increase in initial concentration of BPO and incubation temperature may have contributed to more extensive grafting of PHA that resulted in lower porosity percentages of the resulting materials.

4.4 Effects of Starting Initiator Concentration and Incubation Temperature

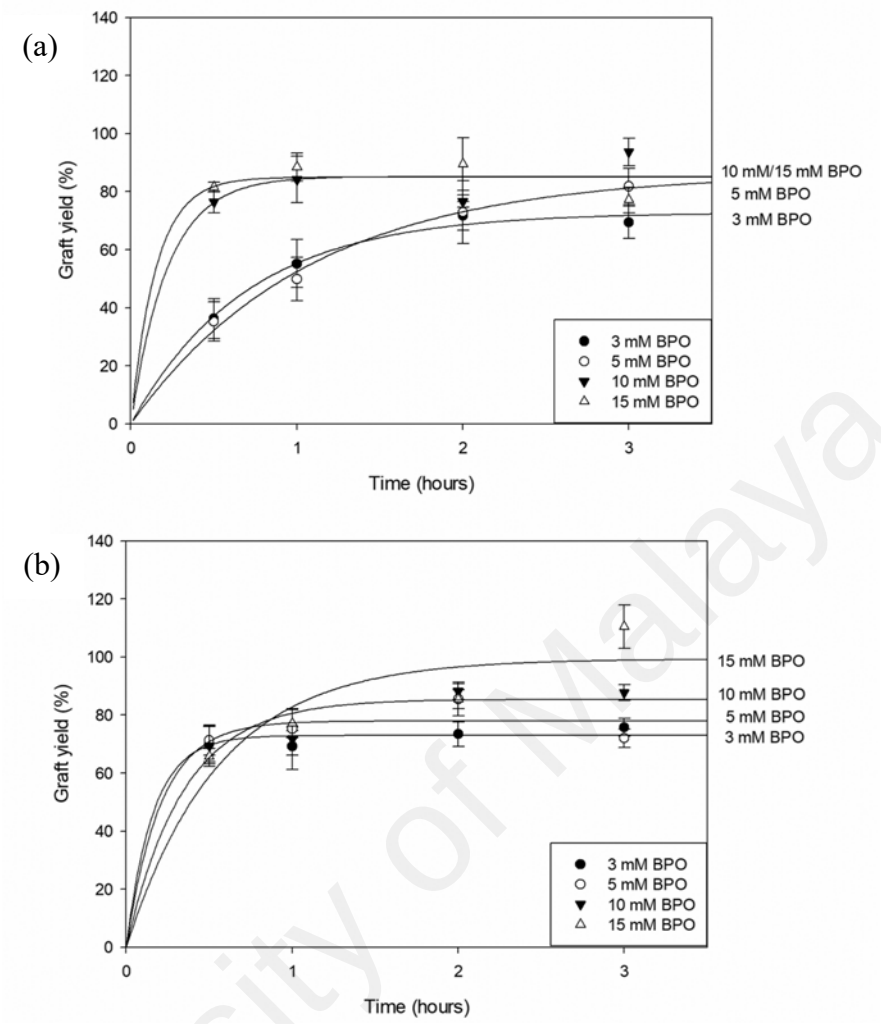


Figure 4.10: Graft yield as a function of incubation time, for (A) 70 ° and (B) 90 °C incubation temperature. Initial BPO concentrations at 3-, 5-, 10- and 15 mM for both temperatures. Initial mcl-PHA and GDD concentrations were 50 g L^{-1} and 0.14 M respectively in 4 ml of acetone

The grafting reaction of mcl-PHA with GDD made use of BPO as the sole microinitiator. Based on Figure 4.10 (a), at 70 °C incubation, graft yield (%) became higher as the starting BPO concentration was increased. Similar trend was observed in Figure 4.10 (b) for incubation temperature 90 °C. At 70 °C incubation, the rate of increase in graft yield was gradual for lower starting initiatior concentration i.e. 3- and 5 mM. When its initial concentration was elevated to 10- and 15 mM, steep increase in graft yield with time was observed (Figure 4.10 (a)). On the other hand, when

incubation temperature was at 90 °C, similar fast rate of graft yield was observed for all starting initiator concentrations used (Figure 4.10 (b)). Nevertheless, for both temperatures, the grafting reaction eventually reached a plateau indicating termination of reaction following exhaustion of grafting sites and/or depletion of radical initiator.

4.5 Effect of initial GDD concentrations

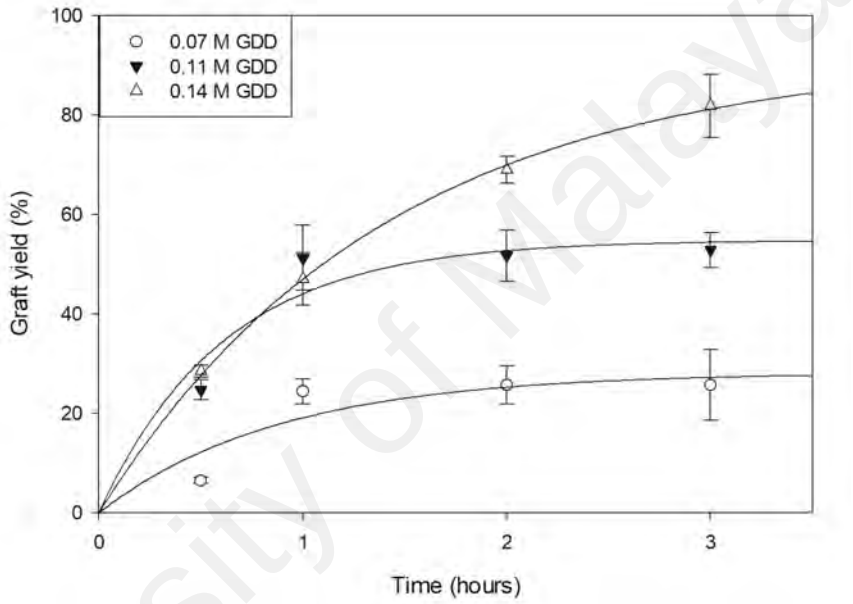


Figure 4.11: Graft yield as a function of incubation time for different GDD concentrations at 70 °C incubation. Initial mcl-PHA and BPO concentrations were 50 g L^{-1} and 10 mM respectively in 4 ml of acetone

The plots in Figure 4.11 showed the graft yields for different GDD concentrations as a function of time. Micro-initiator reaction is considered a fast one hence it is expected to enter termination phase as the concentrations of the radical and/or reactive sites started to deplete. With higher initial GDD concentration, higher graft yields were also observed. Similar trend was evident for different starting initiator concentrations as shown in Figure 4.11.

4.6 Initial Rate of Reaction

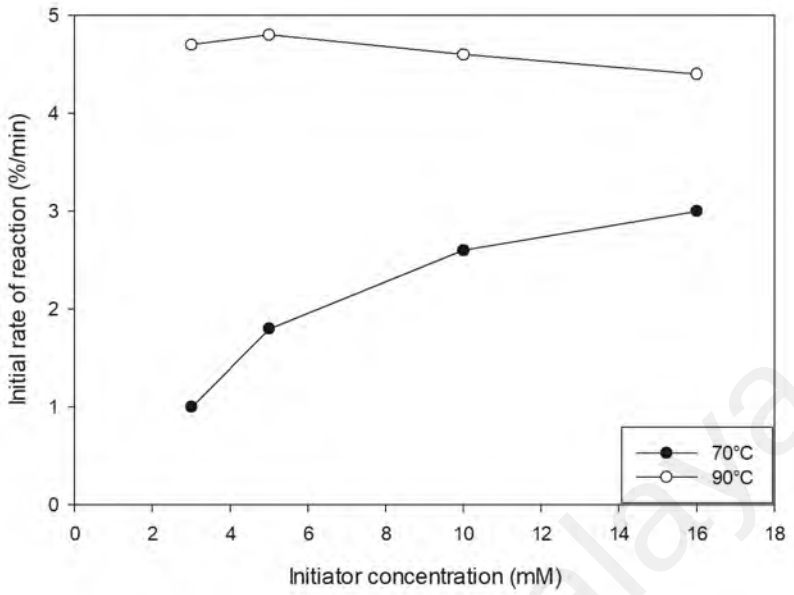


Figure 4.12: Initial rate of reaction for GDD grafting of mcl-PHA as a function of starting initiator concentration. The initial mcl-PHA and GDD concentrations were 50 g L^{-1} and 0.14 M respectively in 4 ml of acetone

Figure 4.12 showed the initial rates of grafting at two different temperatures i.e. 70 °C and 90 °C for different starting initiator concentrations. At 70 °C, the initial rate was increasing gradually from 1.0 % min^{-1} to 3.0 % min^{-1} as BPO concentration was increased, suggesting that the BPO dissociation has yet to reach its maximum level. However, at 90 °C, the initial rate of reaction was almost constant within a narrow range of 4.4 % min^{-1} to 4.8 % min^{-1} for all initial BPO concentrations studied (Figure 4.12). It is suggested that at this temperature, generation of radical initiator from its parent molecule was relatively faster than at lower temperature, hence the higher initial rate of grafting. This is supported by the fact that half-life of BPO is one hour at 91 °C.

CHAPTER 5: CONCLUSION

The graft copolymerization of mcl-PHA with GDD was successfully carried out using benzoyl peroxide as the sole micro-initiator. Elucidation of its mechanism indicates that both species could be incorporated into the same backbone of mcl-PHA polymer consisting α-β carbon linkage due to the random nature of radical polymerization involved. The grafted product yields an amphiphilic copolymer with improved wettability, thus potentially refining its facility for cellular interaction. In addition, grafting of natural biopolyester to yield P(3HO-*co*-3HHx-*co*-3HD-*co*-3HDD)-*g*-GDD add to the available repertoire of functional materials.

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1) Mohamed, S. M. D. S., Annuar M. S. M, Heidelberg. T., Ansari, N. F., & Ismail, N. H. (2019). Porous amphiphilic biogel from facile chemo-biosynthetic route. *Journal of Serbian Chemical Society, 84*(0), 1-15.