A STUDY OF THE GENOTOXICITY OF POLYHYDROXYBUTYRATE: A SYNTHETIC BIOMATERIAL

by

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In the name of Allah most Compassionate most Merciful

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BIOMATERIAL SINTETIK: KAJIAN GENOTOKSISITI KE ATAS POLIHIDROKSIBUTIRAT

ABSTRAK

Tujuan kajian ini adalah untuk memastikan genoketoksisti ke atas polihidroksibutirat (PHB) yang dihasilkan dalam bentuk fiber pendek dan padu dan dikeluarkan oleh Pusat Pengajian Sains Biologi, Universiti Sains Malaysia, Pulau Pinang, Malaysia, dengan menggunakan 3 jenis ujian yang berbeza: Ujian Kemutagenan *Salmonella* (ujian Ames), Ujian *In Vitro* kromosomal keaberanan mamalia dan analisis pernyataan gen.

PHB telah diuji pada kepekatan yang berbeza-beza (0.3125, 0.625, 1.25, 2.5 dan 5 mg/ml) dalam ketiga-tiga ujian. Untuk ujian Ames, PHB telah dieram dengan varian genotip khusus bakteria, *Salmonella typhimurium*, yang membawa mutasi dalam beberapa gen. Lima penguji strain (TA1535, TA1537, TA1538, TA98 dan TA100) telah digunakan, dengan dan tanpa menggunakan campuran sistem pengaktifan metabolik S9 dan ujian ini telah dinilai berdasarkan kepada jumlah koloni revertant. Pada masa yang sama, kawalan positif atau negatif. Serentak, kawalan negatif ujian dengang menggunakan air suling yang steril dan kawalan positif ujian dengang menggunakan 9-aminoacridine hydrochloride monohydrate (98%) and 4-Nitro-O-phenylenediamine (98%) tanpa menggunakan campuran sistem pengaktifan metabolik S9 dan 2-aminoanthracene dengan S9 telah membawa keluar. Untuk menilai keaberanan kromosom, garis sel osteoblas manusia (HOS, CRL-1543) dari jenis sel kultur Amerika telah didedahkan kepada PHB, air suling yang steril (kawalan negatif) dan Mitomisin (kawalan positif), dengan dan tanpa menggunakan campuran sistem pengaktifan metabolik S9. Untuk mengkaji analisis pernyataan gen, garis sel fibroblas MRC-5 telah dirawat dengan PHB pada kepekatan yang berbeza dan dieram untuk 1, 12, 24 dan 48 jam secara berasingan untuk setiap

kepekatan. Jumlah RNA diasingkan dan dianalisa untuk pernyataan gen-gen p53, c-myc, bcl-xl dan bcl-xs.

Keputusan ujian Ames menunjukkan bahawa jumlah purata koloni-koloni revertant per plat yang dirawat dengan PHB (min koloni-koloni revertant adalah dalam lingkungan 28 hingga 344 koloni/plat tanpa S9 dan dari 150 hingga 499 koloni/plat dengan S9) adalah kurang dari sekali ganda jika dibandingkan dengan yang kawalan negatif (147 hingga 346 koloni/plat tanpa S9 dan 340 hingga 443 koloni/plat dengan S9). Ketiadaan penambahan bilangan koloni-koloni secara sekurang-kurangnya sekali ganda dengan bahan ujian menandakan yang PHB adalah tidak mutagenan.Untuk ujian keaberanan kromosom, tidak terdapat petunjuk sebarang mutagenan disebabkan oleh PHB pada garis sel HOS seperti yang ditunjukkan oleh nilai indeks mitotik (min indeks-indeks mitotik adalah dalam lingkungan 3.55(0.06) hingga 4.95 (0.77) peratus tanpa S9 dan dari 3.10 (0.14) hingga 5.20 (0.98) peratus dengan S9) juga tidak terdapat keaberanan kromosom. Ini menunjukkan yang PHB adalah tidak sitotoksik dan tidak menghasilkan keaberanan kromosom pada garis sel HOS. Dalam analisis pernyataan gen, garis sel fibroblas (MRC-5) yang dirawat dengan PHB pada kepekatan yang berbeza dan pada tempoh masa berbeza menunjukkan peningkatan di atas atau di bawah penyataan penurunan pada gen-gen p53 (kedekut IDV banjaran dari 36100 hingga 36295), c-myc (kedekut IDV banjaran dari 33110 hingga 33270), bcl-xl (kedekut IDV banjaran dari 31230 hingga 31443) dan bcl-xs (kedekut IDV banjaran dari 33103 hingga 33290) jika dibandingkan dengan yang kawalan negatif (kedekut IDV banjaran dari 31230 hingga 36240).

Ujian-ujian yang berbeza yang dijalankan dalam kajian ini menunjukkan bahawa PHB (yang dihasilkan oleh Pusat Pengajian Sains Biologi, Universiti Sains Malaysia, Pulau Pinang, Malaysia) adalah tidak genotoksik dalam keadaan ujian sekarang.

A STUDY OF THE GENOTOXICITY OF POLYHYDROXYBUTYRATE:
A SYNTHETIC BIOMATERIAL

ABSTRACT

The purpose of this study is to determine the genotoxicity of polyhydroxybutyrate (PHB), produced in short solid fibre form and manufactured by School of Biological Sciences, Universiti Sains Malaysia, Penang, Malaysia, using three different tests: The *Salmonella* mutagenicity test (Ames test), *in vitro* mammalian chromosomal aberration test and gene expression analysis.

PHB was tested at various concentrations (0.3125, 0.625, 1.25, 2.5 and 5 mg/ml) in all the three tests. For the Ames test, PHB was incubated with special genotype variants of the bacterium *Salmonella typhimurium*, which carry mutations in several genes. Five tester strains (TA1535, TA1537, TA1538, TA98 and TA100) were used, both with and without metabolic activation system S9 mix and the test was assessed based on the number of revertant colonies. Simultaneously, negative control tests using sterile distilled water and positive control tests using sodium azide, 9-aminoacridine hydrochloride monohydrate (98%) and 4-Nitro-O-phenylenediamine (98%) without metabolic activation system (S9) and 2-aminoanthracene with metabolic activation system (S9) were carried out. To assess the chromosomal aberrations, human osteoblast cell lines (HOS, CRL-1543) from American type cell culture were exposed to PHB, sterile distilled water (Negative control) and Mitomycin C (Positive control), both with and without metabolic activation system S9 mix. For the gene expression analysis, the fibroblast cell lines MRC-5 were treated with PHB and incubated for 1, 12, 24 and 48 hours separately for each concentration. Total RNA was isolated and analysed for the expression of p53, c-myc, bcl-xl and bcl-xs genes.

The results of Ames test showed that the average number of revertant colonies per plate treated with PHB (28 to 344 colonies/plate without S9 and 150 to 499 colonies/plate

with S9) was less than double as compared to that of negative control (147 to 346 colonies/plate without S9 and 340 to 443 colonies/plate with S9). The absences of increase in the number of revertant colonies by at least double with the test material indicate that PHB was non-mutagenic. In the case of chromosome aberration test, there was no indication of any mutagenicity due to PHB on the HOS cell line as revealed by the mitotic index values [3.55(0.06) to 4.95(0.77) per cent without S9 and 3.10(0.14) to 5.20(0.98) per cent with S9]. Also, no chromosome aberrations were observed in the HOS cell lines treated with PHB. The results of gene expression analysis carried out on fibroblast cell line (MRC-5) treated with PHB at different timings did not show over or under expression of the genes, p53 (Mean IDV range from 36100 to 36295), c-myc (Mean IDV range from 33110 to 33270), bcl-xl (Mean IDV range from 31230 to 31443) and bcl-xs (Mean IDV range from 33103 to 33290) as compared to that of negative controls (Mean IDV range from 31230 to 36240).

Hence, the present study indicates that PHB produced by School of Biological Sciences, Universiti Sains Malaysia, Penang, Malaysia is non-genotoxic under the present test conditions.

CHAPTER ONE

INTRODUCTION

1.1 Biomaterials

A biomaterial is a non-viable material used in medical devices intended to interact with biological systems (Williams, 1987). They may be distinguished from other materials in that they possess a combination of properties, including chemical, mechanical, physical and biological properties that render them suitable for safe, effective and reliable use within a physiological environment. Biomaterial is a term used to indicate materials that constitute parts of medical implants, extracorporeal devices and disposables that have been utilized in medicine, surgery, dentistry and veterinary medicine as well as in every aspect of patient health care. The National Institutes of Health Consensus Development Conference defined biomaterial as ''any substance (other than a drug) or combination of substances, synthetic or natural in origin, which can be used for any period of time, as a whole or as a part of a system which treats, augments or replaces any tissue, organ or function of the body'' (Boretos and Eden, 1984).

Biomaterials can be naturally or semi synthetically produced. Numerous natural and semi synthetic materials are available in the form of xenografts (from a different species), allografts (from the same species) or autografts (from the same organisms) and function well in facial, plastic and reconstructive surgery. Any synthetic biomaterial can also be referred to as an alloplasts. There are a wide variety of alloplasts, most of which are specifically designed for either soft tissue or skeletal applications (Costantino *et al.,* 1993).

When a synthetic material is placed within the human body, tissue reacts towards the implant in a variety of ways depending on the material type. The mechanism of tissue interaction depends on the tissue response to the implant surface. Biomedical materials can be divided roughly into three main types which

governed by the tissue response. In broad terms, inert (more strictly, nearly inert) materials which elicit no or minimal tissue response. Active materials that encourage bonding to surrounding tissue with, for example, new bone growth being stimulated. Degradable or resorbable materials which are incorporated into the surrounding tissue, or may even, dissolve completely over a period of time. Metals are typically inert, ceramics may be inert, active or resorbable and polymers may be inert or resorbable (Czernuszka, 1996).

1.2 Polyhydroxybutyrate

Polyhydroxybutyrate (PHB) was the first polyhydroxyalkanoates (PHAs) to be discovered and was also the most widely studied and best characterized PHA. PHA is polyesters of various hydroxyalkanoates that are synthesized by many gram-positive and gram-negative bacteria from at least 75 different genera. These polymers are accumulated intracellularly to levels as high as 90% of the cell dry weight under conditions of nutrient stress and act as a carbon and energy reserve (Madison and Huisman, 1999).

PHB is accumulated as a membrane enclosed inclusion in many bacteria at up to 80% of the dry cell weight and has mechanical properties very similar to conventional plastics. PHB was discovered by Lemoigne in the bacterium *Bacillus megaterium* in 1926. In subsequent years, it was also found in other species of bacteria, where it acts as a source of carbon and energy. PHB is a non-toxic, insoluble in water thermoplastic displaying chemical and physical properties similar to polypropylene. PHB is used as a biodegradable, ecologically friendly alternative to conventional plastics (i.e. polypropylene) matrix component of composites reinforced particularly with fibres of natural origin (Peijs, 2002).

A large number of review papers are available, which give a detailed description about the general features of PHAs (Byrom, 1987; Brandl *et al*., 1990; Steinbuchel, 1991), characterization of PHA polymers (Brandl *et al*., 1990; Bonthrone

et al., 1992) and their biodegradation (Steinbuchel, 1991; Mergaert *et al.,* 1992). However, there are no recent reviews which explain the genotoxicity of PHB.

The bacteria that produce PHB can be divided into two groups. The first one includes *Ralstonia eutropha* (*Alcaligenes eutropha*) and consists of bacteria that produce short-chain PHA with C3–C5 monomers, while the second, which contains for example, *Pseudomonas oleovorans*, involves microorganisms that produce medium-chain PHA with C6–C14 monomers (Anderson and Dawes, 1990; Steinbuchel, 1991; Poirier *et al.,* 1995).

Several papers appeared on PHB synthesis in transgenic plants (Nawrath *et al.*, 1994; Poirier *et al.*, 1995; Bohmert *et al.*, 2000). All of them have been pointed to cause the accumulation of PHB in various plant organs with the expectation that the crops will produce the polymer for industrial use. The best result was obtained with *Arabidopsis thaliana* plants where PHB content was enhanced up to 42 mg/g (Bohmert *et al.*, 2000); however the growth of the plants was markedly reduced.

PHB has found more interesting applications as an implant material due to its biocompatibility and resorbability (Miller and Williams, 1987; Boeree *et al.,* 1993). PHB was appeared ideal for use as temporary stents**,** bone plates, patches, nails and screws (Malm *et al.*, 1992; Peng *et al.*, 1996), though in some cases, its brittle mechanical properties limit its use.

1.3 Biocompatibility and genotoxicity

Biocompatibility denotes acceptance of the implant to the tissue surface. The toxicological properties of new substances must be examined in the form in which they are introduced into the market i.e. normally with considerable amounts of impurities. On the basis of the results obtained, the substances are classified and labelled in respect of their dangerous properties (Broschinski *et al.*, 1998). Biocompatibility may generally be regarded as the ability of a material to interact with living cells/tissues or a

living system by not being toxic, injurious or causing immunological reactions while performing or functioning appropriately (Joel *et al*., 2004).

Genotoxicity is the study of chemicals which can damage the genetic structure of living organisms (including humans) and thus cause problems such as mutations, cancer and birth defects. The primary function of genotoxicity testing is to investigate, using test cells or organisms, the potential of products to induce mutations in man that may be transmitted via the germ cells to future generations. Scientific data generally support the hypotheses that DNA damage in somatic cells is a critical event in the initiation of cancer. Such damage can result in mutations and tests to detect mutagenic activity may also identify chemicals that have the potential to lead to carcinogenesis. Thus, some of the tests are useful for the investigation of putative carcinogenic activity (ISO 10993 -3, 1998).

Based on the recommendations of the International Standard Organization (ISO 10993-3, 1992) and the American Society for Testing and Materials (ASTM, 1987), therefore, three different representative test methods for the determination of genotoxic and mutagenic effects of the synthetic implant material PHB are applied: The *Salmonella* mutagenicity test (Ames test), i*n vitro* mammalian chromosomal aberration test and gene expression analyses.

1.4 The *Salmonella* mutagenicity test (Ames test)

Testing of chemicals for mutagenicity in *Salmonella typhimurium* is based on the knowledge that a substance which is mutagenic in the bacterium is likely to be a carcinogen in laboratory animals and thus, by extension, present a risk of cancer to humans. The ease, rapidity and low cost of the test make it an important tool for screening substances for potential carcinogenicity. Several strains of the *Salmonella typhimurium* bacterium may be used for testing. Each is genetically different and hence using several strains in a test increases the opportunity of detecting a mutagenic chemical. All strains of *Salmonella typhimurium* used for mutagenicity testing carry a

defective (mutant) gene that prevents them from synthesizing the essential amino acid histidine from the ingredients in standard bacterial culture medium. Therefore, these "tester" strains can only survive and grow on medium that contains excess histidine. However, in the presence of a mutagenic chemical, the defective histidine gene may be mutated back to the functional state, allowing the bacterium to grow on standard medium that does not contain supplemental histidine (Mortelmans and Zeiger, 2000).

Many chemicals are not mutagenic (or carcinogenic) in their native forms, but they are converted into mutagenic substances by metabolism in the liver. Since the *Salmonella* bacterium does not have the same metabolic capabilities as mammals, some test protocols utilize extracts of rat or hamster liver enzymes (S9) to promote metabolic conversion of the test chemical. This permits the investigator to determine if a chemical must be metabolized to express mutagenic activity. Some mutagenic chemicals are active with and without metabolism, while others are active only under one condition or the other (Malling, 1971; Ames *et al*., 1973b).

Several doses (at least 5) of the test chemical and multiple strains of *Salmonella typhimurium* are used in each experiment. In addition, cultures are set up with and without added S9 at varying concentrations and a variety of culture conditions are employed to maximize the opportunity to detect a mutagenic chemical (Waleh *et al*., 1982). In analyzing data, the pattern and the strength of the mutant response are taken into account in determining the mutagenicity of a chemical. All observed responses are verified in repeat tests. If there is no increase in mutant colonies is seen after testing several strains under several different culture conditions, the test chemical is considered to be non-mutagenic in the *Salmonella* test (Katzer *et al*., 2003).

1.5 *In vitro* mammalian chromosomal aberration test

Chromosomal mutations and related events are the cause of many human genetic diseases and there is substantial evidence that chromosomal mutations and

related events causing alterations in oncogenes and tumors suppressor genes of somatic cells are involved in cancer induction in humans and experimental animals (Ishidate and Sofuni, 1985).

The purpose of the *in vitro* chromosomal aberration test is to identify agents that caused structural chromosome aberrations in cultured mammalian cells (Evans, 1976; Galloway *et al*., 1987). Structural aberrations may be of two types, chromosome or chromatid. With the majority of chemical mutagens, induced aberrations are of the chromatid type, but chromosome type aberrations also occur (Hilliard *et al*., 1998).

This test is used to screen for possible mammalian mutagens and carcinogens. Many compounds that are positive in this test are mammalian carcinogens. The chromosome aberration test using cultured mammalian cells is one of the sensitive methods to predict environmental mutagens and/or carcinogens and is also a complementary test to the *Salmonella* / microsome assay (Ames test) (Ishidate *et al.*, 1998).

Several short-term mutagenicity assays are being widely used for screening chemicals for carcinogenic potential. Gene mutations and chromosome aberrations, major endpoints for the evaluation of mutagenicity, are intimately involved in the initial and subsequent steps of carcinogenesis, oncogenes being commonly activated by gene mutations or chromosome aberrations in various types of cancers (Land *et al.,* 1983). Moreover, some mutagens are reported to cause chromosome aberrations alone, whereas, others solely induce gene mutations (Zeiger *et al*., 1990).

1.6 Gene expression analyses

In each and every organism, different genes are expressed in different cell and tissue types (spatial differences) and at different developmental stages (temporal differences). Analyses of these variations in gene expression will lead to a better understanding of disease states, targetting of drugs to specific cells, tissues or individual's development of agricultural products (Narayanan, 2001).

Studies of gene expression changes induced by genotoxins have focussed on agents where deoxyribonucleic acid (DNA) is the initial primary target, i.e. "direct-acting genotoxins" like methylmethane sulfonate. However, it is well known that chemicals induce genotoxicity by interaction with a number of cellular targets in addition to DNA crosslinking. Indirect effects through inhibition of cellular enzymes, like ribonucleotide reductase and topoisomerase, as well as inhibition of critical macromolecules like tubulin can result in genotoxicity (Hurley, 2002).

In addition to identification of novel pathways involved in cellular response to DNA damage, a number of studies have now demonstrated distinct responses in gene expression changes in cells exposed to different DNA damaging agents, suggesting the possibility of identifying fingerprints for different types of DNA damaging agents (Chang *et al*., 2002; Park *et al*., 2002; Heinloth *et al*., 2003). Studies of individual genes have demonstrated that transcript levels can change as a result of biomaterial contact (Lafrenie *et al.,* 1998; Breen *et al*., 1999, Cukierman *et al.,* 2001; Lam *et al*., 2001).

Huang *et al*. (2003) used the primary human gingival fibroblasts to examine the effect of six dentin bonding agents on the expression of c-*fos* and c-*jun* proto-oncogene to evaluate the genotoxicity/mutagenicity and carcinogenicity potential of the dentin bonding agents. An important requirement for biomaterials agent is biological compatibility and they also should not induce an inflammatory or immune response. Numerous investigators of PHB have focused on the chemistry on bonding strength or on their effects on microleakage. However, there is very limited information available on biocompatibility tests, especially genotoxicity/mutagenicity and carcinogenicity of PHB agents.

1.7 Objectives

1.7.1 General objectives

The purpose of this study was to determine the genotoxicity of polyhydroxybutyrate (PHB) (produced in short solid fibre form and manufactured by School of Biological Sciences, Universiti Sains Malaysia, Penang, Malaysia).

1.7.2 Specific objectives

1. To determine the mutagenic effect of PHB on *Salmonella* strains using the *Salmonella* mutagenicity test (Ames test).

2. To determine the cytotoxic and mutagenic effects of PHB on the chromosomes of human osteoblast cell line.

3. To determine whether PHB altered the expression of proto-oncogenes (p53 and c-myc) and anti-apoptotic genes (bcl-xl and bcl-xs) in the human fibroblast cell line.

CHAPTER TWO

LITERATURE SURVEY

2.1 Background

In the past, there was no targeted development of biomaterials based on scientific criteria. Instead, devices consisting of materials that had been designed, synthesized and fabricated for various industrial needs (for example, the textile, aerospace and defense industries) were tested in a trial-and-error fashion in the bodies of animals and humans. These unplanned and sporadic attempts had (at best) modest success. Most frequently, the results were unpredictable, mixed and confounded both in success and in failure (Cooke *et al.,* 1996).

Because of the continuous and ever-expanding practical needs of medicine and health care practice, there are currently thousands of medical devices, diagnostic products and disposables in the market. In fact, the range of applications continues to grow. To date, tens of millions of people have received medical implants. Undoubtedly, biomaterials have had a major impact on the practice of contemporary medicine and patient care in both saving and improving the quality of lives of humans and animals. Modern biomaterial practice still takes advantage of developments in the traditional, non medical materials field but is also (actually, more so than ever) aware of and concerned about the biocompatibility and biofunctionality of implants (Peppas and Langer, 1994).

There is a necessity for replacing bone substance which has been lost due to traumatic or non-traumatic events. The lost bone can be replaced by endogenous or exogenous bone tissues, which is connected with several problems. The use of endogenous bone substance involves additional surgery (Parsons, 1988); moreover, the endogenous bone is available only in limited quantities (Willmann, 1993). The major disadvantage of exogenous bone implants is that they may be rejected by the human body and disease may be transmitted together with the implant (Willmann,

1993). Also, the clinical performance of exogenous bone is considerably inferior to fresh endogenous graft material (Parsons, 1988). For these reasons, there is a growing need for fabrication of artificial hard tissue replacement implants.

Autogenous bone graft has been considered the gold standard for bone repairing procedures as it contains triggering ingredients necessary for bone formation in bone defect. However, the availability of the autograft bone is limited and the harvesting of autografts bone causes morbidity at the donor site. Bone defects resulting from congenital defects, inflammatory or tumorous destructive processes, trauma or bone gaps arising from osteotomy procedures are conventionally repaired using bone grafts. At present, the optimal grafting material is autologous bone, which provides the essential features of a graft material such as mechanical strength and living osteoblasts (Mulliken and Glowacki, 1980). Therefore, allografts are used as an alternative to autografts. Eventually, alternative bone substitute materials or biomaterials have been developed (Tuominen *et al.*, 2000).

The use of synthetic biocompatible, bioresorbable materials is increasing in orthopedic, plastic and dental surgery. Such materials are typically needed to augment autologous bone grafts or to fill bone voids or augment bone loss (e.g. bone loss caused by periodontal disease, bone defect or cavity due to trauma, cancer, disease or surgery and spinal fusion). The quality of a bone graft substitute is determined by its osteoconductive, osteoinductive and osteogenic properties (Bucholz, 2002).

2.2　Biomaterials

2.2.1　Definitions

Biomaterial is a term used to indicate materials that constitute parts of medical implants, extracorporeal devices and disposables that have been utilized in medicine, surgery, dentistry and veterinary medicine as well as in every aspect of patient health care. The National Institutes of Health Consensus Development Conference defined biomaterial as ''any substance (other than a drug) or combination of substances,

synthetic or natural in origin, which can be used for any period of time, as a whole or as a part of a system which treats, augments or replaces any tissue, organ or function of the body (Boretos and Eden, 1984). The common denominator in all the definitions that have been proposed for ''biomaterials'' is the undisputed recognition that biomaterials are distinct from other classes of materials because of the special biocompatibility criteria they must meet.

2.2.2 Classifications

Synthetic materials currently used for biomedical applications include metals, alloys, polymers and ceramics. Because the structures of these materials differ, they have different properties and, therefore, different uses in the body. When a synthetic material is placed within the human body, tissue reacts towards the implant in a variety of ways depending on the material type. The mechanism of tissue interaction depends on the tissue response to the implant surface. In general, there are three terms in which a biomaterial may be described into representing the tissues responses. These are bioinert, bioresorbable and bioactive (Heness and Ben-Nissan, 2004).

2.2.2(a) Bioinert biomaterials

The term bioinert refers to any material that once placed in the human body has minimal interaction with its surrounding tissue; examples of these are stainless steel, titanium, alumina, partially stabilised zirconia and ultra high molecular weight polyethylene. Generally, a fibrous capsule might form around bioinert implants and hence its biofunctionality relies on tissue integration through the implant.

2.2.2(b) Bioresorbable biomaterials

Bioresorbable refers to a material that upon placement within the human body starts to dissolve (resorbed) and slowly replaced by advancing tissue (such as bone). Common examples of bioresorbable materials are tricalcium phosphate [$Ca_3(PO_4)_2$] and polylacticpolyglycolic acid copolymers. Calcium oxide, calcium carbonate and

gypsum are other common materials that have been utilised during the last three decades.

2.2.2(c) Bioactive biomaterials

Bioactive refers to a material, which upon being placed within the human body interacts with the surrounding bone and in some cases, even soft tissue. This occurs through a time dependent kinetic modification of the surface, triggered by their implantation within the living bone. An ion - exchange reaction between the bioactive implant and surrounding body fluids - results in the formation of a biologically active carbonate apatite (CHAp) layer on the implant that is chemically and crystallography-ically equivalent to the mineral phase in bone. Prime examples of these materials are synthetic hydroxyapatite [$Ca_{10}(PO_4)_6(OH)_2$], glass ceramic and bioglass (Park *and* Bronzino, 2003).

2.2.3 Biological and synthetic biomaterials

2.2.3(a) Biological biomaterials

They are materials that are naturally produced. It is subdivided into autografts, allografts and xenografts. Autografts are grafts from the same organism, allografts from same species and xenografts are those made with grafts from other species.

2.2.3(b) Synthetic biomaterials

They are materials that are produced synthetically. It can be subdivided into metals, ceramic and glass and polymers.

Metallic biomaterials have been used almost exclusively for load-bearing implants, such as hip and knee prostheses and fracture fixation wires, pins, screws and plates. Metals have also been used as parts of artificial heart valves, as vascular stents and as pacemaker leads. Although pure metals are sometimes used, alloys (metals containing two or more elements) frequently provide improvement in material properties, such as strength and corrosion resistance. Three material groups dominate biomedical metals: stainless steel, cobalt-chromium-molybdenum alloy and pure

titanium and titanium alloys. The main considerations in selecting metals and alloys for biomedical applications are biocompatibility, appropriate mechanical properties, corrosion resistance and reasonable cost (Ratner, 1993).

Ceramics and glasses biomaterials are used as components of hip implants, dental implants, middle ear implants and heart valves. However, these biomaterials have been used less extensively than either metals or polymers. Some examples of ceramics that have been used for biomedical applications are alumina (Al_2O_3), zirconia (ZrO_2), pyrolytic carbon, bioglass, hydroxyapatite [$Ca_{10}(PO_4)_6(OH)_2$] and tricalcium phosphate [$Ca_3(PO_4)_2$] (Cooke *et al.*, 1996).

Polymeric biomaterials are the most widely used materials in biomedical applications. They are the materials of choice for cardiovascular devices as well as for replacement and augmentation of various soft tissues. Polymers are also used in drug delivery systems, in diagnostic aids and as a scalding material for tissue engineering applications. Examples of current applications include vascular grafts, heart valves, artificial hearts, breast implants, contact lenses, intraocular lenses, components of extracorporeal oxygenators, dialyzers and plasmapheresis units, coatings for pharmaceutical tablets and capsules, sutures, adhesives and blood substitutes (Peter *et al.*, 1997). Examples of polymers are polyethylene, polyvinylchloride, polyester, PHB, silicone rubber, polyethyleneterephthalate, polytetrafluoroethylene, polymethyl-methacrylate, polylactidecoglycolide, polylactic acid, polyglycolic acid and polylactide-co-lycolide.

2.2.4 Applications of biomaterials

2.2.4(a) Orthopaedic applications

Metallic, ceramic and polymeric biomaterials are used in orthopedic applications. Metallic materials are normally used for load bearing members such as pins and plates and femoral stems. Ceramics such as alumina and zirconia are used for wear applications in joint replacements, while hydroxyapatite is used for bone

bonding applications to assist implant integration. Polymers such as ultra high mole-cular weight polyethylene are used as articulating surfaces against ceramic components in joints (Cordingley *et al*., 2003).

2.2.4(b) Dental applications

Metallic biomaterials have been used as pins for anchoring tooth implants and as parts of orthodontic devices. Ceramics have found uses as tooth implants including alumina and dental porcelains. Hydroxyapatite has been used for coatings on metallic pins and to fill large bone voids resulting from disease or trauma (Thamaraiselvi and Rajeswari, 2004).

2.2.4(c) Cardiovascular applications

Many different biomaterials are used in cardiovascular applications depending on the specific application and the design. For instance, carbon in heart valves and polyurethanes for pacemaker leads. (Czernuszka, 1996).

2.2.4(d) Cosmetic surgery

Materials such as silicones have been used in cosmetic surgery for applications such as breast augmentation.

2.2.4(e) Others

Although biomaterials are primarily used for medical applications, they are also used to grow cells in culture, to assay for blood proteins in the clinical laboratory, in processing biomolecules in biotechnology, for fertility regulation implants in cattle, in diagnostic gene arrays, in the aquaculture of oysters and for investigational cell-silicon "biochips" (Williams *et al*., 1999). The commonality of these applications is the interaction between biological systems and synthetic or modified natural materials.

2.2.5 Biomaterials Act

In August of 1998, USA Congress passed the Biomaterials Access Assurance Act to insulate biomaterials suppliers from liability in civil actions when their raw materials or component parts are used in implanted medical devices

(www.devicelink.com). An existing federal statute is designed to shield biomaterials suppliers from potential lawsuits. A recent case suggests that implant manufacturers can also assert the statute to protect their suppliers. Harm is defined as any injury to or damage suffered by an individual (Melissa and Julia, 2004) by any illness, disease or death resulting from that injury or damage and any loss to that individual or any other individual, resulting from that injury or damage. This definition of harm would appear to cover all damages and types of injury that could conceivably be alleged under any tort theory, including strict product liability. In short, the act insulates biomaterials suppliers for any type of injury to an individual in any court on the basis of any legal theory with regard to the suppliers' products used in implanted medical devices (Ishihara, 2000).

2.3 Plastics

Plastics have versatile qualities of strength, lightness, durability and resistance to degradation. They have become an important commodity to enhance the comfort and quality of life. Plastics are an essential part of almost all industries and have replaced glass and paper in packaging, but these very desirable properties have now become their greatest problem. Accumulation of recalcitrant plastics in the environment has become a world-wide problem. Solutions to plastic waste management include source reduction, incineration, recycling and bio- or photo-degradation. However, most of these have problems associated with them. Incineration of plastics is potentially dangerous and can be expensive. During the combustion of plastic waste, hydrogen cyanide can be formed from acrylonitrile-based plastics and may cause potential health hazards. Recycling can be done but is very tedious. The sorting of the wide variety of discarded plastic material is also a very time-consuming process. Moreover, the presence of a wide variety of additives such as pigments, coatings, fillers, limits the use of the recycled material. In such a scenario, biodegradable plastics offer the best solution to the environmental hazard posed by conventional plastics (Brandl *et al*., 1990).

2.3.1 Biodegradable plastics

2.3.1(a) Chemically synthesized polymers

Which include polyglycollic acid, polylactic acid, polycaprolactone, polyvinyl alcohol and polyethylene oxide. These materials are susceptible to enzymic or microbial attack. Since there is little information about the properties of these plastics therefore, they are not commercially available as substitute for plastics (Bonthrone *et al*., 1992).

2.3.1(b) Starch-based biodegradable plastics

The starch is added as filler and cross-linking agent to produce a blend of starch and plastic (starch-polyethylene). Soil micro-organisms degrade the starch easily, thus breaking down the polymer matrix. These properties are significant in reduction of degradation time but such plastics are only partially degradable. The fragments left after starch removal are recalcitrant and remain in the environment for a long time (Shilpi and Ashok, 2005).

2.3.1(c) Polyhydroxyalkanoates

Polyhydroxyalkanoates (PHAs) are the only 100% biodegradable polymers. They are polyesters of various hydroxyalkanoates (HAs) which are synthesized by numerous microorganisms as energy reserve materials when an essential nutrient such as nitrogen or phosphorus is available only in limited concentrations in the presence of excess carbon source. PHAs possess properties similar to various synthetic thermoplastics like polypropylene. They are also completely degraded to water and carbon dioxide under aerobic conditions and to methane under anaerobic conditions by micro-organisms in soil, sea, lake water and sewage (Mergaert *et al*., 1992).

2.4 Polyhydroxybutyrate

Polyhydroxybutyrate (PHB) was the first of the PHA discovered and is the most widely studied and also best characterized of the PHA. It is accumulated as a

membrane enclosed inclusion in many bacteria at up to 80% of the dry cell weight. It has mechanical properties very similar to conventional plastics like polypropylene or polyethylene and can be extruded, moulded, spun into fibres, made into films and used to make heteropolymers with other synthetic polymers (Holmes, 1988). In spite of its numerous advantages, PHB has yet not been able to replace conventional plastics on a large scale because of its high cost (Shilpi and Ashok, 2005).

Bacterial PHB is a crystalline polyester of great technological interest, because it is a truly biodegradable and highly biocompatible polymer (Doi *et al*., 1990). PHB is an aliphatic polyester synthesised by wide variety of microorganisms as an intracellular store of carbon and an ion sink (Dawes, 1986).

PHB, first characterized in *Alcaligenes eutrophus*, is a biodegradable polymer, which can be completely degraded by enzymatic activities in the soil (Poirier *et al*., 1992). PHB belongs to a class of polyesters of 3-hydroxy acids that are synthesized in various bacterial genera (Schubert *et al*., 1988; Hai *et al.,* 2001).

2.4.1 History of polyhydroxybutyrate

The presence of sudanophilic, lipid-like inclusions material was observed by Meyer, 1903 (cited by Sudesh *et al.,* 2000) which were soluble in chloroform (Stapp, 1924) was initially observed in *Azotobacter chroococcum* bacterium early last century. The chemical composition of similar inclusions in *Bacillus megaterium* bacterium was identified as poly(3-hydroxybutyric acid) P(3HB) by Lemoigne (1926) and Lemoigne (1927). By the end of the 1950s, enough evidence was already accumulated from studies on the genus *Bacillus* to suggest that P(3HB) functions as an intracellular reserve for carbon and energy in these bacteria (Williamson and Wilkinson, 1958; Doudoroff and Stanier, 1959). In fact, it was also demonstrated that the occurrence of this reserve polymer is a widespread phenomenon in gram-negative bacteria (Forsyth *et al*., 1958). In a review on the role and regulation of energy reserve polymers in microorganisms published in 1973 by Dawes and Senior, PHB received its first

extensive coverage as a bacterial storage material analogous to starch and glycogen. However, the 3-hydroxybutyric (3HB) unit wa, thought to be the only hydroxyalkanoate (HA) constituent that forms the building block for this microbial reserve polymer.

In 1974, Wallen and Rohwedder reported the identification of hydroxyalkanoates other than 3HB. Among the HA units that were noted in chloroform extracts of activated sewage sludge, 3-hydroxyvalerate (3HV) and 3-hydroxyhexanoate (3HHX) were the major and the minor constituents, respectively. About a decade later, following the identification of the heteropolymers, the analyses of marine sediments by capillary gas chromatography revealed the presence of 3HB and 3HV as the predominant components among 11 other HA units (Findlay and White, 1983). In the same report, it was also shown that batch-grown *Bacillus megaterium* cells accumulated a polymer which consisted of 95% 3HB, 3% 3-hydroxyheptanoate (3HHP), 2% of an 8-carbon HA and trace amounts of three other HA compounds. At around the same time, an interesting finding was made by Witholt and coworkers when they cultivated *Pseudomonas oleovorans* on *n*-octane (De Smet *et al.,* 1983). Elemental analyses of the storage polymers thus accumulated by this bacterium showed that it consisted of principally 3-hydroxyoctanoate (3HO) units (De Smet *et al.,* 1983) and small amounts of 3HHX units (Lageveen *et al.,* 1988).

The identification of HA units other than 3HB in microbial PHA proved to have major impact on the research and commercial interest for this bacterial reserve polymer. While the homopolymer of P(3HB) is a brittle material with limited applications, the incorporation of a second monomer unit into P(3HB) can significantly enhance its useful properties. This finding is therefore highlighted as a landmark which signifies the beginning of the second developmental stage of research on PHA. It was in this stage that the first industrial production of a copolymer consisting of 3HB and 3HV took place despite the fact that the potential of P(3HB) as a biodegradable thermoplastic was realized much earlier and patents were originally filed in 1962. At this stage, the research trend was to identify and characterize all the various potential

HA units that could possibly be a constituent of this bacterial polyester. This ultimately resulted in the discovery of numerous HA constituents (Doi *et al.*, 1990) including 4HA (Kunioka, 1988) and 5HA (Doi *et al.*, 1987) by the end of the 1980s. By this time, it was already clear that these storage polymers are synthesized not only in gram-negative bacteria but also in a wide range of gram-positive bacteria, aerobic (cyanobacteria) and anaerobic (non-sulfur and sulfur purple bacteria) photosynthetic bacteria, as well as in some archaebacteria (Anderson and Dawes, 1990; Steinbuchel, 1991).

However, an astounding number of approximately 125 different HAs are known to occur (Rehm and Steinbuchel, 1999) and therefore a more general name comprising all these constituents, i.e. polyhydroxyalkanoates, has been used to designate this family of bacterial reserve polymers.

Detailed studies on the bacterium *Ralstonia eutropha* (formerly known as *Alcaligenes eutrophus*) had revealed that only three enzymes are involved in the biosynthesis of P(3HB) from acetyl-CoA and that the regulation of P(3HB) synthesis in this bacterium is achieved at the enzyme level. The enzyme which carries out the polymerization reaction was identified as the key enzyme and it was designated as PHA synthase. To date, about 38 PHA synthase structural genes from more than 32 different bacteria have been cloned. Highly conserved amino acids have been identified based on alignment analysis of the primary structures of these genes and also by site-specific mutagenesis studies (Gerngross *et al.*, 1994).

2.4.2 Manufacture of PHB

Manufacturing process of PHB begins with sunlight. Through photosynthesis, carbon dioxide from the atmosphere is converted to carbohydrates via sugar beets or sugar cane. These carbohydrates are the raw material for the manufacture of PHB. PHB can be produced from glucose as a raw material or agricultural wastes like, for instance, molasses, which is refined from the processing of sugar beets and lactose. The sugar splits up in the metabolism to C2 building blocks, which are converted, over

several steps, to C4 monomers. Finally, PHB is polymerized (Kessler and Witholt, 2001).

Biosynthesis pathway of PHB has been studied in detail in *Ralstonia eutropha* (Schubert *et al.*, 1988; Slater *et al.*, 1988: Peoples and Sinskey, 1989). In this bacterium, the biosynthesis is initiated by the condensation of two acetyl-CoA molecules to acetoacetyl-CoA catalyzed by the enzyme β -ketothiolase. The gene for this enzyme is designated *phbA*. Acetoacetyl-CoA is then reduced to (*R*)-3-hydroxy-butyryl-CoA by the NADPH-dependent acetoacetyl-CoA reductase; the gene was designated *phbB*. These enzymes have been found and studied in several PHB-accumulating bacteria (Rehm and Steinbuchel, 1999).

Advances in the biochemistry and molecular biology of PHA biosynthesis have resulted in the cloning of PHA biosynthesis genes from various bacteria (Steinbuchel, 1991; Rehm and Steinbuchel, 1999). This has allowed for the development of recombinant bacteria which can produce PHA under the direction of heterologous genes (Lee, 1997). The biosynthetic pathway of PHB is shown in Figure 2.1 as described by Reddy *et al*. (2003). P (3HB) is synthesized by the successive action of b-ketoacyl-CoA thiolase (phbA), acetoacetyl-CoA reductase (phbB) and PHB polymerase (phbC) in a three step pathway. The genes of the phbCAB operon encode the three enzymes. The promoter upstream of phbC transcribes the complete operon (phbCAB).

2.4.3 PHB fermentation

2.4.3(a) Pre-fermentation

Selected bacteria are used and the cells are grown at 30°C in a stream of air in a mineral medium with glucose as a carbon-source. The cells multiply after 24 hours and reach approximately a cell density of 20 g/l. This is called pre-fermentation (Kim *et al*., 1994).

2.4.3(b) Fermentation

The cells continue to multiply and after 40-80 hours, reach a cell density of 100 g/l. PHB occurs as discrete granules with diameters of 0.3 -1 μm in cells as storage material. At the end of the fermentation process, the cell has a dry mass 80 % in the form of PHB. For 1 kg of PHB, approximately 2.8 kg of sugar are needed (Yamane, 1993).
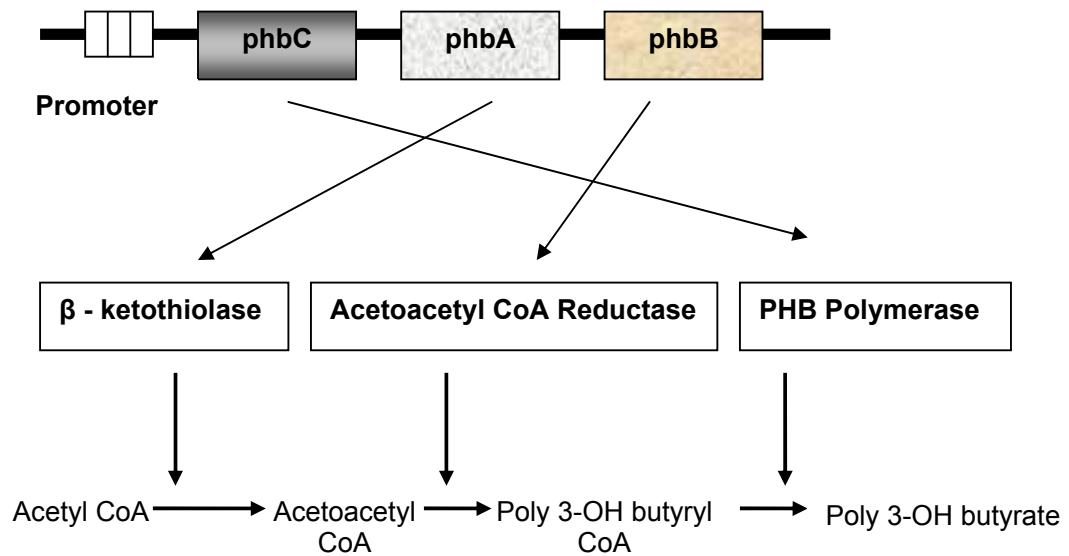
Fig 2.1 Biosynthetic pathway of poly (3-hydroxybutyrate)

2.4.4 Physical properties of PHB

The molecular weight of PHB produced from wild-type bacteria is usually in the range of $1 \times 10^4 - 3 \times 10^6$ g/mol with a polydispersity of around two (Doi *et al*., 1990). The glass transition temperature of PHB is around 4°C while the melting temperature is near 180°C, as measured by calorimetric analysis (Kobayashi *et al*., 2000). The densities of crystalline and amorphous PHB are 1.26 and 1.18 g/cm^3 respectively. The extension to break (5%) for PHB is however, markedly lower than that of polypropylene (400%). Therefore, PHB appears as a stiffer and more brittle plastic material compared to polypropylene (Sudesh *et al*., 2000).

The recombinant *E. coli* harboring PHA biosynthesis gene from *R. eutropha* can produce ultra-high molecular weight PHB homopolymer. The weight-average molecular weight values were found to be in the range of 3×10^6 - 1.1×10^7 under special fermentation conditions. The chemical structure of PHB is - {O- CH (CH3) – CH2 –(C = O)} n – (Kusaka *et al.,* 1998).

PHB is a fully biodegradable polyester with optical activity, piezoelectricity and very good barrier properties. PHB is a thermoplastic, belonging to the family of PHAs. It has physical and mechanical properties comparable to those of isotactic polypropylene. PHB is stiff and brittle. The degree of brittleness depends on the degree of crystallinity, glass temperature and microstructure (Song *et al*., 2001). At room temperature, the longer it is stored the more brittle it becomes. PHB does not contain any residues of catalysts like other synthetic polymers. It is not water-soluble but is 100% biodegradable. PHB has low permeability for O_2, H_2O and CO_2 but PHB has the disadvantage of high cost and is thermally unstable during processing, therefore the viscosity and molar mass decrease (Valentin and Dennis, 1997).

2.4.5 Applications of PHB

2.4.5(a) Applications in medicine

PHB is compatible with the blood and tissues of mammals. The monomer of PHB is a normal metabolic in the human blood. As the body reabsorbs PHB, it might be used as a surgical implant, in surgery, as seam threads for the healing of wounds and blood vessels.

2.4.5(b) Applications in pharmacology

PHB can be used as microcapsules in therapy or as materials for cell and tablet packaging.

2.4.5(c) Applications in industry

In packaging for deep drawing articles in the food industry, for example, bottles, laminated foils, fishnets, potted flower, hygiene, fast food, one-way cups, agricultural

foils and fibres in textile. PHB was found more interesting applications as an implant material due to its biocompatibility and resorbability (Miller and Williams, 1987; Boeree *et al.,* 1993). PHB appeared ideal for use as temporary stents**,** bone plates, patches, nails and screws (Malm *et al*., 1992; Peng *et al*., 1996). In some cases, its brittle mechanical properties limit its use.

2.5 Problem description

Nowadays, plastics and synthetic polymers are mainly produced from petro-chemical elements, which do not decompose, thus resulting in environmental pollution. During combustion, water and carbon dioxide are released into the atmosphere, i.e. an increase in the carbon dioxide concentration in the atmosphere occurs. By recycling polymers, the material quality decreases. Biological polymers are part of a cycle, i.e. water and carbon dioxide are used during the photosynthesis in the plant. The bacteria used carbohydrates by fermentation in the manufacture of PHB. In the USA, Europe and Japan it is expected that biodegradable materials will be important due to their material properties being suitable for a wide range of fields. Waste is currently causing serious environmental problems in many countries, especially in industrialized countries. In household waste, 30% are all types of packaging foil, i.e. packaging foils for foods, bags and coated foil on paper. Making eco-friendly products such as bioplastics is one such reality that can help us overcome the problem of pollution caused by non-degradable plastics (Reddy *et al*., 2003).

2.6 Biomaterials and cancer

Since breast implants first came on the market in 1962, between 1.5 and 2 million women in the United States have had breast augmentation surgery. About 80% of the surgeries were for cosmetic reasons while 20% were for breast reconstruction following breast cancer surgery (Brinton *et al*., 2001).

The Food and Drug Administration (FDA) banned the use of silicone implants for breast enlargements in 1992 because there was little information on their long-term

safety. The FDA now only permits silicone implants to be used in controlled clinical trials of women seeking breast reconstruction (www.cancer.gov).

Baird and Rea (1999) reported 14 patients with temporomandibular joint (TMJ) who had alloplastic implants and exhibited chronic signs and symptoms of chemical sensitivity. These patients were well before their implantation whereas they had memory loss, confusion, imbalance, dizziness, non-immune vasculitis, petechiae, spontaneous bruising, edema, Raynaud's phenomenon, pain and autoimmune dysfunction after implantation. Laboratory data also showed immunological abnormalities, including positive autoantibodies and altered T and B lymphocyte function and provocation skin testing showed reaction to their implant material. The symptoms of patients with jaw implants were similar to those patients who experience complications from their breast implants (Baird and Rea, 1999).

2.7 Biocompatibility testing

Any medical device that comes into direct or indirect contact with a patient must be tested for biocompatibility. Even if a device does not physically touch the patients, in some cases it may release chemical constituents that could be harmful. The degree of concern about a material depends on its composition, the nature and duration of its contact with the patients. Biocompatibility is the ability of a material to perform with an appropriate host response in a specific application (Williams, 1999).

Recognition of an implant material as biocompatible nowadays depends on a large number of factors such as absence of cytotoxicity, mutagenicity, carcinogenicity, exclusion of allergenic properties, physical, chemical and biological "inertia" and stability in its biological environment. Therefore, before new materials are approved for medical use, mutagenesis system to exclude cytotoxic, mutagenic or carcinogenic properties is applied world wide. There are more than one hundred different testing methods for collecting evidence of carcinogenic and mutagenic activity. Many of them are based on the principle that genotoxicity or mutagenicity serves as an indicator for