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PHA applications: addressing the price performance issue I. Tissue engineering

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Abstract

This paper describes the development of medical applications for polyhydroxyalkanoates (PHAs), a class of natural polymers with a wide range of thermoplastic properties. Methods are described for preparing PHAs with high purity, modifying these materials to change their surface and degradation properties, and methods for fabricating them into different forms, including tissue engineering scaffolds. Preliminary reports characterizing their in vivo behavior are given, as well as methods for using the natural polymers in tissue engineering applications. © 1999 Elsevier Science B.V. All rights reserved.

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1. Introduction

1.1. Polyhydroxyalkanoates

Polyhydroxyalkanoates (PHAs) are a class of natural polyesters that are produced by numerous organisms [1,2]. They are one of the most fascinating and largest groups of thermoplastic polymers known, with over 100 different types currently produced from a variety of different monomer types as shown in Fig. 1. Their properties span a wide range, including materials that resemble polypropylene and others that are elastomeric. Their different properties arise chemically, either from the length of the pendant groups which extend from the polymer backbones, or from the distance between the ester linkages in the polymer backbones. Typically, PHAs with short pendant groups are hard crystalline materials, whereas PHAs with longer pendant groups are elastomeric. As well as offering a wide range of mechanical properties, the PHAs are also biodegradable.

At Metabolix in Cambridge, MA, researchers have taken new approaches to producing PHAs [3]. Using both genetic engineering and new fermentation techniques, scientists have successfully developed new routes to prepare a range of PHAs, while advances in downstream processing and subsequent treatments have led to new methods for their purification and modification. The ultimate goal of the transgenic approach is to produce PHAs in plant crops to make them price competitive with traditional oil-derived plastics. While this goal is still some years away, the efforts so far have broadened the range of PHAs available, and are expected to meet target pricing for specialty polymer applications through new fermentation routes. In addition to the development of specialty applications, these advances in PHA production have opened up the opportunity to further evaluate and develop medical applications for these natural polymers. Prior studies by others in this area had been primarily restricted to two commercially available PHA polymers, namely, polyhydroxybutyrate (PHB) and polyhydroxybutyrate-*co*-valerate (PHBV). These materials have been evaluated

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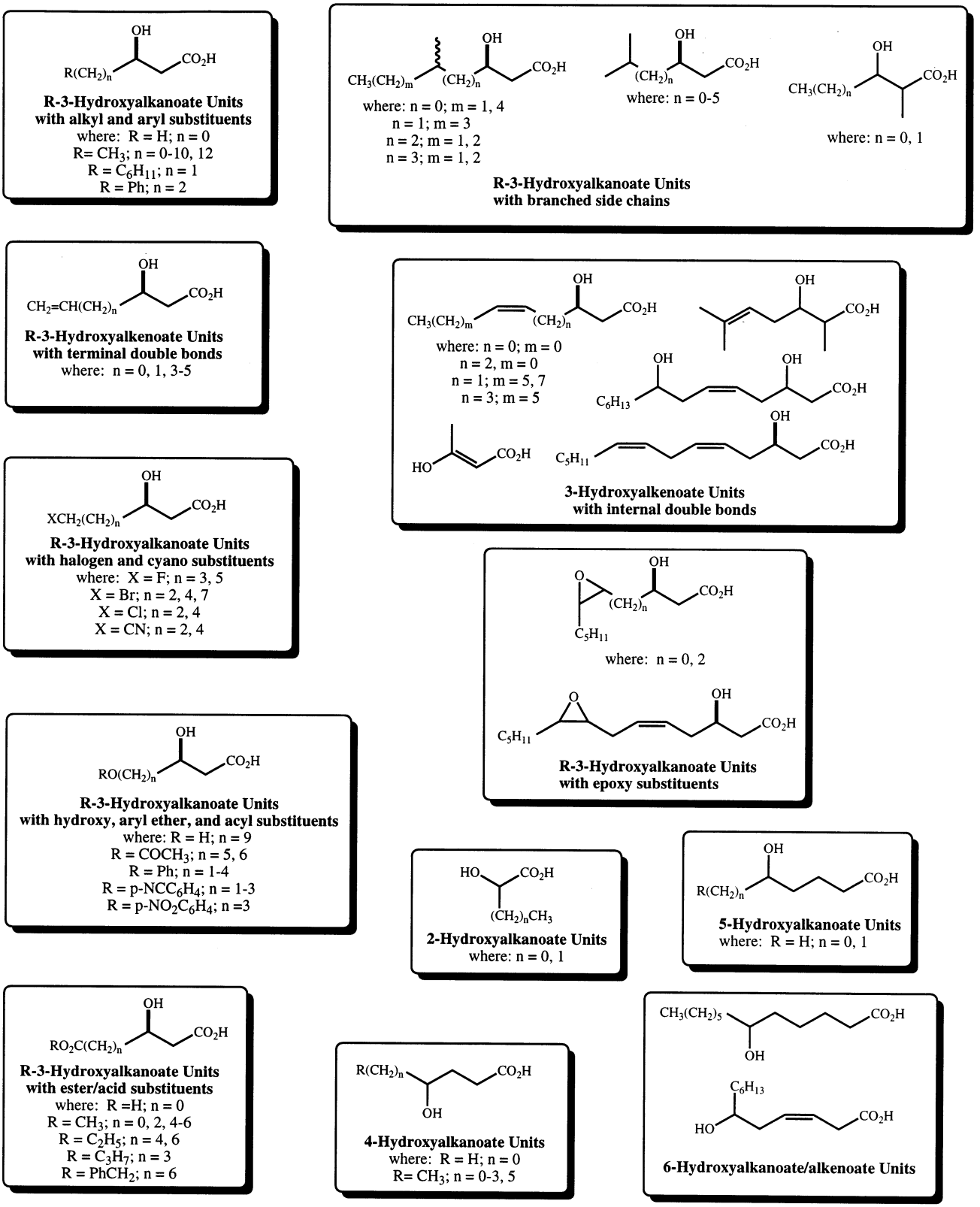


Fig. 1. Examples of monomers that can be incorporated into PHA polymers.

for a variety of medical applications, which include controlled release, surgical sutures, wound dressings, lubricating powders, orthopedic uses [4] and as a pericardial substitute [5]. This paper describes some of our progress in developing PHAs for use as biopolymer scaffolds in tissue engineering applications.

1.2. Tissue engineering

Tissue engineering has emerged as a multi-disciplinary field combining biology, materials science, and surgical reconstruction, to provide living tissue products that restore, maintain, or improve tissue function [6]. The need for this approach has arisen primarily out of a lack of donor organs and tissues, but also because it offers the promise of being able to dramatically expand our ability to repair tissues, develop improved surgical procedures, and significantly improve the quality of life.

In general, there are three distinct approaches currently being used to engineer new tissue. These are (1) infusion of isolated cells or cell substitutes, (2) use of tissue-inducing materials, and (3) implantation of cells seeded in scaffolds (either prior to or subsequent to implantation) [6]. Our recent efforts focus on this latter case.

Tissue engineering scaffolds are designed to provide a support structure for the engineered tissue and may be configured either in a closed manner to protect the implanted cells from the body's immune system, or in an open manner so that the new cells can eventually be incorporated into the body. Rejection of the implant can be minimized by using autologous cells, and/or by administering immunosuppressive drugs. In the open scaffold systems, some of the early pioneers fabricated lattices from protein polymers based on, for example, collagen [7–9], while others employed degradable synthetic polymers [10]. As well as providing a means to deliver cells to a designated anatomic location, the scaffolds can play a critical role in serving as templates for the reorganization of the transplanted cells as they form new tissue.

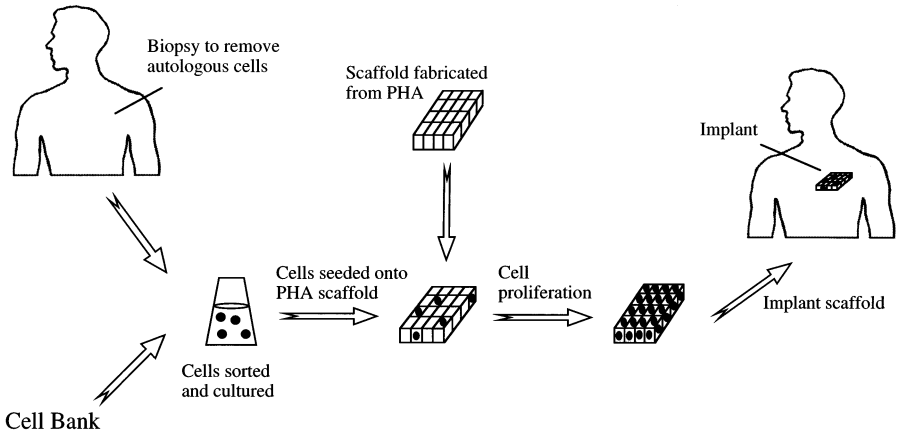
Using open scaffolds, tissue engineers have attempted to engineer virtually every mammalian tissue, including liver, cartilage, bone, uroepithelial–smooth muscle structures, tracheal epithelium, tendon, and heart valve leaflets [6–11]. Open scaffolds must have at least five key properties. First and foremost, the scaffold must be biocompatible. Second, it must be able to support cell growth. Most mammalian cells only proliferate while adherent to a surface, and therefore it is necessary for the tissue engineering scaffold to have a surface which promotes or is conducive to cell adhesion. Once attached, the scaffold must allow the cells to continue to grow and function normally. Third, the scaffold must be able to guide and organize the cells in the desired manner and, fourth, it must have the capacity to deliver or permit ingrowth of a significant number of cells, maintained in a viable state by proper diffusion of nutrients and passage of waste. Finally, once the scaffold has fulfilled its purpose in providing a natural tissue replacement, it is no longer needed, and thus it is desirable for the scaffold to be degradable. The products of degradation must, however, be non-toxic and well tolerated. In addition to these five key properties, other properties specific to particular tissue engineering applications may be desirable or even essential. For example, certain tissues may have specific mechanical requirements that need to be provided by the scaffold until the new tissues are ready to take over these functions. Other tissue engineering applications may require scaffolds which can be derivatized by bioactive molecules. As such, no single biomaterial is able to fulfil all these needs, and thus there exists a need to have available a range of scaffold materials able to satisfy the particular requirements of specific tissue engineering applications.

2. PHAs in tissue engineering

To be useful as tissue engineering scaffolds, PHAs must possess the five key properties described above. They must be biocompatible, support cell growth, guide and organize the cells, allow tissue ingrowth and, ultimately, degrade to non-toxic products. If these key properties are met by the PHAs, then the additional benefits of using PHAs in tissue engineering are enormous. For example, unlike many other degradable polymers, the properties of PHAs can be tailored with a wide range of building blocks, various treatments can be used to attach bioactive factors, alter surface and mechanical properties, and a number of methods can be used to provide a range of degradation rates. Furthermore, scaffolds can be made using a wide range of fabrication techniques, which when coupled with the range of materials available provides an ability to design scaffolds that have different properties in different regions of the scaffold-like real tissue.

The biocompatibility of two PHA polymers, namely, PHB and PHBV [12] have been studied by a number of different research groups. The polymers have been reported to induce prolonged acute inflammatory responses as well as more severe chronic inflammatory responses when implanted in vivo [13]. Separately, it has also been reported that when a highly porous well-interconnected PHBV structure was seeded with fibroblasts, it sustained a cell proliferation rate similar to that observed in collagen sponges for 35 days, with a maximum cell density being observed on day 28 [14].

The potential use of PHAs in tissue engineering is illustrated in Fig. 2. Our approach to the development of PHAs for this application has begun with the development of methods to prepare PHAs suitable for use in tissue engineering and medical applications, biocompatibility testing, fabrication of the naturally occurring polymers into tissue engineering scaffolds, cell seeding of the scaffolds, and preliminary evaluation of the potential uses for PHAs in tissue engineering. Here, we describe some of our progress towards these ends.



Stages
1. PHA scaffold fabricated for application
2. Tissue specific cells obtained from biopsy or cell bank
3. Cells sorted, cultured and seeded into scaffold
4. Cells proliferate on scaffold and are implanted at tissue engineering site

Fig. 2. Role of PHAs in tissue engineering.

2.1. Production of medical grades of PHAs

Tissue engineered products comprising PHAs are likely to be classified by regulatory agencies, such as the US Food and Drug Administration (FDA), as medical devices, although it is possible that they could be regulated as biological products or drugs under certain circumstances. In any event, the specifications for PHAs used in these products will be extremely demanding.

Commercially available PHAs and the procedures described for their purification, which of course were not developed specifically for medical implant applications, fall short of the standards necessary to meet regulatory approval in the medical device industry for this class of applications. For example, these materials can contain residual protein, surfactants and/or high levels of endotoxin, a potent pyrogen. Foreign proteins present in PHAs may induce immune reactions, certain surfactants used in PHA processing are not approved for in vivo use, and exposure to endotoxin, an integral component of the outer cell surface of Gram-negative bacteria, can induce fever. In fact, the presence of bacterial endotoxin, a lipopolysaccharide, in medical polymers is reported to be one of the biggest concerns of suppliers [15]. The presence of endotoxin is known to be responsible not only for pyrogenicity, but also for complement activation which regulates the immune response, and mediates an acute inflammatory response. The FDA, for example, requires that the endotoxin content of medical devices should not exceed 20 US Pharmacopeia (USP) endotoxin units (KU) per device, except those devices that contact cerebrospinal fluid, where the content must not exceed 2.15 USP endotoxin units per device. Despite the voluminous literature describing production, purification, and applications development of PHAs, there are currently no reported methods specifically for depyrogenating PHA polymers.

At Metabolix, we have developed methods to prepare PHAs in high purity which we believe are suitable for use in medical applications such as tissue engineering. The goal of this effort was to devise processes for preparing a range of different PHAs which would meet the requirements set for biocompatibility by medical regulatory agencies. To this end, we have developed methods to depyrogenate PHAs and provide materials of high purity.

A typical procedure we have developed for preparing a high purity PHA for medical use involves depyrogenating the material using oxidizing agents. The use of oxidizing agents in depyrogenation is well known [15]; however, in the case of PHAs this method of treatment is only partially effective if it is applied to solid crystalline polymer. This is probably because the polymer entraps endotoxin during the extraction process, and effectively protects it from the oxidizing agent. Thus, although oxidizing agents have sometimes been used for other reasons in the processing of PHAs, these treatments fail to be effective as depyrogenation procedures because the endotoxin is sequestered. We have discovered, however, that if the PHA is in latex form, exposure to oxidizing agents is highly effective in removing endotoxin. The PHA latex is preferably derived directly from whole cells, before the PHA crystallizes, but may also be reconstituted from crystalline material. The choice of oxidizing agents is wide, and includes both inorganic and organic peroxides, like hydrogen peroxide, sodium hypochlorite, and benzoyl peroxide.

Certain oxidants, especially ozone, are very effective at eliminating the color of the extracted polymers, thought to be due to contaminating ubiquinones. Our preferred method at present employs hydrogen peroxide. Using this procedure we have reduced the endotoxin content of an elastomeric PHA copolymer, known as PHOH (which comprises both *R*-3-hydroxyoctanoate and *R*-3-hydroxyhexanoate units, in a ratio of approximately 9:1, and a third monomer, *R*-3-hydroxydecanoate, present at a fraction of a percent), from over 1 million EU/g to less than 6 EU/g, measured using the *Limulus* amebocyte lysate (LAL) test with the copolymer in latex form. We have also depyrogenated PHB, a representative semi-crystalline PHA prepared by fermentation, with this method, and obtained polymer with an endotoxin content of 0.12 EU/g, measured as a powder. This compares to a commercial sample of PHB with an endotoxin content greater than 120 EU/g.

In addition to devising methods to depyrogenate PHAs, we have also developed purification steps to remove other contaminants commonly found in PHA samples. Many reports describe the extraction of PHA polymers with chlorinated solvents. We have found that alternative solvents, particularly in the extraction of the more elastomeric PHA materials, such as PHOH (often simply referred to as polyhydroxyoctanoate), can often provide superior results. For example, when PHOH is extracted with hexane or acetone instead of a chlorinated solvent, and subsequently precipitated from a solvent solution like acetone by the addition of a non-solvent for the PHA, such as methanol, the resulting polymer can be obtained in high purity. Residual lipids, feedstocks, and other contaminants, are typically removed by this procedure. Any particulate matter present can be removed prior to these steps very effectively using glass microfiber filters.

Supercritical fluids are also a promising tool for the extraction of PHAs for medical use. Supercritical carbon dioxide is used increasingly in industry, for example for the extraction of fragrances and flavors or caffeine, the deinking of waste paper, or the removal of residual solvents from pharmaceutical preparations. Carbon dioxide is benign and easily removed from the product. We found that pure supercritical CO_2 is highly effective at extracting lipids and other hydrophobic contaminants from PHA-containing cells. Supercritical mixtures of CO_2 with conventional solvents or 'modifiers' may subsequently be used to extract the PHA cleanly and in good yield. Suitable modifiers can include ethyl acetate, hexane, THF, methylene chloride, and acetylene dichloride. PHAs have very high solubilities, e.g. 9%, in the supercritical mixtures. Supercritically extracted PHOH reached 100% purity in a single step and contained 25–150 times less endotoxin than PHOH prepared by conventional solvent extraction and recrystallization.

Using various combinations of these procedures it is possible to obtain PHA polymers with very high purities, as measured by gas chromatography and elemental analysis. The ultimate test of a PHA's biocompatibility, of course, is its behavior in vivo.

2.2. Biocompatibility testing of PHAs

In some initial studies, we have evaluated host–biomaterial interactions for the copolymer PHOH. The polymer was purified by solvent extraction methods, and several different types of implants were fabricated, including microspheres, tubes, and pellets (5 mm diameter and 2 mm thick). The PHOH devices were sterilized, and implantations were performed aseptically. Five cohorts of four mice were anesthetized and each animal received four subcutaneous implants. Implants were removed at 2, 4, 8, 12, and 40 weeks post-implantation, examined by SEM to assess the presence of microscopic degradation, and prepared for histological examination. Biocompatibility of the implants was evaluated by determining the degree of fibrotic reaction surrounding the samples and the presence of inflammatory cells.

After 2 weeks, all implants showed minimal tissue reaction. Histological analysis revealed that the pellets and tubes were encapsulated by a thin layer (four to six cell layers thick) of fibroblasts surrounded by collagen. This loose connective tissue could easily be removed. There was no evidence of any macrophages at the implant sites. At 4 and 8 weeks post-implant, the host reaction to the implants continued to be very mild, the implants were still intact with no visible signs of degradation. At 12 weeks, the amount of connective tissue adherent to the tubes and microspheres had not increased, and in particular the pellets proved to be especially inert as they were surrounded by an easily removable thin layer of loose connective tissue observed at earlier times. At 40 weeks, all implants were still intact but were somewhat more pliable upon handling. The amount of tissue adherent to the tubes and microspheres had not increased.

As well as determining the response of the host to an implant, we have also evaluated the sensitivity of the skin to PHA exposure. This would obviously be important in any tissue engineering applications involving skin, but it is also relevant to other applications. One of the standard test methods for assaying skin sensitization is described by ASTM F270, and this method was used to evaluate a representative PHA polymer, namely a sample of PHOH. In the procedure, a 60-cm^2 sample of the PHA polymer, 3 mm in thickness, was extracted with 20 ml saline at 50°C for 72 h. Guinea pigs, previously subjected to two-stage induction (21 days) were exposed to patches soaked in the saline extract, pure saline, or a positive control. After 24 h, the

patches were removed, and the guinea pigs were checked for a skin reaction after further time intervals of 1, 24 and 48 h. There was no discernible erythema/eschar formation with the PHOH extract or the pure saline; positive controls all showed well defined to severe erythema/eschar formation within 1 h. Overall, the results are consistent with the notion that the PHA polymers can be prepared in hypoallergenic forms.

2.3. Biodegradation of PHA biopolymers in vivo

The PHOH implants described above were examined for degradation during the course of the study using SEM. At 12 weeks, it was apparent, however, that the rate of degradation of the polymer implants in the subcutaneous position was slow. At 40 weeks, it was therefore decided that the molecular weight of two implants should be compared to a control sample which had not been implanted. After 40 weeks, the weight average molar masses, M_w, of two implants were 68 000 and 65 000, compared to 137 000 for the unimplanted control. The number average molar masses, M_n, of the implanted samples were both 31 000, compared to 58 000 for the control. Additional measurements were made to compare the molecular weights at the surface versus the interior of the implants. Notably, no significant differences were observed, suggesting a slow, homogeneous hydrolytic breakdown of the polymer.

For certain tissue engineering applications, a relatively slow degradation of the polymer may well be ideal. In other cases, a more rapid breakdown may be preferable. It would therefore be desirable if we could control the rate of PHA degradation in any location such that the rate could be anywhere from a matter of weeks to months to several years. Towards this end we are developing methods to prepare PHAs with more labile monomers that will hydrolyze faster and/or be more susceptible to enzymatic attack. For example, we have recently prepared a PHA copolymer comprising a 2-hydroxy acid, namely 2-hydroxybutanoic acid (Skraly F, Metabolix, unpublished results). We are also exploring subsequent polymer treatments and the use of additives to accomplish these same ends.

2.4. Fabrication of PHA scaffolds for tissue engineering

The fabrication of a suitable scaffold is a critical part of any tissue engineering application that incorporates the use of a degradable cell seeded material [16]. A significant amount of work has been focused on identifying suitable scaffold configurations for various applications which provide not only adequate sites for cellular attachment, but also allow adequate diffusion of nutrients and gases. In general, porous scaffolds which limit nutrient and gas diffusion distances through cell masses to less than 200–300 μm are considered ideal [17]. Greater diffusion distances tend to reduce cell viability, although optimum configurations are likely to vary on a case by case basis. It should also be noted that it may be desirable for only certain parts or surfaces of the scaffold to be porous.

It is well known that PHAs can be fabricated using a wide range of conventional polymer processing techniques which include solvent casting, fiber spinning, foaming, and melt processing techniques such as extrusion, injection molding, and in certain cases blow molding [12]. It is also possible to fabricate articles using PHAs in latex form. The fabricated articles can include molded shapes, films, fibers, woven and non-woven materials, tubes, composites, and so forth. If necessary, the PHAs may also be combined or blended with other polymers or materials to further improve properties, shape and/or performance. Together with the range of polymer options, the PHAs therefore provide a wealth of design space for fabricating tissue engineering scaffolds, and can be packed with useful chemical and physical information to provide independent control of the biological and mechanical properties of the scaffold.

We have used these techniques to fabricate porous PHA scaffolds, not only to optimize cell viability and growth under different circumstances, but also to provide PHA scaffolds tailored for specific tissue engineering applications. In one method, we have fabricated porous PHA scaffolds using leaching techniques [18]. This approach generally involves dispersing a leachable material within a scaffolding material, fabricating the required material shape, and then removing the leachable material to leave a porous scaffold. An example of a porous PHOH tube fabricated in this manner is shown in Fig. 3 together with a PHOH tube derived from a film. The PHOH material may also be subjected to the leaching technique prior to fabrication, and the scaffold subsequently processed into the desired device from the porous material. (Alternatives to the leaching technique for preparing porous PHA materials

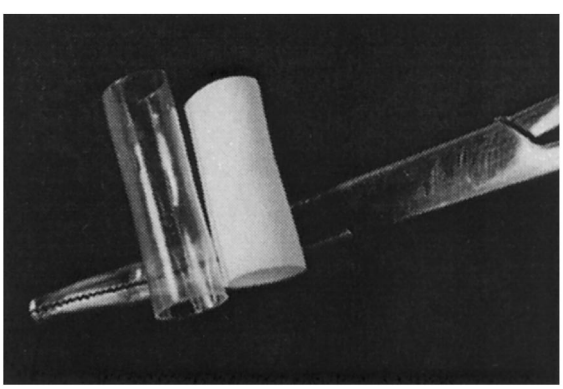


Fig. 3. Tubes derived from PHOH film (left) and porous PHOH (right).

include foaming, spinning, and fiber processing methods.)

In a typical procedure used to prepare a porous PHA tube using the leaching method, a PHOH polymer was melted and mixed with sieved salt particles in a weight ratio of 1:2 to yield a homogeneous mixture. The salt particle sizes in this instance were 80–180 μm; however, the particle size, distribution and weight percent may all be varied according to the desired pore size and density. The PHOH/salt mixture was pressed into a thin film, allowed to crystallize, and molded around a cylindrical Teflon™ support to produce a tube. The diameter of the support can be adjusted to produce tubes of any desired size. Finally, the tube was subjected to exhaustive leaching of the salt to yield a porous PHOH tube.

Other approaches to preparing suitable PHA tubes include extrusion, using leachable or nonleachable processing agents with the PHA if necessary, as well as spinning and fiber-based techniques which can be employed to make, for example, non-woven porous PHA fabrics for subsequent processing into tubes, or woven tubes directly. In the former case, for example, PHOH combined with a leachable material has been successfully extruded directly into a tube.

2.5. Surface modification of PHAs

Surface properties of any medical device are extremely important, since it is this surface that interacts with the host. In tissue engineering approaches that employ cell seeded scaffolds, however, it is not only necessary for the scaffold to be biocompatible and biodegradable, it is also essential that the surface is conducive to cell attachment and subsequent tissue growth. In the ideal case, it is therefore desirable to be able to adjust surface properties to suit the intended application, often without altering other properties of the scaffold, like its mechanical strength or thermal properties. Useful surface modifications could include changes in chemical group functionality, surface charge, hydrophobicity, hydrophilicity, and wettability. For instance, it may be necessary or desirable to modify the surface to improve or maximize cellular attachment. It could also be desirable to make a modification that would provide a selection for the desired cell type or types. The latter modifications, aimed at improving and optimizing cellular attachment, can be accomplished, for example, by attaching or coating the surface with a bioactive compound or peptide which promotes cellular attachment. The coating or bioactive compound may be attached to the surface either covalently or non-covalently as required.

One procedure we have employed to alter the surface of a PHA polymer, for the purposes described above, involves the use of gas plasma, a technique which typically results in covalent modification of the material's surface with the introduction of new functional groups. Thus, when a film of PHOH was treated with an ammonia gas plasma at 250 μm of ammonia with a flow of 350 SCCM and 220 W of power for 10 min, ESCA analysis indicated that the surface had been modified and stable incorporation of about 8% nitrogen was determined. Measurement of water contact angles indicated that the wettability of the surface had been increased significantly as a result of the gas plasma treatment. Before treatment the contact angle of the PHOH polymer was high (approximately 95°), whereas after treatment the angle had decreased dramatically to about 20–30°. Subsequent measurements of the contact angles of the treated and untreated samples and ESCA analysis confirmed that the surface modification was stable.

In subsequent experiments, the PHOH polymer modified by gas plasma treatment was further modified by attachment of biologically active compounds. Compounds which can be used in this procedure include growth factors designed to stimulate cell and tissue growth, cellular attachment factors to promote cell and tissue attachment, labeling agents to assist in locating and monitoring the implant, drug molecules to aid in tissue repair, as well as agents to improve biocompatibility, such as anti-coagulants to prevent thrombogenesis. To demonstrate the approach in general, a gas plasma-modified PHOH was treated with an activated form of biotin, a biologically active compound, comprising an *N*-hydroxysuccinimide ester able to undergo acylation reactions at the polymer surface with amino groups. After treatment the film surface was washed, quenched, blocked, and assayed using a biotin-specific chemi-luminescent assay to demonstrate that surface modification with biotin had taken place. A PHOH polymer which had not been modified by gas plasma treatment served as a control and was not modified by the activated biotin under the same conditions.

In addition to using gas plasma to modify the surface of PHA polymers, particularly to incorporate new functionality, it is of course possible to directly prepare PHA polymers with functionalized pendant groups [19,20]. These groups can be subsequently modified using similar procedures as those described above. In addition to gas plasma treatment, it is also possible to expose the surface of the polymer to other reactive reagents, including acids, bases, and other chemical reagents, for example, nucleophiles, to induce surface modification. For example, we and others [21] have used pH treatments to modify the surface of PHA polymers to liberate acid groups or charged species which can promote cellular attachment and cell proliferation. We have also used amines to modify the surfaces of PHA polymers. Whatever the chosen method, it is apparent that the surfaces can be readily modified and tailored to suit the particular needs of a given tissue engineering or medical application.

2.6. Cell seeding of PHA scaffolds

Depending upon the chosen approach, PHA scaffolds can either be seeded with cells prior to use or simply implanted directly. In the former case, cell seeding can enhance biocompatibility, promote the growth of the desired tissue and generally be beneficial in helping to provide and restore the lost function. The cells used to seed the PHA scaffolds should be chosen from the appropriate type of tissue and are preferably harvested from the patient to minimize tissue rejection and reduce the likelihood of disease transmission. However, cells may also be obtained from a cell bank or derived by genetic engineering techniques. Prior to seeding, the PHA polymers may be coated with bioactive compounds such as cell attachment proteins as described previously. This can help to assist cell attachment and tissue growth. After seeding, the cells can be grown in vitro until the desired viability for a particular application is obtained, at which point the seeded scaffolds are ready for implantation. In an example, a porous PHOH scaffold was prepared as described herein, sterilized, and seeded with ovine smooth muscle cells. The seeded scaffold was incubated in cell culture medium in vitro at 37°C with 5% carbon dioxide. Subsequent microscopic examination of the scaffold indicated good biocompatibity of the scaffold and good cellular attachment to the scaffold. Similarly, a wide range of different cell types can be seeded onto PHA scaffolds.

3. Applications for PHAs in tissue engineering

Sufficient data has now been generated to indicate that the PHA biopolymers promise to have a significant role in tissue engineering and the development of living tissue products for therapeutic applications. On account of their varied and diverse properties, the PHA biopolymers provide a means to target a wide range of tissues with potential product applications for the cardiovascular system, cornea, pancreas, gastrointestinal system, kidney and genitourinary system, musculoskeletal system, nervous system, teeth and oral cavity, skin, and so forth.

Our approach to developing PHAs for these applications is based on an examination of the scaffold requirements, our ability to meet these requirements from the PHA family, the uniqueness of the opportunity, and related considerations. In some cases, for instance, it is apparent that certain PHA biopolymers, such as the elastomeric materials and/or those with high tensile strength, have properties that are not only well suited or perhaps critically important to the application, but are unmatched and unmet by other alternatives. The elastomeric materials, for example, are currently thought to be well suited to tissue engineering applications of cardiovascular tissue. In these types of applications, the tissue scaffolds, particularly in the form of tubes or films, must have good flexibility and be able to resist the mechanical forces exerted either from the surrounding tissue or from the body fluids contained within. In addition to resisting mechanical forces, these types of scaffolds must also provide good barrier properties to fluids, particularly on luminal surfaces. Two of the potential opportunities for PHA polymers in the cardiovascular area are described below in more detail. Studies in both these areas are currently being undertaken at Children's Hospital in Boston.

3.1. Vascular grafts

Vascular grafts are currently inserted to repair or replace compromised blood vessels, in the arterial or venous systems, that have been subject to damage or disease such as atherosclerosis, aneurysmal disease, and traumatic injury. Currently, there are three grafting options, namely, an autograft, a synthetic graft, or a cryopreserved graft when an autograft is not available. The choice between an autograft and a synthetic graft depends upon a number of factors. In general, synthetic grafts are restricted to applications involving the replacement of large and medium size vessels, where high flow rates and low outflow resistance provide 85–95% 5-year patency (open to blood flow) rates.

For coronary artery bypass grafting (CABG), the most common open-heart surgical procedure, small diameter vascular grafts are required. Currently, there are no synthetic vascular grafts approved for this procedure since failure typically results from graft occlusion. When synthetic vascular grafts cannot be used, the preferred procedure involves the use of an autograft, which entails a second traumatic surgical procedure to harvest a suitable artery or vein from the patient. In the case of CABG, surgeons harvest two main types of grafts from the patient: arterial grafts, primarily the internal mammary and radial arteries; and, venous grafts, normally saphenous veins, from the legs.

It has been estimated that 40% of CABG patients receiving saphenous vein bypasses will require subsequent intervention within 10 years of the original operation [22]. For this reason, and the severe morbidity affiliated with harvesting the saphenous vein (an operation involving a long incision from the groin area to the knee), there is a strong need to develop a small diameter vascular graft product particularly for CABG procedures, and below the knee grafting procedures, which will remain patent.

One approach to the problem has been to seed synthetic grafts with endothelial cells to provide a more natural graft/blood interface [23]. Normal endothelial cells, which line blood vessels, produce various factors

which contribute to the endothelial cells' anticoagulant activity, and possess a negative outer charge on the cells that repel platelet adherence. This approach has been successful at decreasing platelet deposition but has not reduced the pseudointimal hyperplasia, which is believed to result in part from a time-limited inflammatory response by the body to the foreign graft implant. A PHA-based tissue engineered vascular graft could well address this issue since the prosthetic material in the degradable graft will eventually be absent.

To achieve this end, PHA tissue engineered grafts are being developed that can provide the necessary near term structural and barrier support, and comprise confluent endothelial linings to maximize acceptance of the graft upon implantation. One of the main issues in this undertaking is the need to balance timely tissue ingrowth with the requirement for time-dependent structural stability. If degradation proceeds too fast, the in-growing cellular and protein matrix may not be able to assume the load bearing and blood transporting functions ultimately required. If degradation is too slow, the degradable prosthetic material may be recognized as foreign, leading to an inflammatory response. Through careful screening of PHA options, we are developing materials which meet these requirements, as well as the need for the graft to be flexible, able to withstand bursting pressures in the aortic system, and prevent aneurysmal dilation.

The approach is anticipated to yield small diameter tissue engineered PHA grafts which can improve the current dismal patency rates of existing synthetic grafts in this size range, and provide a better alternative to the use of autologous and cryopreserved grafts. These new grafts may also ultimately facilitate controlled angiogenesis in providing a blood supply to artificial organs.

The potential demand for a small diameter vascular graft is considerable. For example, it has been estimated that there were 404 000 CABG procedures performed in the US in 1996, and 621 000 worldwide [22]. In addition to the CABG procedures, there are around 120 000 peripheral vascular reconstructive procedures performed in the US each year. It is estimated that about 70 000 of these procedures are carried out below the knee, and that 30% (22 000) of these procedures are secondary or reoperations requiring a substitute graft [24].

3.2. Heart valves

The unidirectional flow of blood through the entire circulatory system is controlled by the heart's valves. Humans have a total of four heart valves. These valves are known as the tricuspid valve, the pulmonary valve, the mitral valve, and the aortic valve. With the exception of the mitral valve which has just two cusps (or leaflets), each valve has three cusps which are forced open and shut by differences in pressure within the heart.

Valvular heart disease, which is characterized by a defective heart valve, impairs the ability of the heart to function properly. This can be caused by degenerative processes, congenital defects, bacterial endocarditis, or rheumatic fever, and results in oscillations of a patient's blood pressure and circulation, leading to heart murmurs, heart failure, or myocardial infarction.

Currently, there are a number of different methodologies employed to treat heart valve disease, including drug treatments, valve repair and valve replacement. In non-life-threatening situations, drugs used in the treatment of congestive heart failure are usually employed first to make the heart work harder and pump blood throughout the body. However, once valvular disease progresses to the point at which the heart's ability to pump blood is significantly impaired, surgery is usually recommended to repair or replace the diseased valve. Many surgeons prefer to repair a heart valve when possible; however, in many cases this is either not possible or the benefits are short lived.

Valvular replacement surgery is a traumatic procedure which involves placing a patient on cardiopulmonary bypass while the diseased valve is replaced with an artificial valve prosthesis. The procedure is only undertaken after it has been decided that a native heart valve is beyond repair. There are currently two primary types of artificial valve prostheses: mechanical heart valves and tissue heart valves [25]. Each type has benefits and drawbacks. Mechanical valves, for example, are noted for their durability and reliability. However, a major drawback is the need for the recipient to be placed upon a lifelong anticoagulant therapy which involves continuous monitoring of anticoagulant levels. (Often recipients must have their anticoagulant levels checked by a physician every 3–4 weeks.) Other disadvantages of the mechanical heart valve include turbulent flow, noise and catastrophic failure modes. Current tissue valves, derived from heterologous sources, on the other hand, do not require anticoagulant therapy, they are quiet, provide physiological flow patterns, and typically have slowly developing rather than catastrophic failure modes. The major problem associated with these valves is their lack of durability. Most of the current tissue valves generally last between 5 and 15 years before they need to be replaced due to a gradual deterioration of the tissue resulting from calcification, which leads to a loss in tensile strength of the material. It should be noted that these tissue valves comprise non-living tissues, normally derived from bovine or porcine sources, which have been crosslinked by treatment with glutaraldehyde to decrease their immunogenicity. Despite such treatments, these valves can, however, be a potential source of disease transmission, passing viruses from the donor to the recipient.

Most experts agree that if the durability problem can be solved, tissue valves would be the clear choice for treatment of valvular heart disease—no synthetic material has proven to have the properties needed to endure bi-directional flexing some 40 million times a year without producing thrombosis. Furthermore, mechanical valves cannot be used to repair valve leaflets.

The advantages of developing a tissue engineered heart valve would be several-fold. First, the ultimate product would be a durable living heart valve able to withstand the demands of the body. It can be derived from non-immunogenic tissue obviating the need for anticoagulant therapy, furthermore, the tissue can be derived from an autologous source virtually eliminating the risk of disease transmission. In the case of infants and children where growth is a concern, the use of a living tissue valve would remove the need to replace the valve as the patient grows. Finally, in cases where repair rather than replacement is preferable, the tissue engineering solution would potentially provide a source of suitable living tissue.

Researchers have already begun to use tissue engineering as a means to develop living tissue heart valves [26,27]. In representative procedures used so far, the two types of cells which make up a heart valve, namely myofibroblasts and endothelial cells, have been derived by culturing and sorting tissue derived from a patient's femoral artery. The myofibroblasts were subsequently seeded onto a synthetic degradable fiber scaffold and overlaid with a grid of synthetic degradable material. The myofibroblasts proliferated, spreading through the scaffold, and achieved full permeation of the scaffold within about 2 weeks. Endothelial cells were seeded on top and multiplied quickly to form a single layer. The scaffolds were then implanted in juvenile sheep. While these experiments established the feasibility of the approach and represent a major breakthrough in the development of a tissue engineered heart valve, the scaffolds used were not well suited to the task, being relatively inelastic materials unable to support the near-term functions of the heart valve leaflets.

The use of PHA polymers to develop a living tissue heart valve could provide, in the short term, the properties required to substitute for the native valve's properties, providing for example, the valve with sufficient mechanical strength to endure the repetitive bi-directional flexing. Ultimately, these PHA biomaterials will degrade as the new tissue regenerates leaving a new living replacement valve. The ideal PHA biomaterial will thus have a finely tuned flexibility, durability, and tensile strength, which phases out by degradation, as the mechanical properties of the new tissue phase in, leading to a functioning new living heart valve.

As with vascular grafting, the potential markets for a PHA-based tissue engineered heart valve are substantial. The total worldwide market for all heart valves in 1995 was $550 million, representing approximately 220 000 procedures.

4. Conclusion

Until now, the range of biomaterials available for use in tissue engineering as temporary three-dimensional scaffolds, which can support cell growth and then degrade away leaving viable tissue, has been limited. To a degree, this has slowed the more widespread application of tissue engineering, particularly in areas where there is a significant mismatch between the physical and mechanical properties of the target tissue and the available biomaterials. The PHA polymers promise to extend significantly the range of biomaterials suitable for tissue engineering.

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