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POLY(HYDROXYBUTYRATE) AND NORBIXIN AS BIOMATERIALS IN BIOLOGICAL APPLICATIONS

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Abstract. This review presents the survey of performance of biomaterials prepared using norbixina and poly(hydroxybutyrate) as biomaterials in biological applications. Biomaterials can replace and/or stimulate biological systems that no longer have their functions, such as the restoration of tissue functions. In the field of biomaterials, norbixin is an antioxidant dicarboxylic carotenoid found in *Bixa orellana L*. and poly(hydroxybutyrate) (PHB) is a natural polymer that has characteristics of biodegradability and biocompatibility. There is a shortage of studies involving such constituents together as biomembranes in tissue healing with repair of cutaneous wounds or bone regeneration, although both present promising research that suggests its effectiveness as a guide for tissue regeneration.

1. INTRODUCTION

Progress and scientific research in the area of Material Science and Engineering leads to the acquisition and discovery of new materials, new properties and new applications, optimizing the cost / benefit ratio, improving performance and quality of materials in several sectors from automotive to pharmaceuticals [1].

In recent years, such progress has reached the area of health, which allied to Materials Engineering from research that sought to develop materials and use them in biological environment (biomaterials). This has allowed a great evolution of the techniques of total or partial reconstitution of organs and tissues [2].

The biomaterials science has undergone great evolution, thanks to the multidisciplinary scientific and technological development of several areas such as Medicine, Dentistry, Biology, Engineering, Physics and Chemistry. Examples of materials used in biomedical applications are biosensors, blood circulation tubes, hemodialysis systems, implantable materials (sutures, plaques, heart valves, teeth), devices for the controlled release of drugs, artificial organs and dressings [2,3].

When applied in cutaneous wounds, biomaterials can act in the substitution and/or stimulation of tissues that have lost functions due to loss of skin continuity. And these substances must have physical, chemical and biological properties necessary for healing treatments that lead to better repair and regeneration guided in a satisfactory manner. For this, it is necessary to know about the interactions between biomaterials, biological tissues and performance in clinical use [1,2,4].

The good performance of biomaterials in living organisms depends on important properties such as biocompatibility, biofunctionality, biodegradabil-

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ity and the capacity of dissolution in the organism, in order to obtain good tissue interaction consistent with the healing process [5].

In this context, poly (hydroxybutyrate) (PHB), a natural polymer with a unique biodegradable thermoplastic characteristic, produced by bacteria from renewable carbon sources such as sugarcane, has been used in the manufacture of membranes for use in biological systems [1]. PHB is a linear, partially crystalline saturated polyester with properties similar to petroleum-based plastics such as polyethylene and polypropylene. The poly (hydroxybutyrate) has consistency as a biomaterial and bioreactorable bioreabsorbable polymeric plasticizer action and can be used in biological membrane models [6-9].

PHB in membranes may be associated with several biomaterials with important properties in tissue healing and bone repair. Poly (hydroxybutyrate) may be associated with hydroxyapatite, collagen and other natural materials, such as norbixin (dicarboxylic acid extracted from Bixa orellana).

Norbixin, an apocarotenoid extracted from annatto (*Bixa orellana* L.), a plant native to tropical America, has great potential for use in biodegradable and biocompatible polymer membranes, due to antioxidant, antimicrobial and antitumor properties. Compounds extracted from Bixa orellana L. are widely used in the food and textile industry as natural dyes and in dyeing fabrics. Additionally, carotenoids have the ability to react against free radicals and animal studies have shown the absence of genotoxic, teratogenic or mutagenic effects of these materials [10-13].

This review presents the survey of performance of biocomposites prepared using norbixina and poly(hydroxybutyrate) as biomaterials in biological applications.

2. USE OF BIOMATERIALS IN BIOLOGICAL APPLICATIONS

Materials engineering has as main objective to develop materials that can be used in the most diverse areas of human activity. Materials or devices that come into contact and / or perform some function in a biological environment are called biomaterials [1]. Biomaterials are devices that come into contact with biological systems, with diagnostic, vaccine, surgical or therapeutic applications, and are composed of compounds of natural or synthetic origin, as well as chemically modified natural materials, having a great diversity of these products used in the health area [3].

Regarding the applications of biomaterials, in general, when they are used to stimulate a certain function, it is important to know its intrinsic properties and internal structure. For example, when a biomaterial is used to regenerate a functional tissue, in addition to the ability to replace damaged tissue, it must be capable of providing adequate mechanical support to the patient, as well as being biocompatible with tissue and presenting low implant rejection by the organism [14,15].

Because of this, the idealization of new biomaterials aimed at facilitating the tissue healing process, in the field of materials used for biological purposes, requires a control over the biomaterial-tissue interface, that is, between living and non-living tissues. The knowledge of the toxicity of the material, or minimal toxic response during replacement of the host tissue is also essential and can be assessed by tissue culture testing or short-term implantation [3].

The use of implants to replace or repair parts of the human body developed in recent years is related to the increase in life expectancy and consequences of aging, traffic accidents, sports injuries, among others, seeking to replace them, repair them or reconstitute them. Tissue engineering aims to create and improve new biomaterials resulting in reduced hospital costs and better quality of life [15,16].

For Oréfice, Pereira and Mansur [17], the biomaterial can respond to living tissue in different ways, either in an almost inert form (the implant is encapsulated by fibrous tissue and there is no direct interaction), with a porous surface (associated with cellular supply and tissue vitality), reabsorbable (the invading material is replaced by tissues) or material with superficial reactivity (controlled chemical reactivity in the physiological system, such as in the release of drugs).

The physical properties of most tissues can be determined according to the careful selection of materials isolated or combined for the purpose that will be employed. Some problems or variations in this biomaterial-tissue interface may be associated with different properties of the biomaterials, especially with respect to their microstructure, such as the presence of pores and their size, and the chemical interactions that occur in the material reabsorption process (if there is) [14,16].

Polymeric biomaterials (biopolymers) are macromolecules made up of mere (constituent units of the polymers) which usually have low density, low temperature resistance and low electrical and thermal

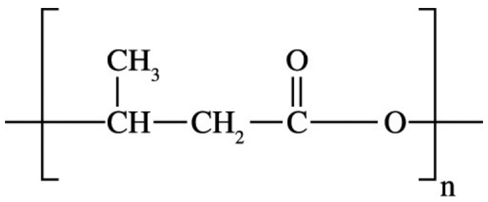

Fig. 1. The chemical structure of the PHB.

conductivity. Due to the physicochemical characteristics of biopolymers, structural versatility and origin, they can be classified into natural biopolymers (polymers derived from proteins and plants) or synthetic biopolymers (which in turn may be non-biodegradable synthetic thermoplastics, such as polyethylene and polypropylene, or biodegradable synthetic thermoplastics, such as poly (lactic acid) and poly (hydroxybutyrate) [17,18].

There is a wide variety of biopolymers in biomedical applications, whether natural or synthetic, biodegradable or stable in the body environment. The scientific environment evolves and new studies seek to modify other biomaterials from existing ones, enhancing their properties, or using other purposes.

A biomaterial from a non-synthetic biopolymer (PHB) incorporated with a natural material (norbixin), starts from the need to synthesize new biomaterials from natural resources with promising application in the healing of cutaneous wounds and bone repair, if necessary the knowledge of their behavior in biological environment.

2.1. Physico-chemical characteristics of poly(hydroxybutyrate)

Among the biopolymers used in biomedical applications, poly(hydroxybutyrate) (PHB) ($C_4H_6O_2$), is a linear, partially crystalline saturated polyester polymer with the unique characteristic of being a biodegradable synthetic thermoplastic, which has been widely employed as a matrix in systems [6,8]. Polymers of this nature, when implanted in living systems, do not require subsequent removal of the body and do not cause undesirable effects in the long term, since they present a reduction of molar mass when in contact with the body environment [17]. The chemical structure of PHB is set forth in Fig. 1.

The main properties of PHB polymer are: totally non-polar constitution; insolubility in water; crystallization around 55-80%; glass transition temperature of about 5 °C and melting temperature, 175 °C; low thermal stability with decrease in molecular weight above 170 °C; total degradation in a single step between 225 and 300 °C in thermogravimetric analysis [19].

PHB belongs to the family of polyhydroxyacids, polymers with high capacity to be used as biomaterials and with high potential for biocompatibility and bioreabsorption [6,7]; is also included in the class of polyhydroxyalkanes, which are polymers synthesized by bacteria in the presence of renewable carbon sources, such as sugarcane, with properties similar to petroleum-based plastics such as polyethylene and polypropylene. Therefore, the production of biodegradable plastics, such as PHB, can be a response to problems related to the accumulation of plastic waste in the environment [7].

There are increasing researches involving the substitution of conventional synthetic polymers with biodegradable polymers. In applications related to the environment and sustainable development, PHB is commonly used in biocomposites as a matrix for bioplastics and, because it is hard and brittle, is generally reinforced with treated plant fibers for better adhesion and optimization of its properties, as in studies by Machado et al. [19] and Melo et al. [8].

As for the application as biomaterial, biodegradable synthetic thermoplastic materials such as PHB have been used in sutures, as devices for controlled drug distribution, in the fixation of orthopedic devices (screws and osteosynthesis plates), in adhesion prevention, as a matrix for engineering of tissues (supporting growth of new tissues, guiding tissue response, promoting cell attachment and proliferation, controlling the inflammatory and immune response) [17,20].

2.2. Physico-chemical characteristics of norbixin

Norbixin and bixin are the main pigments extracted from the pericarp of annatto seeds (Bixa orellana), a tree native to Central and South America. Bixin ($C_{25}H_{30}O_4$) accounts for 80% of the carotenoid pigments found in annatto and is chemically characterized as chain isoprene of 25 carbons containing a carboxylic acid and a methyl ester at the ends. However, norbixin ($C_{24}H_{28}O_4$) is found in smaller quantities and, because of this, is generally obtained from the saponification of bixin in the form of its demethylated derivative, that is, its chain has only 24 carbons [21-23]. The chemical structures of these two pigments are shown in Fig. 2.

The active pigments of Bixa orellana L., in particular bixin and norbixin, are also known as

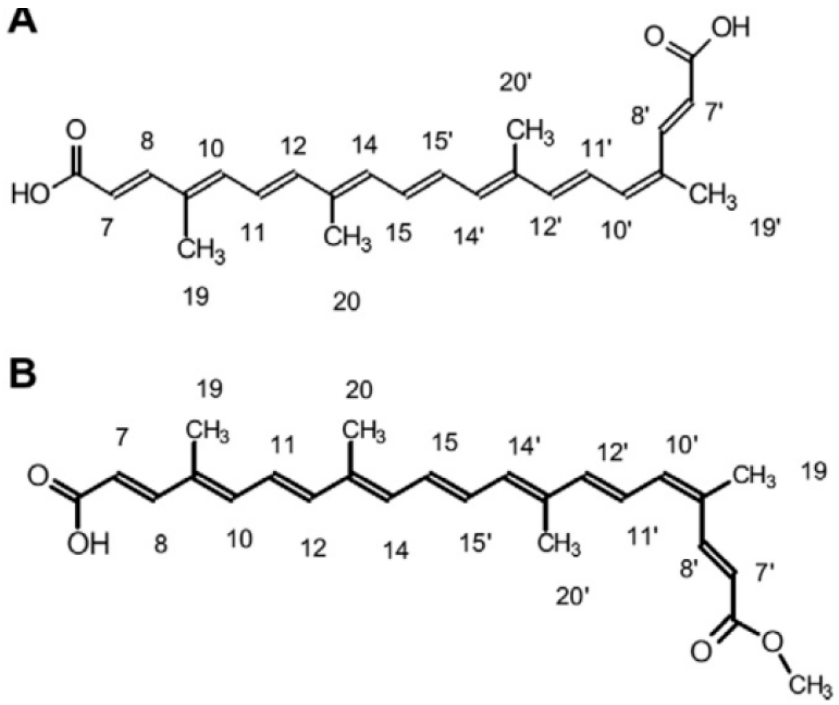

Fig. 2. Chemical structure of carotenoids present in Bixa orellana L.: a)cis-norbixin and b)cis-bixin.

carotenoids or apocarotenoids, that is, they have molecular organization of less than 40 carbons with conjugated double bonds with the potential to extinguish free radicals, and form a commercial dye known as annatto, which is widely used in the food and textile industry as a natural dye and for dyeing fabrics [13]. On the other hand, there are increasing studies about their potential for biological purposes and against certain microorganisms [22,23].

Norbixin is a naturally occurring, water-soluble (dicarboxylic acid) apocarotenoid that has antioxidant, antimicrobial and antitumor properties, which can influence and optimize tissue healing processes [11]. In the literature, several studies have been reported on the evaluation of the genotoxic, teratogenic or mutagenic effects of this pigment, which aimed to evaluate the properties of its interactions with biological media in animals [11,12,24,25].

2.3. Biological applications of poly (hydroxybutyrate)

Genotoxicity studies are in vitro and in vivo tests used to detect the potential of substances under investigation to cause gene mutations and chromosomal changes and must be performed prior to biological applications. These studies are very important in materials that will be used for health in an organism. Genotoxicity tests should be able to assess the potential damage to DNA. These damages can be observed in the form of genetic mutations and numerical or structural chromosomal changes, that is, they allow the prediction of carcinogenic potential. For in vivo tests, micronucleus test, comet assay and chromosomal aberrations are examples of recommended tests [26,27].

In the literature, research involving biological applications of polymers, such as poly (hydroxybutyrate), in a biological environment, including genotoxicity evaluation, is available, considering the importance of evaluating its biocompatibility [25,28]. However, there is a shortage of studies involving such constituent as biomembrane in tissue and / or bone healing.

In this context, regarding the evaluation of the genotoxicity of poly (hydroxybutyrate) as biomembrane in biological environment, was observed in the study of Sousa et al. [25], in which the 5% PHB / Norbixin membrane (with addition of ethylene glycol as regent) was tested, that such membrane did not present genotoxic effect by means of the micronucleus and comet assay, respectively, in the bone marrow and peripheral blood of *Rattus novergicus*, after 72 hours of the experiment, which involved a laparotomy with implant of the biomaterial in the peritoneum of these animals.

Poly (hydroxybutyrate-co-hydroxyvalerate) (PHBV) scaffolds associated with adipose tissue derived stem cells (ASCs) were implanted into cutaneous wounds on the backs of 27 rats in the study of Zonari et al. [28], and enabled bioactive plaques necessary to improve wound healing and reduction of cutaneous scar thickness, as well as demonstrating sufficient mechanical properties to support the contraction of the wound, with complete degradation of scaffolds at 28 days. The dermis formed had a more complex collagen structure, with presence of growth factors, mainly with the presence of ASCs. The combination of these two elements promoted bioactive clues: PHBV was able to guide the healing process of wounds, as well as the presence of ASCs optimized vascularization and skin quality.

In the study by Ali et al. [29], PHB was considered non-genotoxic and did not alter the expression of proto-oncogenes and antiapoptotic genes; different concentrations of PHB (not exceeding 5 mg/ plate) were incubated and used in in vitro mutagenicity assays in Salmonella and human fibroblast strains for 1, 12, 24, and 48h, separately for analysis of gene expression.

Researchers such as Esposito et al. [7] performed in vitro assay with PHB. Poly (p-dioxane) / poly (hydroxybutyrate) (PPD / PHB) blends were developed and tested with fibrochondrocytes for application in cartilage tissues and had no cytotoxicity. It was verified by the MTT analysis that the blends allowed adhesion and proliferation of fibrochondrocytes in several variations of their composition, as well as the ability to maintain the synthesis of the extracellular matrix of collagen. They concluded that such blends may be suitable for cell culture.

Gredes et al. [30] evaluated the osteogenic potential of pure PHB patches in surgically created cranial defects. This work has demonstrated that PHB membranes can act as matrices for cell migration,proliferation,differentiation, and vascularization in the process of bone healing. After 4 weeks, the PHB patches were completely embedded in connective tissue. Eight weeks after PHB insertion, bone regeneration proceeding from bearing bone was found in 50% of all treated animals, whereas all PHB treated cavities showed both bone formation and embedding of the patches in bone 12 weeks after surgery and all slices showed pronounced development of blood vessels. The RT-PCR analyses showed significantly lower expressions of genes encoding growth factors and osteogenic differentiation markers (Alpl, Col1a1 and VEGFA) in cranium defects after treatment with PHB patches compared to untreated bony defects.

PHB has been promising in the osteoinduction and engineering of bone tissue and tissue healing, especially when associated with the hydroxyvalerate copolymer (PHBV). Composites of PHBV, silk fibroin and nanohydroxyapatite (*n*HAp) were made using the electrospun technique. The Scanning Electron Microscopy (SEM), Energy Dispersive X-Ray (EDX) and Raman analyzes confirmed the deposition of nHAp with formation of apatite layer on the surface of PHB fibers and coating them, after 28 days of immersion in body fluid simulation solution, proving their bioactivity. *In vitro* tests, biological activity was demonstrated after 1 and 3 days of culture of human osteoblasts in the synthesized scaffold, since the cells presented flat and elongated morphology on the surface of scaffolds fibers [31].

In the work by Sadat-Shojai et al. [32], a novel hybrid scaffold system with electrospun mat based on biodegradable polyhydroxybutyrate (PHB) and hydroxyapatite (HAp) was combined with a protein based hydrogel in a single tri-layered scaffold and were observed bone cells inside the scaffold highly viable and infiltrated into the electrospun center after 14 days of encapsulation, besides increasing the mechanical properties of the scaffold.

However, in the study by Celarek et al. [33], we observed the mechanical stability deficit of PHB compared to metal alloys and questioned their suitability for use in osteosynthesis implants. Biodegradable materials such as pure PHB, PHB with addition of ZrO_2 and bone graft substitute, and crystalline and amorphous magnesium alloys were tested and compared for their suitability for use in osteosynthesis implants. Traction tests were performed after several implantation times on the femoral metastasis of rats to evaluate the shear forces between the implant material and the bone. The most promising materials were WZ21 (MgYZnCa crystalline alloy with slow degradation rate) and BMG (MgZnCa amorphous alloy), because they had high shear forces and tensile energies. PHB exhibited insufficient mechanical properties: it degraded very slowly and with low shear forces, in addition to unsatisfactory discharge energy levels.

2.4. Biological applications of norbixin

Bixa orellana L., and its derivatives, such as apocarotenoids bixin and norbixin, are widely used as food additives, especially as condiments and dyes. The literature has few studies aimed at biological applications of norbixin in terms of tissue healing and bone repair, but its potential for interaction with DNA with promising natural therapeutic effect of antioxidant protection is well known [34].

Furthermore, according to Costa [21], norbixin has no deleterious systemic or toxic effects on hepatic, renal and bone marrow cells. In this context, regarding the evaluation of the genotoxicity of norbixin as biomembrane in biological environment, in the study of Monte et al. [24], the polystyrene / collagen / norbixin membrane did not present a genotoxic effect by means of the micronucleus and comet assays, respectively in the bone marrow and peripheral blood of Rattus novergicus, after 72 hours of the experiment, which involved a laparotomy with biomaterial in the peritoneum of these animals.

Sousa Júnior et al. [22] studied the effect of norbixin on the response of Escherichia coli cells to DNA damage induced by ultraviolet (UV) radiation, hydrogen peroxide (H_2O_2) and superoxide (O_2) anions. It was observed that cells cultured in norbixin have greater protection against these agents, with an increase in survival of at least 10 times more. Antimutagenic properties of norbixin were observed with the 87% inhibition in the *Salmonella* mutagenicity test.

In the study by Muthukumar et al. [11], a composite with collagen microspheres and extracts of Bixa orellana L. (obtained from the leaves) was prepared, which was characterized in physicochemical properties and in vitro application in MCF-7 cell lines. Regarding the spectroscopic analysis with Fourier Transformed (FTIR), the collagen microspheres presented amides (protein-specific peaks); the *Bixa orellana* L. extract presented alcohol, alkane, phenolic and aliphatic amines groups; the collagen microspheres with *Bixa orellana* L. extracts incorporated showed characteristic peaks of both, indicating their incorporation. The SEM also confirmed the findings of FTIR when revealing coating the extract on the surface of collagen microspheres by the simple adsorption method.

Still in relation to the study of Muthukumar et al. [11] for biological applications, antimicrobial properties of this composite were observed with collagen microspheres and extracts of Bixa orellana L. against *Staphylococcus aureus* and *Escherichia coli*; for the anticancer properties, the MCF-7 cell line presented 83.46% cell death in 1000 μg/field.

Barcelos et al. [35] aimed to evaluate the protective effect of carotenoids present in Annatto seeds (urucum extract) against DNA damage caused by metals, such as mercury, in rats. The animals were divided into 14 groups (6 animals/group) according to the treatment with mercury (30 μg/kg), bixin (0.1-10 mg/kg), norbixin (0.1-10 mg/kg) applied at different concentrations within the 45-day interval to determine glutathione (GSH) levels, catalase activities, and DNA damage of hepatocytes and leukocytes by cell-gel electrophoresis assays. Metal treatment reduced GSH levels and induced DNA data; already the intermediate and higher concentrations of the carotenoids showed protective effect against damages to the same. They concluded that the consumption of carotenoids, bixin and norbixin, can protect the animals from adverse health effects caused by exposure to organic mercury.

Norbixin has shown in another study a reduction of A2E accumulation in retinal cell culture of orally treated pigs for 3 months, and may be a promising antioxidant in the treatment of macular degeneration [36].

In investigating the effect of bixin on DNA damage and pre-neoplastic lesions induced by 1,2-dimethylhydrazine (DMH) on the liver and colon of Wistar rats treated with daily doses of bixin per gavage for 7 days, Bixin reduced the damage to hepatocyte DNA, but there were no conclusive results regarding the protective effect against colon carcinogenesis [37].

In order to determine the potential for elimination of free radicals and their antimalarial activity, samples of the ethanol extract of *Allamanda cathartica* and *Bixa orellana L*. leaves were administered in albino rats for 2 days by gavage and their effects were compared with placebo and vitamin E (positive control). *A. cathartica*, presented phytochemicals that recorded important antioxidant defense activities for blood and liver tissues. However, *B. orellana* did not record similar results [38].

The cytotoxic and antibacterial effects of methanolic extracts of *Bixa orellana* L. seeds and leaves were tested in vitro against *Streptococcus mutans* (ATCC 25175) (*S. mutans*) and *Streptococcus sanguinis* (ATCC 10556) (*S. sanguinis*). The experimental findings demonstrated the antibacterial effect of the methanolic extract of *B. orellana* (achiote) on *S. mutans* and *S. sanguinis*, especially of the extract from the leaves. However, at high concentrations, leaf and seed extracts are cytotoxic [39].

In terms of skin protection, we know of the inhibitory effect of high concentrations of bixin and norbixin against excessive expression of tyrosinase (metalloenzyme that acts on melanin synthesis) and consequent diseases of hyper pigmentation [40]. In addition, Tao et al. [41] observed the cutaneous cytoprotection of bixin in the regulation of the expression of genes in human keratinocytes as a function of the transcription factor NRF2, constituting a molecular photoprotective strategy of carcinogenesis.

However, in the histomorphometric evaluation of the aqueous extract of urucum (containing only 2.5% norbixin) in the healing process of cutaneous wounds of rats (skin wounds were induced on the back of 32 Wistar rats, which were treated topically with extract of the *annatto*), the authors concluded that this extract is not innocuous to the cutaneous tissues and that it has pro-inflammatory and pro-angiogenic effects during the tissue repair process, being able to interfere negatively in the physiological process of cicatrization, but there is no delay in the process of reepithelialization and fibroplasia [12].

In this study Santos et al. [12], the use of a commercial dye with a low norbixin content probably interfered with the results, since it is known that the use of biologically active natural substances in tissue repair, such as extracts of plant components, is an alternative source and not -limiting for research.

3.CONCLUSION

In the field of biomaterials, norbixin is an antioxidant dicarboxylic carotenoid found in the species Bixa orellana L. and poly (hydroxybutyrate) (PHB) is a natural polymer that has characteristics of biodegradability and biocompatibility. In the literature, research involving biological applications of norbixin and poly (hydroxybutyrate) in biological environment, including genotoxicity evaluation, is available, considering the importance of the biocompatibility evaluation of the components in question. However, there is a shortage of studies involving such constituents together as biomembranes in tissue healing with repair of cutaneous wounds or bone regeneration, although both present promising research that suggests its effectiveness as a guide for tissue regeneration.

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