

# Biodegradable Polymers for Bone Tissue Engineering

M. Susana Cortizo and M. Soledad Belluzo

## 1 Introduction

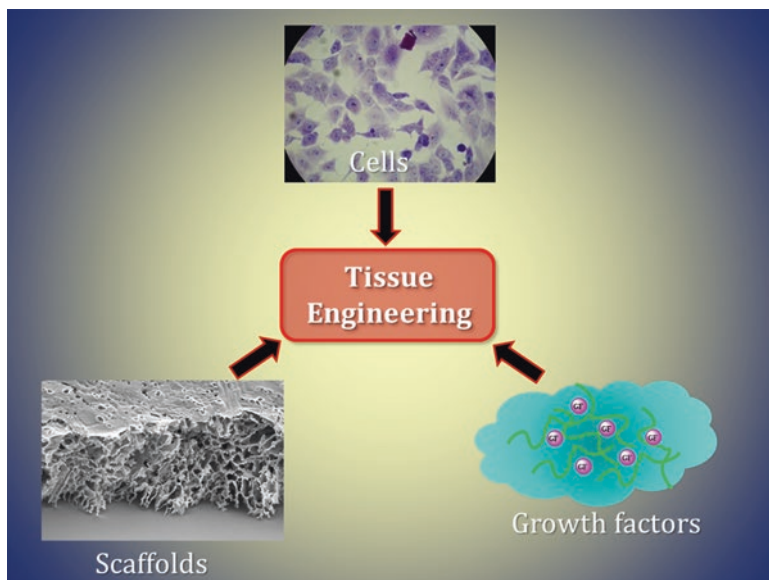
The destruction of bone tissue due to disease (osteonecrosis, tumors, osteoporosis) or inefficient healing posttraumatic injury is a problem affecting the world population. The repair of small defect may be mediated by the osteoblast and osteoclast activity which ensures a balanced control of bone resorption and formation, allowing the bone repair, renewal, and growth. However, when the defect reaches a crucial size, it is necessary to appeal to the promising field of tissue engineering in order to develop a new methodology of bone regeneration. Tissue engineering was defined as “an interdisciplinary field of research that applies the principles of engineering and the life sciences towards the development of biological substitutes that restore, maintain or improve tissue function” (Langer and Vacanti 1993). This strategy has been exponentially developed in the last years and currently constitutes an expansive field of research.

Tissue engineering is based on three fundamental pillars, as can be represented in Fig. 1. Porous 3D scaffolds, made of adequate biomaterial, act as a template for tissue formation and have the capacity to support cell adhesion and proliferation induced by growth factors, together promoting tissue regeneration.

Scaffolds used for tissue engineering not only provide a temporary three-dimensional support during tissue repair but also regulate the cell behavior, such as cell adhesion, proliferation, and differentiation (Guo et al. 2015). Thus, this three-dimensional matrix mimics the extracellular matrix, providing structural and mechanical integrity to tissue while communicating with the cellular components it supports to help facilitate and regulate daily cellular processes and wound healing.

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**Fig. 1** Fundamental pillars of the tissue engineering: interrelationship between scaffold, cells, and growth factors

However, the appropriated design of scaffold strictly may meet a series of properties that make it suitable for tissue engineering applications. In particular, scaffolds which will be applied to bone tissue regeneration should be biocompatible (well integrated in the host's tissue without eliciting an immune response); possess a highly porous structure with interconnected pores of adequate size which allows cell penetration and nutrient and waste transportation; have good surface properties (chemical and topographic) which favor the cell adhesion and proliferation; be osteoinductive (be able to recruit immature cells and to stimulate these cells to develop into pre-osteoblasts), with sufficient mechanical strength to withstand the hydrostatic pressures and to maintain the space required for cell growth and matrix production; and finally exhibit a degradation rate in line with the growth rate of the neo-tissue, so that the time of the injury site is totally regenerated, the scaffold is totally degraded (Salgado et al. 2004). Based on these considerations and taking into account the important advances achieved along the years, it is clear that one of the most critical issues in tissue engineering is the design of the scaffold with the appropriated characteristics to efficiently regenerate the target tissue. Numerous materials have been used as scaffolds to satisfy the above requirements; among them are ceramics and natural or synthetic polymers, as well as blend and composite biomaterials (Mano et al. 2007; Tian et al. 2012; Goonoo et al. 2016).

This chapter presents the main developments in the area of biodegradable biomaterials, a brief description of the biodegradation mechanisms, and the biomaterial features and more relevant properties, currently developed for bone tissue engineering.

## 2 Biodegradation of Polymeric Materials

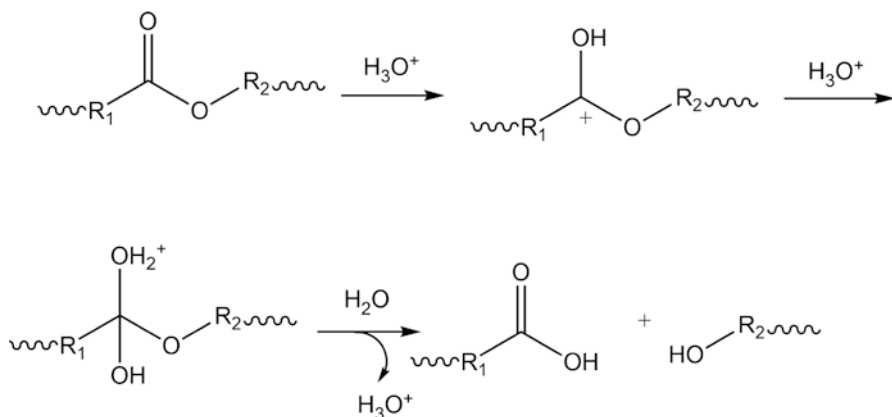
The polymer scaffold material has to be chosen that will degrade and resorb at a controlled rate at the same time as the specific tissue cells seeded into the 3D construct attach, spread, and increase in quantity as well as in quality (Hutmacher 2000). The degradation process consists of cleavage of chemical bond leading to polymer chain scission, decrease of polymer molecular weight, and ultimately producing the loss of mechanical stability of the biomaterial. The biodegradation is the degradation process which is carried out in biological environment which included body fluid, cellular activities, and enzymatic reactions. The mechanisms of biodegradation depend on the chemical nature of the material as well as the physical and morphological properties of polymers. For example, hydrophobic polymers limit water accessibility and typically have decreased hydrolytic degradation rates compared to their more hydrophilic counterparts (Gopferich 1996). Further, amorphous polymers or polymers with lower glass transition temperature ( $T_g$ ) usually degrade faster than semicrystalline or with high  $T_g$  polymers. This degradation behavior is applied to both natural and synthetic polymers.

It has been recognized that the biological environment is surprisingly harsh and can lead to rapid or gradual breakdown of many materials (Coury et al. 2004). The mechanism involved in these processes may be considered through synergic pathways due to different factors that converge and contribute to the aforementioned biodegradation process, for example, superficial cracks, swelling, water uptake, plasticization, or alteration of local pH induced by the presence of degradation products, among others.

Two different mechanisms are accepted as responsible for the polymer biodegradation “in vivo”: hydrolytic and oxidative process.

### 2.1 Hydrolytic Biodegradation

The hydrolytic degradation is the scission of chemical bonds of functional group susceptible to reaction with water, which can be favored by different catalytic conditions (acids, bases, or enzymes). Between the polymers more vulnerable to hydrolytic degradation, polyanhydrides, polyesters, polycarbonates, polyamides, and acetals must be mentioned. In particular, the mechanism of degradation of polyglycolic acid (PGA) and polylactic acid (PLA), two of the most widely used polyesters in biomedical application, was extensively studied (Chu 1989). This mechanism takes place in two stages, the first being associated with the attack on amorphous regions, releasing some of glycolic acid. The second phase of degradation starts more slowly than the first because of the difficulty of hydrolyzing the crystalline regions, and at the end of this step, glycolic acid is released rapidly. Hydrolytic degradation mechanism of this kind of polymers can be represented as is shown in Fig. 2.

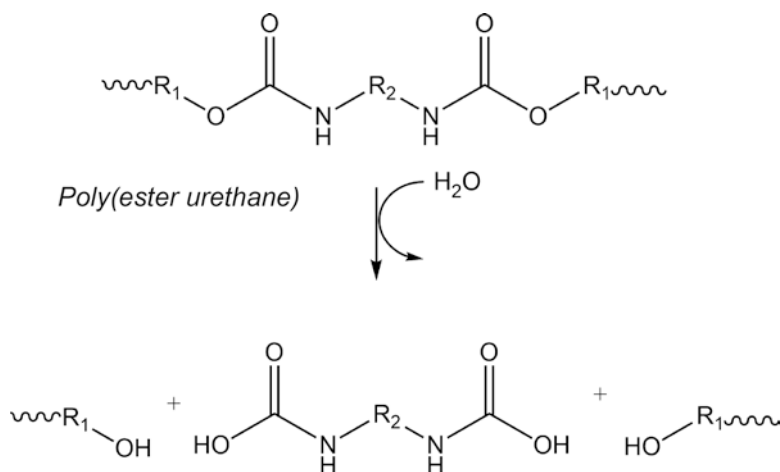


**Fig. 2** Reaction mechanism of the polyester hydrolysis

The hydrolytic degradation of aromatic polyesters exhibited important differences; due to that, the water diffusivity is very low, as was demonstrated for polyethylene terephthalate (PET), the major aromatic polyester used medically, with extensive application in vascular prostheses (Williams 1989).

Polymer with other heteroatoms, such as amino group in chain polymer, i.e., nylon or polyamino acids, exhibited variable behavior of hydrolytic degradation, depending on their hydrophilicity. So, nylon 6 hydrolyzes faster than nylon 11, although the reaction mechanism followed a similar step (Zaikov 1985). Also the influence of enzymes on the rate of degradation was demonstrated; papain, trypsin, and chymotrypsin degrade nylon 66, while esterase had no effect (Smith et al. 1987). The biodegradability of polyamino acids and the role of enzymes, in particular, have been known for some years and have been discussed by Dickinson et al. (1981).

In biomedical applications, polyurethanes (PU) are usually classified as either poly(ester urethane)s or poly(ether urethane)s, based on the nature of the soft segments (ester or ether group included in this segment). The hydrolytic degradation of both kinds of PU is different and so do their biomedical applications. Hafeman et al. (2011) studied the degradation mechanism of poly(ester urethane) scaffolds prepared from lysine trisocyanate or a trimer of hexamethylene diisocyanate under hydrolytic, esterolytic, and oxidative conditions. They proposed that the primary mechanism of degradation was hydrolysis of ester bonds to yield  $\alpha$ -hydroxy acids, together with other unidentified but water soluble products. Similar pathways for hydrolytic degradation were suggested for polyurethane copolymers which were prepared from 1,6-diisocyanatohexane (HDI), polycaprolactone diol (PCL), 2,2-bis(hydroxymethyl) propionic acid (DMPA), and ethylene glycol (EG). In the case of poly(ester urethane), the hydrolytic degradation rate of ester group is significantly faster than urethane, urea, or amide functional group. This results in relatively high percentage of oligomeric products due to the preferential degradation of



**Fig. 3** Scheme of main pathways for hydrolytic degradation of poly(ester urethane)

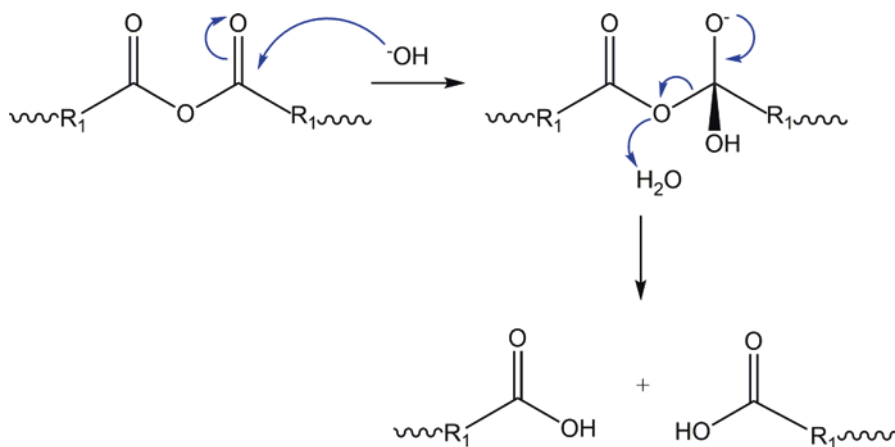
ester group within the PU structure, particularly during the early stages of the degradation (Gunatillake and Adhikari 2011). Figure 3 shows the schematic pathways of PU degradation based on a generalized structure.

In the last years, new polyurethanes have been developed which included blended soft segments, in order to control the degradation rate and mechanical properties of scaffolds applied in tissue regeneration. For example, the partial replacement of the polyester units with polycarbonate (PC) or polycaprolactone (PCL) with polyethylene glycol (PEG) fragments in the soft segment has resulted in polymers with better modulated degradation kinetics of the materials (Zhang et al. 2016).

Due to the relatively faster hydrolysis of polyanhydrides in comparison with polyesters, they are a main class of polymers used in drug delivery (Murthy et al. 2012). Polyanhydrides can be formulated from a variety of monomer units, which allow engineers to design materials that can degrade and/or release therapeutics at a particular rate that is appropriate for the desired application. They are hydrolyzed predominantly by base- and water-catalyzed hydrolysis. The overall hydrolysis mechanism is similar to that of polyesters, as can be seen in Fig. 4. The first step is the addition of base to the carbonyl carbon, followed by generation of a tetrahedral intermediate. The tetrahedral intermediate formed during polyanhydride hydrolysis generally results in the leaving of the attached ester.

Numerous reports have demonstrated that the hydrolysis of polyanhydrides is proportional to the pH of the surrounding medium (Leong et al. 1985; Park et al. 1996; Santos et al. 1999). The results of these studies demonstrated that polyanhydrides degrade more rapidly at high pH which is in accordance with a base-catalyzed hydrolysis mechanism.

A class of synthetic polymers which can be considered biodegradable are poly(alkylcyanoacrylates) (Coury et al. 2004). These class of polymers were extensively studied as tissue adhesives for the closure of skin wounds, as surgical glue,



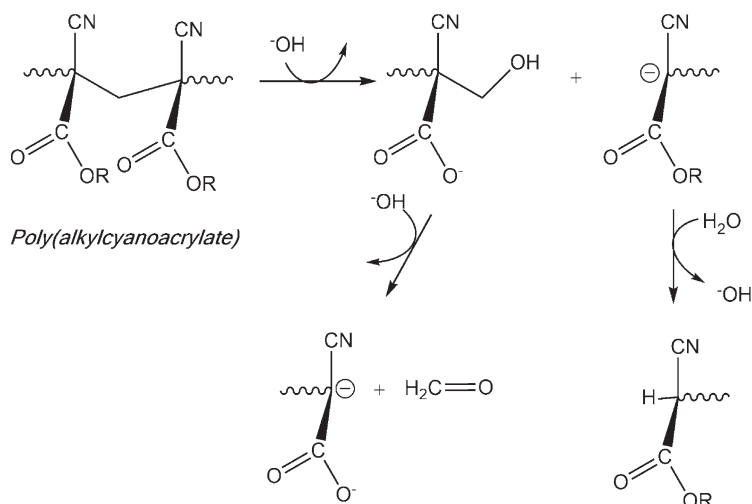
**Fig. 4** Base-catalyzed anhydride hydrolysis (Adapted from Murthy et al. 2012)

and as embolitic material for endovascular surgery (Vauthier et al. 2003). Different hydrolysis mechanisms were proposed for these kinds of polymers that include C—C bonds in the main chain. One of them is through a “reverse Knoevenagel” reaction, as can be represented in Fig. 5 (Leonard et al. 1966). It was proposed that this reaction occurs because the methylene ( $-\text{CH}_2-$ ) hydrogen in the polymer is highly activated inductively by electron-withdrawing neighboring groups. In vivo, the water associated with the tissue could be inducing the polymer hydrolysis, as well as basic or enzymatic process. Other degradation mechanisms described in the literature consist of the hydrolysis of the ester bond of the alkyl side chain of the polymer (Lenaerts et al. 1984). Degradation products consist of an alkyl alcohol and poly(cyanoacrylate), which are soluble in water and can be eliminated in vivo via kidney filtration. However, the first of the two mechanisms mentioned is too slow to compete with the other, much more rapid, mechanisms occurring in vivo catalyzed by enzymes (Vauthier et al. 2003).

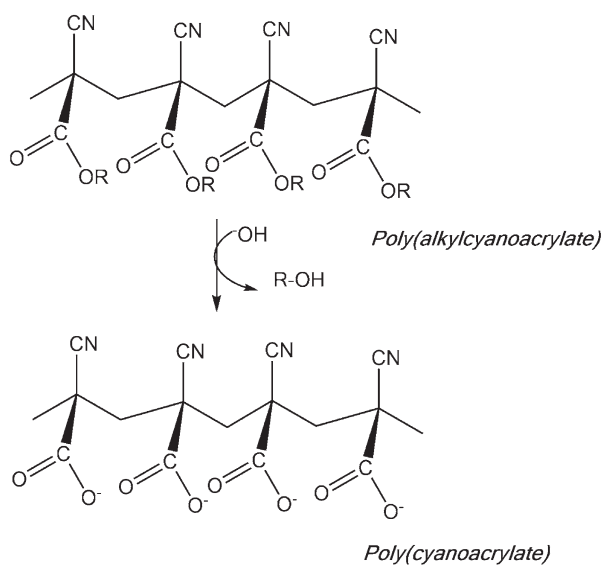
## 2.2 Oxidative Biodegradation

The biodegradation of polymer “in vivo” is a process mediated by enzymes, which can be hydrolytic or oxidative. The hydrolytic mechanism involves enzyme that attacks on susceptible and specific chemical bond, as previously described. The oxidative biodegradation involves reactive molecules that are derived from activated phagocytic cells (neutrophils and monocytes) responding to the injury and the properties of the foreign body at the implant site (Coury et al. 2004). Sites favored for initial oxidative attack, consistent with a homolytic or heterolytic pathway, are those that allow abstraction of an atom or ion and provide resonance stabilization of the resultant radical or ion. Thus, different kinds of polymers could be susceptible to

(A)



(B)



**Fig. 5** Degradation pathways of poly(alkylcyanoacrylate): (a) “Reverse Knoevenagel” mechanism (Adapted from Leonard et al. 1966). (b) Basic hydrolysis mechanism (Adapted from Vauthier et al. 2003)

oxidative biodegradation, such as polyolefins, polymers including aromatic ring, polyethers, polyacrylic or polymethacrylic acids, and polyols, to mention a few. It is considered that neutrophils and macrophages metabolize oxygen to form a superoxide anion ( $O^{2-}$ ). This intermediate can undergo transformation to more powerful oxidants and conceivably can initiate homolytic reactions on the polymer through a radical ( $R^\bullet$ ) or heterolytic mechanism (Coury et al. 2004). The oxidation processes induced by phagocytes are the result of oxidants produced by general foreign-body responses. The macrophages are activated by the presence of released product of polymer degradation, such as monomer or oligomers. Figure 6 shows the proposed

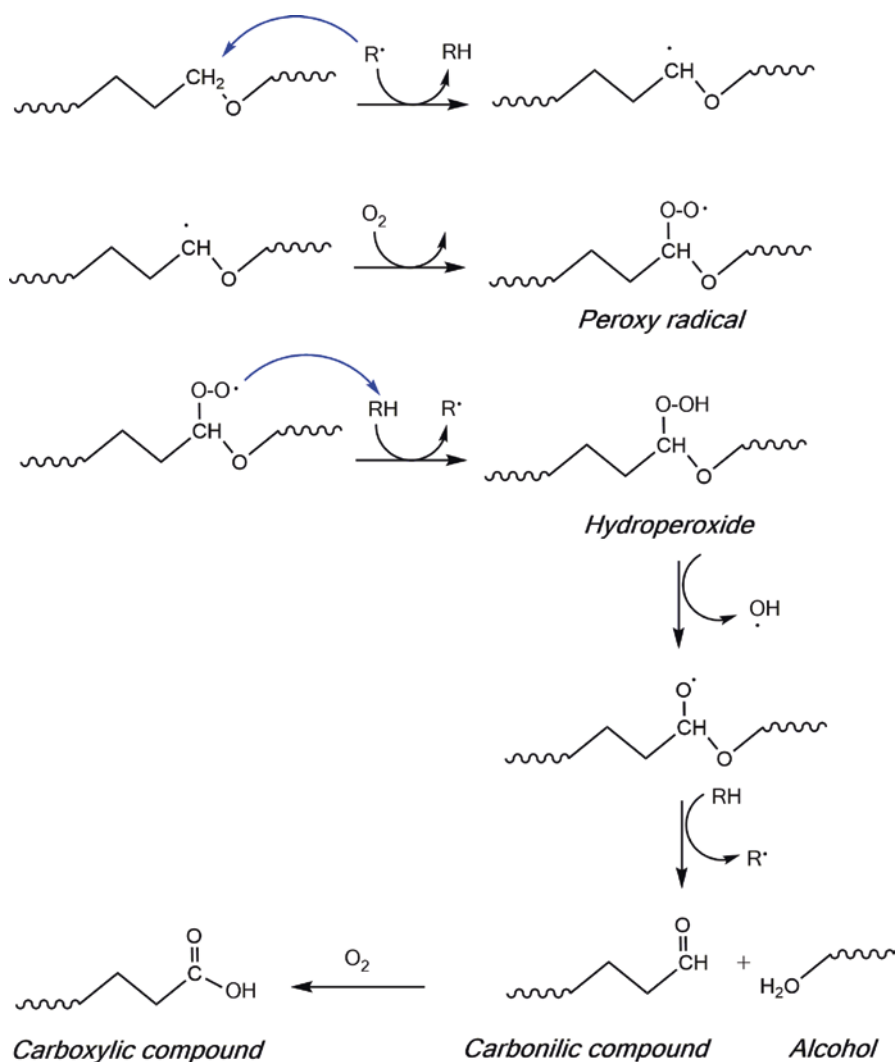


Fig. 6 Pathway for radical oxidative fragmentation of polyethers (Adapted from Coury et al. 2004)



pathways for oxidative fragmentation of polyethers mediated by radical species, as was suggested by Schubert et al. (1997) and Coury et al. (2004).

The consequence of this process is the formation of more polar molecular species with lower average molecular weight, which will be more soluble and therefore have greater speed of diffusion. This characteristic will facilitate the process of phagocytosis and then the biodegradation of the polymeric material “in vivo.”

### **3 Biodegradable Polymer Used as Biomaterials for Bone Tissue Engineering**

Biodegradable polymers used as scaffold materials for bone tissue engineering can be divided, considering their origin, in two groups: synthetic and natural. The main advantage of the synthetic polymers is that they can be produced under controlled conditions and therefore exhibit in general predictable and reproducible mechanical and physical properties such as tensile strength and elastic modulus and degradation rate (Rezwani et al. 2006). On the other hand, the benefits of biomaterials based on natural polymers are their low immunogenic potential, the potential bioactive behavior, and the capability of interacting with the host's tissue, chemical versatility, and in some cases their source, as in starch and chitosan, which is almost unlimited (Salgado et al. 2004).

#### **3.1 Synthetic Polymer for TE**

In addition to the previously mentioned advantages of synthetic polymers, it must be mentioned that they can be fabricated into various shapes with desired pore morphology and conductive features and designed with chemical functional groups that can induce tissue ingrowth (Gunatillake and Adhikari 2003).

##### **3.1.1 Polyesters**

Biodegradable synthetic aliphatic polyesters are the most extensively used polymers for bone tissue engineering, such as the poly(glycolic acid) (PGA), the stereoisomer forms of poly(lactic acid) (PLA), and their copolymer poly(lactic-co-glycolide) (PLGA). Their properties and application were exhaustively described in several papers and reviews (Guo et al. 2015; Gunatillake and Adhikari 2003). As was noted, with the exception of PGA, the polymers in this family are soluble in many common organic solvents, and thus it can be processed by a variety of thermal and solvent-based methods. However, the degradation products of these polyesters caused some drawbacks because it reduces the local pH value, which in turn may accelerate the polyesters' degradation rates and induce an inflammatory reaction.

These inflammatory processes are often ascribed to acidosis caused (chemically unavoidable) by the release of acidic degradation products (monomeric or oligomeric hydroxycarboxylic acids) (Martin et al. 1996; Winet and Bao 1997).

Nanocomposites based on nano-sized hydroxyapatite (HA) and bioactive glass (BG) fillers in combination with biodegradable polyesters as biomaterials for applications in bone regeneration were described by Allo et al. (2012).

Poly( $\epsilon$ -caprolactone) (PCL) is another aliphatic polyester that has been intensively investigated as a biomaterial. This polymer exhibited low melting point (59–64 °C), high thermal stability, biocompatibility, and biodegradability although with slower degradation rate than the previously mentioned polyesters (Mondrinos et al. 2006). The addition of HA increased the compression modulus of composite toward bone fixation, but until some level, the failure mechanism of the composites changes from plastic to brittle (easily rupture), hence lowering the mechanical properties of PCL and other biodegradable polymers (Razak et al. 2012). In recent years, new nanocomposites based on PCL were designed by different techniques and studied as potential scaffolds for biomedical regeneration (Mkhabela and Ray 2014).

Polypropylene fumarate (PPF) is an unsaturated linear polyester whose degradation products (i.e., propylene glycol and fumaric acid) are biocompatible and readily removed from the body (Peter et al. 1997). This polymer can be cross-linked by reaction of the double bond using photochemical or thermal radical polymerization. The mechanical properties can be regulated by appropriate molecular weight control as well as cross-linking conditions and the incorporation of reinforcement (Lalwani et al. 2013; Horch et al. 2004).

Recently, there has been a growing interest in the development of bio-polyesters from renewable resources due to limited fossil fuel reserves, rise of petrochemical price, and emission of greenhouse gasses (Zia et al. 2016). Between them, novel materials such as poly(1,8-octanediol-co-citrate) (POC) combined with hydroxyapatite (HA), polyhydroxyalkanoates (PHA)/isosorbide copolyesters, and polyesters based on citric and tartaric acid, among others, are included (Qiu et al. 2006; Zhang et al. 2013; Jiang et al. 2012). Blends and composites of polyesters and hydrophilic natural polymers have been receiving significant attention, since they could lead to the development of novel biodegradable polyesters with properties suitable for extraordinary biomedical applications (Zia et al. 2016).

### 3.1.2 Polyanhydrides

Polyanhydrides have limited mechanical properties that restrict their use in load-bearing applications such as in orthopedics (Uhrich et al. 1995). To combine good mechanical properties of polyimides with surface-eroding characteristics of polyanhydrides, poly(anhydrides-co-imides) have been developed (Attawia et al. 1995; Uhrich et al. 1997). Anseth et al. developed a new family of photopolymerizable, methacrylated anhydride monomers and oligomers that combine high strength, controlled degradation, and photoprocessibility (Anseth et al. 1999). They also demonstrate, by *in vivo* studies in rats, that these networks possess excellent osteocompatibility.

### 3.1.3 Polymers Including C—C Bond in Main Chain

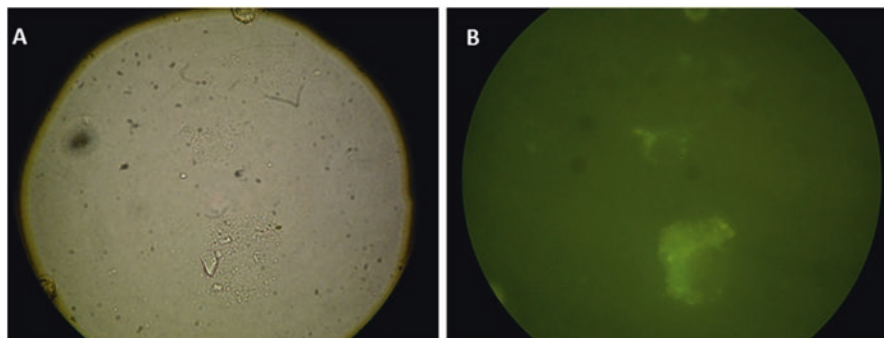
Very few polymers with C—C structure in the main chain were proposed as biomaterial for bone tissue engineering, the majority of which are composite materials. Recently, a polymer scaffold with  $\text{Ca}^{2+}$  was synthesized by copolymerization of acrylamide (AM), 2-hydroxyethyl acrylate (HEA), and calcium methacrylate (CDMA) (Kang et al. 2017). This polymer was combined with calcium phosphate in order to increase the attachment of organic and inorganic interface and greatly enhance the mechanical properties of the composite scaffolds. The biocompatibility of the prepared materials was also improved by minerals coating at a certain degree, as evaluated by L929 cell viability.

Hybrid material scaffolds consisting of methacryloxypropyl trimethoxysilane, zirconium propoxide, and 2-(dimethylamino)ethyl methacrylate (DMAEMA) were obtained as composite scaffolds for bone repair (Chatzinikolaidou et al. 2015). The scaffolds' ring structure exhibited a complex 3D geometry which showed good cell adhesion and proliferation, similar to the polystyrene control.

Some research is being directed to the design of hybrid scaffolds that combine the properties of synthetic and natural polymers. One of them is proposed by Galperin et al., an integrated bilayered scaffold based on degradable poly(hydroxyethyl methacrylate) hydrogel layer coated with hydroxyapatite particles and a second layer that had 200  $\mu\text{m}$  pores with surfaces decorated with hyaluronan (Galperin et al. 2013). The scaffold supported the simultaneous growth of chondrocytes and human mesenchymal stem cells (hMSCs) by providing a suitable environment for cell attachment, infiltration, proliferation, and differentiation of hMSCs to osteoblasts (for the designated bone layer) and retention of chondrocyte phenotype (for the designated cartilage layer).

However, in very few of these studies, the biodegradability of the scaffold was analyzed, which is a relevant property for the applications in tissue engineering.

Fernandez et al. prepared a biomimetic bone scaffold based on PCL and poly(diisopropyl fumarate) (PDIPF) blends obtained by sonication (Fernández et al. 2010). PDIPF was synthesized by microwave radical polymerization and presents a characteristic structure of C—C bond. The mechanical properties of this blend were comparable to those of the trabecular bone, while the biocompatibility studies show that osteoblasts plated on the compatibilized blend adhered to and proliferated more than on either homopolymer. Later, HAP–blend composite, with improved physical, mechanical, and osteoinductive properties, was developed and their non-cytotoxicity was demonstrated (Fernández et al. 2011, 2014). PCL is known to be biodegraded by hydrolytic mechanism, as was previously indicated. Previously, biodegradation studies of PDIPF were performed both in PBS buffer and using an in vitro macrophage degradation assay (Cortizo et al. 2008). The polymer was only degraded in the presence of RAW264.7 macrophages, as was demonstrated by the decrease of the average molecular weight (21 days), and the cells' morphological change was observed, from a rounded monocytic appearance to an activated phagocytic phenotype as can be seen in Fig. 7. These results indicated that the polymer can be degraded by a phagocytic process through an oxidative mechanism and thus could be a good candidate for applications in bone regeneration.



**Fig. 7** RAW264.7 macrophages growing on PDIPF\*. Cells were cultured on a fluorescent PDIPF matrix for 20 days. Light (a) and fluorescent (b) microscopy revealed the presence of fluorescent particles included in the cytoplasm of activated macrophages. Obj. 40×

## 3.2 *Natural Polymer for TE*

### 3.2.1 Polyhydroxyalkanoates

Natural polyesters from the group of polyhydroxyalkanoates (PHAs) have emerged as promising materials for various tissue engineering applications, due to their biocompatibility and biodegradability, as well as their broad range of mechanical properties (Freier 2006). Current methods for PHA production at the industrial scale are based on their synthesis from microbial isolates in either their wild form or by recombinant strains (Dias et al. 2006). The cost of PHA production is still too high for PHA to become a competitive commodity plastic material. The most significant factor in the production costs of PHA is the price of the substrate and the corresponding fermentation strategies. The use of renewable carbon sources based on agricultural or industrial wastes and the development of processes requiring lower investment can contribute to reducing the production costs. Besides it, PHA production processes based on mixed microbial cultures are being investigated as a possible technology to decrease production costs, since no sterilization is required and bacteria can adapt quite well to the complex substrates that may be present in waste material.

Poly(3-hydroxybutyrate), P3HB, is the simplest and most widely studied member of the group of PHAs. The high crystallinity of the isotactic P3HB leads to stiffness and brittleness, as well as slow hydrolysis *in vitro* and *in vivo*, while P4HB films are characterized by low stiffness and high elongation at break (Freier 2006). PHA with elastomeric properties can be obtained from P3HB copolymers containing more than 20% of 4-hydroxybutyrate or medium chain-length (C6–18) 3-hydroxyalkanoate units, as well as medium chain-length PHA homopolymers. This characteristic is very important in tissue engineering applications. Moreover, mechanical stimuli promote the formation of functional tissue, for example, in car-

diovascular or cartilage tissue engineering, and allow for gradual stress transfer from the degrading synthetic matrix to the newly formed tissue.

Scaffolds based on P3HB/HA or P3HB/tricalcium phosphate (TCP) composites were found that exhibited better mechanical properties and biocompatibility, which are important for bone tissue engineering (Hayati et al. 2011; Rasoga et al. 2017).

In vitro degradation studies on P3HB films in buffer solution (pH 7.4, 37 °C) showed no mass loss after 180 days but a decrease in molecular weight starting after an induction period of about 80 days, the hydrolysis process being described in two stages (Doi et al. 1989). In vivo studies demonstrated that P3HB is a completely resorbable polymer, with a degradation rate comparable to that of slowly degrading synthetic polyesters such as high molecular weight poly(L-lactide) (Gogolewski et al. 1993).

### 3.2.2 Collagen

Collagen is the most abundant structural protein in the body and is the principal component of extracellular matrix (ECM). There are 28 types of collagen decrypted (Mienaltowski and Birk 2014); collagen types I, II, and III have been commonly found in human tissues like the skin, blood vessel, tendon, cartilage, and bone. These types of collagen receive the name of fibril-forming collagen (O'Brien 2011; Pina et al. 2015; Dong and Lv 2016).

Collagen is a non-cytotoxic, biocompatible, and biodegradable protein, extensively used for a wide range of biomedical applications and considered a valid alternative to synthetic materials due to its inherent biocompatibility involving low antigenicity, inflammation, and cytotoxic responses (Gorgieva and Kokol 2011; Meghezi et al. 2015). This biopolymer has low elasticity and poor mechanical strength but relatively stable structure due to covalent cross-link formation among collagen fibrils (Dong and Lv 2016).

Collagen is easy to obtain from many animals and plat sources (Gómez-Guillén et al. 2011), especially from tissues rich in fibrous collagen such as the dermis, tendon, and bone. The isolation of this protein is mostly from rat, bovine, porcine, and sheep, but the extraction of collagen from fish skin and bones has recently been reported (Yamada et al. 2014). Another source of collagen is the production of recombinant human collagen from yeast, bacteria, or mammalian cells, among others (Yu et al. 2014). This approach is promising due to the possibility for mass production.

The interest in collagen-based scaffolds for bone tissue engineering lies on the ability of this protein in mimicking the ECM with the presence of functional groups that can enhance osteoblast adhesion and migration (Ma 2008; Gorgieva and Kokol 2011) and its excellent physicochemical properties. Collagen can be processed into fibers, films, membranes, sponges (Ferreira et al. 2012; Dong and Lv 2016; Rau 2016), blends (with other polymers), and composites.

Scaffold geometry affects cell adhesion, proliferation, and distribution by affecting cell ingrowths, vascularization, and access of nutrients and oxygen. Scaffolds'

pore size and interconnectivity seem to be able to modulate osteogenesis, due to cell osteogenic response to particular pore dimensions (Polo-Corrales et al. 2014). In this way, 3D micropattern porous collagen scaffolds with controlled pore structure were obtained by Chen et al. (2015). After culturing L6 myoblast in the micro-groove collagen scaffolds, it can be seen that myoblast was well aligned and had high expression of myosin heavy chain and synthesis of muscle extracellular matrix, demonstrating the potential use for implantation to restore disease tissue. In another study, a biomimetic scaffold for tissue engineering using bovine collagen with different topographic characteristics was developed, using matrices with random or parallel-arranged collagen fibers (Cortizo et al. 2012). Adhesion, proliferation, alkaline phosphatase activity, and mineralization were significantly improved when cells were grown on the ordered collagen matrix, and no significant increase in proinflammatory cytokine release was observed.

Although several cross-linking strategies for the enhancement of mechanical properties of collagen scaffold have been reported, these methods may have cytotoxic effects (Dong and Lv 2016). Therefore, the combination of different natural polymers can be used as a strategy for the preparation of polymeric scaffold with better properties. Arakawa et al. (2017) synthesized a photopolymerizable hydrogel consisting of photocross-linkable methacrylated glycol chitosan (MeGC) and semi-interpenetrating collagen (Col) with a riboflavin photoinitiator under blue light, with enhanced compressive modulus and slowed degradation rate. MeGC–Col composite hydrogels significantly enhanced cellular attachment, spreading, proliferation, and osteogenic differentiation of mouse bone marrow stromal cells (BMSCs) seeded on the hydrogels compared with pure MeGC hydrogels, as observed by alkaline phosphatase activity as well as increased mineralization. These findings demonstrate that MeGC–Col composite hydrogels may be useful in promoting bone regeneration. Other collagen natural polymer scaffolds for bone regeneration in combination with silk fibroin (Sun et al. 2015; Sangkert et al. 2016), hyaluronic acid (Zhang 2014; Bornes 2015), and alginate (Bendtsen and Wei 2015) had also been described.

Collagen-blending scaffolds made with synthetic polymers also make it possible to achieve both mechanical and biological optimal properties. Scaffolds composed of collagen and synthetic polymers, such as poly( $\epsilon$ -caprolactone) (PCL), polylactic acid (PLA), poly(ethylene glycol) (PEG), polyglycolide (PGA), poly(lactide-co-glycolide) (PLGA), and polyvinyl alcohol (PVA), have been widely used for tissue engineering (Dong and Lv 2016). A 3D macrochanneled poly- $\epsilon$ -caprolactone (PCL) scaffold, fabricated via the robotic dispensing technique, with the bioactive properties of collagen was prepared by Yu et al. (2012). Rat mesenchymal stem cells (MSCs) were loaded into collagen hydrogels, which were then combined with macrochanneled PCL scaffolds. The cells actively proliferated within the combined scaffold for up to 7 days. MSC-loaded collagen–PCL scaffolds were subsequently cultured under flow perfusion to promote proliferation and osteogenic differentiation. Cells are proliferated to levels significantly higher in flow perfusion culture than that under static conditions during 21 days. The activity of collagen/PCL scaffolds and alkaline phosphatase (ALP), an early osteogenic marker, was also significantly upregulated at 14 days, as well as the expression of the osteogenic genes OPN, OCN, and BSP.



As natural bone is mainly composed of collagen type I and Hap, it is understandable to think that, when aiming to emulate bone tissue regeneration, porous collagen scaffolds are often combined with calcium phosphates (Van Vlierberghe et al. 2011). Several inorganic materials such as hydroxyapatite (HA) and  $\beta$ -tricalcium phosphate ( $\beta$ -TCP) have been used in the field of bone regeneration (Ngiam et al. 2009; Mate Sanchez de Val et al. 2015; Sarikaya and Aydin 2015). These materials show increased mechanical strength as compared to pure collagen scaffolds due to a strong interaction between calcium-binding residues on the polymer macromolecules and the nanoparticle surface (Wahl and Czernuszka 2006). Ngiam et al. (2009) modified electrospun PLLA/collagen scaffolds with HA by an alternating soaking method. They found that HA improved the hydrophilicity of the scaffolds significantly and could enhance the cell capture efficiency of scaffolds to osteoblasts, which was beneficial to early cell capture of bone graft materials. In another study, in vitro osteogenic potential of an electrospun PLLA/collagen/HA scaffold was also studied by Raghavendran (Raghavendran et al. 2014). They indicated that the scaffold exhibited good cytocompatibility and superior osteoinductivity, an upregulated osteogenic lineage gene expression associated with human MSCs. This fact demonstrates that PLLA/collagen/HA scaffolds may be supportive for stem cell-based therapies for bone repair and reconstruction.

Another innovate type of collagen-based scaffolds is carbon nanotube-collagen scaffolds. Since CNT can interact with collagen at a molecular way, these combined scaffolds increased the stiffness due to its rigidity and enhanced the functionality of collagen for biomedical applications (Dong and Lv 2016). Several authors have developed composite and scaffold materials with CNTs (Venkatesan et al. 2014).

Gelatin is the denaturized form of collagen. Despite the lack of structural characteristics of the collagen, it is biocompatible, bioresorbable, and non-immunogenic. For bone tissue engineering uses, it is often combined with ceramics like HA. Azami et al. (2010) designed a gelatin/HAp nanostructured scaffold with mechanical strength comparable to the spongy bone, with an excellent capacity of cell attachment, migration, and penetration into the pores of the nanocomposite. Recently, the same group tested a nano-hydroxyapatite/gelatin (HA/gel) nanocomposite scaffold in vitro using rat mesenchymal stem cells (Samadikuchaksaraei et al. 2016), and in in vivo studies, the HA/gel/OC nanocomposite was implanted in the critical size bone defect created on rat calvarium as well.

### 3.3 *Silk Fibroin*

The uses of silk proteins have gain more interest in the last years due to its properties like elasticity, impressive mechanical strength, morphologic flexibility, biocompatibility, and biodegradability with controllable degradation rates.

Silk is composed of two major proteins, SF (fibrous protein) and sericin (globular protein), and SF can be isolated from several sources in the form of an aqueous protein solution (Melke et al. 2016). Studies have demonstrated that while native fibroin–sericin proteins can activate the adaptive response, purified fibroin does not

(Aramwit et al. 2009), so the isolation of purified SF is essential for biomedical applications and can be achieved by eliminating sericin via boiling in an alkaline solution (Pina et al. 2015).

Silk proteins are produced by an enormous variety of insect and spider species including ants, fleas, and crickets (Thurber et al. 2015). In spite of that, for biomedical applications, the main silk source is natural silk fibroin of the domesticated *Bombyx mori* (Hardy et al. 2016; Melke et al. 2016). Recently, other authors report the obtaining of recombinantly produced silk-inspired proteins, an interesting alternative because it is possible to produce large quantities of such silks with designed primary sequences (Fredriksson et al. 2009; Humenik et al. 2011; Teulé et al. 2012).

For biomedical applications, silk can be fabricated into a wide range of material formats with the possibility to achieve desirable mechanical and degradation properties. SF can be easily modified into different physical forms such as hydrogels, sponges, fibers, particles, microspheres, tubes, and electrospun fibers (Koh et al. 2015; Melke et al. 2016; Yao et al. 2016). Also, a few studies have been conducted using SF as a material for bioprinting processes.

Schacht et al. prepared a 3D printing scaffold without cross-linking with a recombinant spider silk protein eADF4(C16). The adhesion of different cell types which were seeded after the printing process was tested and revealed that osteoblasts showed a much better adhesion than fibroblasts, myoblasts, HeLa cells, or keratinocytes (Schacht et al. 2015).

Silk-based composite scaffolds in combination with components like collagen and CaPs (calcium phosphates) are also reported. He et al. (2016) prepared a silk fibroin/cellulose nanowhiskers–chitosan (SF/CNW–CS) composite scaffold by layer-by-layer assembly and tested in vitro using human MG-63 osteosarcoma cells. The results indicated that the composite scaffold supporting cell proliferation and promoting the levels of biomineralization is a promising candidate for bone generation and implantation. In another study, macro-/microporous silk/nano-sized calcium phosphate was developed, and the new bone formation ability in rat femur of the composite scaffold was evaluated in vivo. New bone growth was observed directly on the scaffolds' surface, demonstrating osteoconductive properties as they can promote de novo bone formation (Yan et al. 2013).

Other silk fibroin blend scaffolds were also prepared with natural polymers as cellulose, gelatin, chitosan, hyaluronan, alginate (Freddi et al. 1995; He et al. 2010; Das et al. 2015; Kapoor and Kundu 2016), and synthetic polymers like acrylic polymer, PVA, PEO (polyethylene oxide), PAA (polyacrylic acid), PU, and PEG (Sun et al. 1997; Hardy et al. 2016; Kapoor and Kundu 2016).

### 3.4 Chitosan

Chitosan is a deacetylated form of chitin, a polysaccharide present in marine crustacean exoskeleton like crab, shrimp, and lobster (Pina et al. 2015; Logith Kumar 2016). Chitosan is a linear polysaccharide composed of glucosamine and



N-acetylglucosamine units linked by  $\beta$ -(1,4)-glycosidic bonds, and different forms of pure chitosan differ by their degrees of deacetylation (DD) and molecular weights (Levengood and Zhang 2014). The degree of deacetylation represents the glucosamine to N-acetylglucosamine ratio and generally falls in the range of 50–95%. Chitosan solutions can easily be prepared by dilution of the polymer in dilute organic acids like acetic or formic acid.

This biopolymer is interesting for biomedical applications due to its low toxicity, non-immunogenicity, biodegradability, ability for cell ingrowth, and intrinsic antibacterial nature. Additionally, chitosan is the only positively charged biopolymer and is able to interact with negatively charged polymers and structural molecules present in the ECM.

Chitosan can support the attachment and proliferation of bone-forming osteoblast cells as well as formation of a mineralized bone matrix *in vitro* and *in vivo* neovascularization (Costa-Pinto et al. 2011; Saravanan et al. 2013). For bone regenerative applications, chitosan can be developed in different forms like sponges, fibers, films, foams, hydrogels, and particles (Croisier and Jérôme 2013; Niranjana et al. 2013; Pina et al. 2015; Logith Kumar et al. 2016) and can be processed by several methods from physical blends (to form polyelectrolyte complexes) to novel techniques as rapid prototyping and electrospinning (Levengood and Zhang 2014). The use of ultrasound to compatibilize chitosan-based scaffolds was also described (Belluzo et al. 2016). Several materials for bone tissue engineering using functionalized chitosan (such as quaternization, carboxyalkylation, hydroxylation, phosphorylation, sulfation, and copolymerization) also have been described (Logith Kumar et al. 2016).

To enhance the properties of the scaffolds for bone remediation, chitosan can be combined with other polymers and inorganic materials. For example, the blending of chitosan with alginate stabilized the system by their electrostatic interaction (Venkatesan et al. 2015). The inclusion of chicken feather keratin nanoparticles within chitosan significantly improved the protein adsorption and probed biocompatibility with human osteoblastic cells (Saravanan et al. 2013). Chitosan/gelatin scaffolds promoted osteoblast proliferation *in vivo*, showing a complete degradation in 8 weeks (Oryan et al. 2016).

Also, incorporation of ceramics can enhance mechanical properties and osteoconductive properties of chitosan composite materials. Kim et al. (2015) prepared a chitosan/alginate matrix with nanoHA and probed to enhance the mechanical property of the scaffold as well as stimulated the differentiation of mouse pre-osteoblastic cells (MC3T3-E1) to osteocytes. Chitosan/hyaluronic acid scaffolds with addition of calcium phosphate cement exhibited a significant increase in ALP activity with no significant change in the rate of osteoblastic cell proliferation (Hesarakı and Nezafati 2014). The addition of nHAp to chitosan/gelatin matrix not only increased the mechanical property of the scaffolds but also stimulated the proliferation and differentiation of induced pluripotent stem cells of gingival fibroblasts to osteocytes (Isikli et al. 2012). The incorporation of chondroitin sulfate into chitosan scaffolds increased apatite deposition which facilitated the spreading of bone marrow stromal cells and significantly enhanced the compressive modulus (Park et al. 2013).

Several materials combining chitosan with synthetic polymer also were used over the year to bone tissue engineering. Ku et al. (2009) designed PLLA/chitosan multilayered membrane composed of the outer layers of chitosan mesh for ease of cell adherence and the middle layer of nanoporous PLLA for sufficient mechanical strength. The membrane maintained its integrity for up to 8 weeks while allowing gradual degradation. Mohammadi et al. (2007) developed a 3D nanofibrous hybrid scaffolds consisting of poly( $\epsilon$ -caprolactone), poly(vinyl alcohol), and chitosan via an electrospinning method and assed the differentiation of mesenchymal stem cells into osteoblasts. The result revealed that cells were well attached, penetrated into the construct, and uniformly distributed. The expression of early and late phenotypic markers of osteoblastic differentiation was upregulated in the constructs cultured in the osteogenic medium. Other groups developed a borax cross-linked scaffold based on fumarate–vinyl acetate copolymer and chitosan for osteochondral tissue engineering. Biocompatibility studies demonstrate the versatility of this material since it allows BMPC osteogenic development and supports primary chondrocyte growth and extracellular matrix deposition, without evident signs of cytotoxicity in the in vitro system (Lastra et al. 2016).

### 3.5 Alginate

Alginate is a naturally occurring anionic polymer typically obtained from brown algae (Phaeophyceae) including *Laminaria hyperborea*, *Laminaria digitata*, *Laminaria japonica*, *Ascophyllum nodosum*, and *Macrocystis pyrifera* through treatment with aqueous alkali solutions (Venkatesan et al. 2015). Alginate is a block copolymer composed of two monomers, (1,4)-linked  $\beta$ -D-mannuronate (M) and  $\alpha$ -L-guluronate (G), and the ratio of guluronate to mannuronate varies depending on the natural source influencing the properties of the alginate (Pina et al. 2015, Lee and Mooney 2012). This polymer has been extensively investigated and used for many biomedical applications, due to its outstanding properties in terms of biocompatibility, biodegradability, nonantigenicity, relatively low cost, abundant source, and chelating ability. The preparation of alginate scaffolds can be achieved by diverse cross-linkers that are calcium based due to the ability of mild gelation by addition of divalent cations such as  $\text{Ca}^{2+}$ . Alginate can be easily modified in any form such as hydrogels, microspheres, microcapsules, sponges, foams, and fibers, by several methods including lyophilization, electrospinning, and cross-linking (Lee and Mooney 2012; Sun and Tan 2013). Among these, alginate gels can be introduced into the body in a minimally invasive way, representing an advantage for bone repairing by filling irregularly shaped defects. The polymer composition, molecular weight, purity, and concentration used in the scaffolds play the biggest role in providing mechanical strength, biocompatibility, cell adhesion, proliferation, and osteogenic differentiation (Venkatesan et al. 2015). Specially, the molecular weight of alginate influences the degradation rate and mechanical properties of the scaffolds – the slower the degradation rate, the higher the molecular weight – because of the decreases in the number of reactive positions available for hydrolysis degradation.

Chemical modifications of alginate as, for example, oxidation or introduction of chemical moieties in the backbone of this polymer also have been used to enhance the scaffold properties (Lee and Mooney 2012).

Alginate-based blends with other natural polymers and ceramic component have interesting properties for bone repair. A nano-sized hydroxyapatite/alginate/chitosan composite scaffold was achieved by Kim et al. (2015), with high strength and controlled pore structures that helped a better differentiation and mineralization of the MC3T3-E1 cells. Bharatham et al. (2014) prepared a novel scaffold combining alginate with a naturally obtained biomineral (nano-cockle shell powder/nCP) and tested it in vitro using MG63 human osteoblast cells. Hydrogels based on methacrylated alginate and collagen were developed, and MC3T3-E1 cells that grow in the scaffolds exhibited a rapid proliferation and a facilitated osteogenic differentiation. This chemical modification of the alginate also provides the capacity to control the degradation rate, swelling, and mechanical properties of this material.

The addition of synthetic polymers onto alginate normally increases the mechanical strength of the composite material (Venkatesan et al. 2015). Chan et al. (2015) described the technique for synthesizing of biocompatible alginate/poly( $\gamma$ -glutamic acid) base gel with potential application as injectable bone repair material. Evaluation of its mechanical properties, swelling behavior, and blood compatibility showed its nontoxicity and use for repairing bone defects.

### 3.6 Cellulose

Cellulose is a polysaccharide consisting of a linear chain of several hundred to over 10,000  $\beta$ -(1-4)-linked D-glucose units. Cellulose is considered as one of the world's most abundant natural and renewable resource of raw material. Natural cellulose is present in a wide variety of living species, being mainly obtained from wood, hemp, cotton, and linen. Intra- and intermolecular hydrogen bonds and high molecular weight give cellulose important characteristics such as chemical stability, mechanical strength, biocompatibility, and biodegradability. Nevertheless, the chemical nature of cellulose makes dissolution in common solvents difficult and complicates tissue engineering use. To overcome this problem, several alternatives like using cellulose derivatives (as carboxymethyl cellulose) or bacterial cellulose have been used for scaffold preparation. Bacterial cellulose can be obtained by biosynthesis from bacteria, *Acetobacter xylinum* being the most efficient and investigated producer of this biopolymer (Gomes de Oliveira Barud et al. 2016). Bacterial cellulose is identical to plant cellulose in chemical structure, but it can be produced without contaminant molecules, such as lignin and hemicelluloses, and does not require intensive purification processes (Novotna 2013). Several studies with BC have been developed in this way, using BC with a mineral phase (i.e., hydroxyapatite [HA]) to emulate bone composition. A membrane composed of BC and hydroxyapatite (HA) was developed as biomaterial for potential bone regeneration, which delivered prone growth of osteoblast cells, high level of alkaline phosphatase activity, and greater bone nodule formation (Tazi et al. 2012). Saska et al. prepared BC-HA

nanocomposites and evaluated the biological properties and performance of the material with respect to bone regeneration in defects of rat tibia (Saska et al. 2011). The composite BC–HA membranes were effective for bone regeneration and accelerated new bone formation. In addition, reabsorption of the membranes was slow, suggesting that it takes longer to this composite to be completely reabsorbed. Pigossi et al. (2015) evaluated the potential of BC–HA composites associated with osteogenic growth peptide (OGP) or pentapeptide OGP (10–14) in bone regeneration in critical-sized calvarial defects in mice analyzed at 3, 7, 15, 30, 60, and 90 days. The researchers found that at 60 and 90 days, a high percentage of bone formation was observed by micro-computed tomography (CT) and a high expression of some bone biomarkers, such as ALP, was also observed. They concluded that the BC–HA membrane promoted a better bone formation in critical-sized mice calvarial defects.

Composite blend constructs with cellulose and different natural polymers also are probed to be interesting for BTE. Liuyun and col (Liuyun et al. 2009) reported the novel composite of nanoHA–chitosan–carboxymethyl cellulose, which was prepared by freeze-drying method. Nanocomposite scaffold with 30% wt. carboxymethyl cellulose had the most ideal porous structure and the highest compressive strength. Cell attachment and proliferation on the scaffold indicate that the nHA–chitosan–carboxymethyl cellulose is nontoxic and has good cytocompatibility. Lee and his group (2013) evaluated in vivo assays by implanting silk fibroin–BC membranes to successfully promote the complete healing of segmental defects of zygomatic arch of rats.

In another work, Aravamudhan reports the fabrication and characterization of cellulose and collagen-based micro-nanostructured scaffolds using human osteoblasts (Aravamudhan et al. 2013). These porous micro-nanostructured scaffolds exhibited mechanical properties in the midrange of human trabecular bone and supported great adhesion and phenotype maintenance of cultured osteoblast as reflected by higher levels of osteogenic enzyme alkaline phosphatase and mineral deposition.

Finally, some works using all-cellulose composites are described elsewhere. He et al. (He et al. 2014) fabricated uniaxially aligned cellulose nanofibers with well-oriented cellulose nanocrystals (CNCs) via electrospinning. The incorporation of CNCs into the spinning dope resulted in more uniform morphology of the electrospun cellulose/CNC nanocomposite nanofibers (ECCNN), and a remarkable enhancement of their physical properties was observed. Cell culture experiments demonstrated that cells could proliferate rapidly not only on the surface but also deep inside the composite material, and the aligned nanofibers exhibited a strong effect on directing cellular organization.

### 3.7 Hyaluronic Acid

Hyaluronic acid is an anionic, non-sulfated glycosaminoglycan, consisting of repeating D-glucuronic acid– $\beta$ -1,3-N-acetyl-D-glucosamine– $\beta$ -1,4 units. Hyaluronic acid can be found in extracellular matrix of all connective tissues in the body and

display several properties like excellent viscoelasticity, water solubility, biocompatibility, and non-immunogenicity (Pina et al. 2015). Another important feature is the capability of hyaluronic acid-based scaffolds to be degraded by enzymatic action. The rate of enzymatic degradation will depend both on the number of cleavage sites in the polymer and the amount of available enzymes in the scaffold biological environment and is catalyzed by hyaluronidase. Recently, Schante et al. have published work on improved enzymatic stability of hyaluronic acid by grafting with amino acids (Schante et al. 2012).

For bone tissue engineering, material with several forms as hydrogels (Bae et al. 2014), fibers (Fischer et al. 2012), meshes (Rhodes et al. 2011), and foams (Dehghani and Annabi 2011) has been created. Also, scaffold of hyaluronic acid derivatives or hyaluronic acid-based composites has been widely used for bone tissue engineering (Collins and Birkinshaw 2013; Sarkar and Lee 2015), aiming to improve mechanical strength, structural integrity, or toughness. For example, photocross-linked methacrylated HA hydrogel loaded with simvastatin or differentiation factor 5 to promote osteogenesis showed better mechanical properties (Bae et al. 2011, 2014). These materials evidence good biocompatibility and higher level of MC3T3-E1 cell proliferation and differentiation in vitro, and in vivo tests using male adult New Zealand white rabbits showed a significant improvement on osteogenesis.

Blending made of hyaluronic acid with natural polymers and bioceramic has also been used as a strategy for bone healing. Hyaluronic acid–gelatin hydrogel loaded into a biphasic calcium phosphate (BCP) ceramic scaffold, with unique micro- and macroporous orientation, was previously obtained (Nguyen and Lee 2014). Both in vitro and in vivo tests were conducted, showing a significant increase in cell proliferation at 3 and 7 days, high alkaline phosphatase activities at 14 and 21 days, and a rapid bone formation (confirmed by histological section) and collagen mineralization after 3 months of implantation. In another study, an injectable nano-hydroxyapatite/glycol chitosan/hyaluronic acid composite hydrogel has been obtained (Huang et al. 2016). In vitro cytocompatibility was evaluated by using MC-3T3-E1 cells to confirm that the developed composite hydrogels were cytocompatible and nontoxic, and cells were found to be attached and well spread out on the hydrogels after 7 days of co-incubation.

## 4 Conclusions

Tissue engineering is a multidisciplinary field of research oriented to the search of new materials whose biodegradation processes are dependent on their applications, focusing in the type of tissue to be regenerated and rate of cell growth. Currently, an ideal biomaterial that meets all the necessary requirements for its application in bone tissue regeneration does not exist. However, the increasing development of TE shows that current trends are focused on composite materials (including nanomaterials), mixing natural and/or synthetic polymers with nanofillers, especially bioceramics. This strategy combines suitable biodegradation and biocompatibility

properties with adequate mechanical strength for each class of tissue to be repaired, together with low cytotoxicity.

Despite advances in the field and the large amount of materials developed, very few of these materials have been tested in clinical trials until today. The study of the interaction between these materials and the tissue to be regenerated, the mechanical strength, the time of degradation optimum to allow the creation of new tissue, and the inclusion of factors that can promote the cellular growth and differentiation are crucial to achieve this goal.

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