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Review Article

Natural biopolymers in bone tissue engineering from aquatic resources: A review

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Abstract: Bone tissue engineering is a rapidly expanding research area that uses an artificial scaffold as a template for new tissue formation by culturing osteoblasts, along with adding regulating factors that promote cell recruitment, growth differentiation, and mineralized bone tissue. Different natural and synthetic materials and their combinations are used for this purpose. However, polymers have many advantages to use as scaffolds in bone tissue engineering, and therefore, their application in tissue engineering has been widened in recent years. In this work, we aimed to review those natural polymers that are used in bone tissue engineering with an emphasis on those originating from natural aquatic resources. In this regard, the recent findings in the application of those natural biopolymers and their characterization *viz.* hydroxyapatite, starch, fibrinogen, silk fibroin, alginate, gelatin, chitosan, and collagen for use as scaffolds in bone tissue engineering were discussed.

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Introduction

Bone tissue engineering is a promising method (Ma et al., 2001; Liu and Ma, 2004) compared to traditional autograft and allograft techniques. It applies autogenous cell/tissue transplantation that can eliminate concerns of donor scarcity, supply limitation, pathogen transfer, and immune rejection (Liu and Ma, 2004). Therefore, it is a rapidly expanding research area (Lysaght and Reyes, 2001; Griffith and Naughton, 2002; Chen et al., 2018) and typically uses an artificial extracellular matrix (or scaffold), osteoblasts or cells that can become osteoblasts, and regulating factors that promote cell recruitment, growth differentiation, and mineralized bone tissue formation. Among them, highly porous scaffolds play a critical role in cell seeding, proliferation, and new tissue formation (Ma and Langer, 1995; Livingston et al., 2002). The scaffold is a 3D substrate for cells and serves as a template for tissue regeneration. Ideal scaffolds should be biocompatible, biodegradable, promote cellular interactions and tissue development, and have proper mechanical and physical properties (Bonadio et al., 1999; Hutmacher, 2001).

A variety of materials have been used for the replacement and repair of damaged or traumatized bone tissues (Thomson, et al., 1995; Langer, 2000; Hench and Polak, 2002; Livingston et al., 2002). These materials are metals, ceramics, polymers (natural and synthetic), and their combinations. Metals and ceramics have two main disadvantages i.e. lack of degradability in a biological environment, and limited processability (Maquet and Jerome, 1997). Whereas, polymers possess design flexibility, and are attractive candidates. Some polymers contain chemical bonds that undergo hydrolysis upon exposure to the body's aqueous environment, and some others can degrade by cellular or enzymatic pathways. Hence, polymeric materials have received considerable attention and are widely studied for bone tissue engineering applications (Chapekar, 2000), especially those obtained from natural resources. Therefore, this review will focus on the selection of polymeric materials obtained from natural aquatic resources to design scaffolds.

Polymers in tissue engineering: In recent years many works focused on developing biomaterials easily produced, offering proper properties and improved

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Table 1. Classification of biomaterials implemented in tissue engineering.

Natural polymers	- Polysaccharides; e.g., chitosan, alginate, hyaluronic acid etc. - Proteins; e.g., collagen, gelatin, albumin, etc.
Synthetic polymers	Polyglycolic acid, polycaprolactone, etc.
Ceramics	Hydroxyapatite, bio glass, _-tricalcium phosphates, etc.
Hybrid	Natural polymers with synthetic polymers; e.g., chitosan and PLGA (poly(D,L-lactide-co-glycolide)). - Natural polymers with ceramics e.g., chitosan and hydroxyapatite - Two or more artificial scaffolds e.g., PEG (polyethylene glycol) and PLGA - Synthetic polymers with bio ceramic e.g., PLGA with hydroxyapatite
Metals	Gold, silver, titanium, etc.

healing rates without any adverse effects. Polymeric materials offer a multipurpose alternative to metallic and ceramic biomaterials which suffer from stress-shielding effects (Fan et al., 2004). Polymeric materials are often biocompatible and are much easier to fabricate; they can be molded into desired shapes and sizes. Their mechanical properties and degradation characteristics can also be controlled, enabling them to be tailor-made and targeted toward a specific purpose once implanted in the host tissue (Pielichowska and Blazewicz, 2010). The first successful clinical application of polymeric materials dates back to using polymethyl methacrylate (PMMA), an acrylic cement, to attach a femoral head prosthesis (Charnley, 1960). Since then, more research has been done on the different natural and synthetic polymers widening their application in tissue engineering (Stone et al., 2004; Lou et al., 2008; Neves et al., 2011).

Natural biopolymers: Biopolymers are produced from natural sources, either chemically or completely biologically synthesized by living organisms (Smith et al., 2016). Biopolymers are chain-like molecules made from repetitive chemical blocks produced from environmentally degradable renewable resources (Mohiuddin et al., 2017). The application of biopolymers from various sources has been studied in pharmaceutical and biomedical uses for many years. Biopolymers have attracted attention due to their varied compositions, tunable physical conduct, and a broad range of products. The comparatively low price and renewable character also attract this material class, especially in the pharmaceutical and biomedical sectors (Smith et al., 2016). A biopolymer can be mixed with another biopolymer, a biodegradable synthetic polymer, or a non-degradable synthetic polymer. Biopolymers can also be mixed to form a composite of a bio-polymer matrix with various reinforcing components such as mineral particles or natural fibers to improve the total time of degradation and the mechanical properties (Rudin and Choi, 2012). Biopolymers are majorly classified into two types based on the source: (1) natural biopolymers that are classified into polysaccharides (starch, alginate, chitin/chitosan, hyaluronic acid derivatives) and proteins (soy, collagen, gelatin, fibrin gels, silk) and (2) synthetic polymers: poly(lactic acid), poly(caprolactone), poly(urethane), poly(propylene fumarate), poly(vinyl alcohol), poly(glycolic acid), poly(hydroxybutyrate) etc., (Imre and Pukánszky, 2012). The classification of biomaterials implemented in tissue engineering is shown in Table 1.

Hydroxyapatite: Hydroxyapatite ($Ca10(PO_4)6(OH)_2$) (HA) is a member of calcium phosphate ceramics, including tricalcium phosphates and bioglass ceramics that are widely used as bone-substitute materials. The preference for HA over other calcium phosphates can be attributed to the structural and functional similarity to mineral composition present in bones and teeth. In the human bone, HA constitutes up to 70% along with 5% water and 25% organic matter (Sommerfeldt and Rubin, 2001; Dutta et al., 2015). Because of its inherent bioactivity, biocompatibility, and ease of fabrication, it is a proper implant material (Ferreira et al., 2012; Felgueiras et al., 2018). Hydroxyapatite can be produced in the form of solid scaffolds with variable porosities, which are ideal for cell attachment, migration, and bone

formation. Moreover, it can be ground into nanoscale powders to be used in combination with other biomaterials, including polymers such as chitosan and collagen for improved bone mineralization (Chen et al., 2000; Li et al., 2002). There is also evidence that HA coatings improve the integration of implants such as titanium to the host bone (Ghalia and Dahman, 2017; Goudarzi et al., 2021).

HA from fish bone and scales are alternatives to substitute synthetic and bovine HA, because of similar chemical properties and simple and inexpensive extraction methods (Hoyer et al., 2012; Venkatesan et al., 2012). Fish source HA is safe and presents low risks of disease transmission (Hoyer et al., 2012). In addition, fishes are easily available, and the application of their byproducts is suitable for the extraction of such bioactive materials as HA since it will decrease environmental pollution and threats of biohazards to humans (Venkatesan et al., 2012) Many fish species have been used to obtain HA, such as salmon (Hoyer et al., 2012) carp, Japanese anchovy, sardine, tilefish, and tuna (Fratzl, 2008). For this purpose, many different protocols of extraction methods for chemical analysis have been proposed (Venkatesan et al., 2012).

Starch: Starch is a natural and recyclable polysaccharide having characteristics of safety, nontoxicity, widespread abundance, low price, cytocompatibility, degradability, and biosafety (Ding et al., 2019). Native starch components include amylose and amylopectin in varying quantities (15-30 and 70-85%) depending on species, growth conditions, and collecting processes. The biochemical nature and physical arrangement of starch components have a serious influence on the material's vast variety of applications (Morrison et al., 1993; Katoch and Choudhury, 2002; Tester et al., 2004). By introducing extra subunits to its networks, starch may be given new functionalities and its utility can be expanded (Nigam and Singh, 1995). Owing to their architecture, enhanced starches have been used to carry diverse nanoparticles with therapeutic treatments against ailments in recent years (Ali et al., 2020). Because of their strong hydrophilicity and viscosity stability, nonionic starch derivatives from hydroxyalkyl starch have excellent application performance. 3D printed hydroxyapatite—Zn2+ functionalized starch composite has enhanced mechanical and biological features, and could be used as a bone filler in low load-bearing defects such as craniomaxillofacial bone (Bhattacharjee and Bose, 2022).

Fibrinogen: Fibrin gel is obtained by the reaction between commercially purified allogenic fibrinogen and thrombin which are the primary proteins in blood clotting (Riedelová-Reicheltová et al., 2016). The use of fibrin as a cell transport material result in autologous and mechanically durable constructs that undergo notable tissue growth *in vivo* (Flanagan et al., 2009). In order to create implantable constructions that may be employed in dental engineering or dentistry, fibrin may also serve as an autologous scaffolding biomaterial. Dental tissue engineering can make use of injectable biomaterials, particularly those made from aqueous solutions, which are ideal transporters for cells and other bioactive ingredients (Kretlow et al., 2009). Fibrin (Fbn) and fibrinogen (Fbg) can be utilized as scaffolds because they have a tendency to achieve uniform cell distribution, a higher cell seeding effectiveness, and consistent cell distribution (Rajangam et al., 2013). They also have the capacity to migrate, proliferate, and differentiate into specific organs and tissues by releasing ECM (extracellular matrix) to produce tissues. As a result, fibrin is the perfect substance to use as a b platform for dental tissue engineering purposes. In recognition of their unique nano and micro features, fibrin (Fbn) and fibrinogen (Fbg) have been created into various scaffolds. Various scaffolds such as hydrogels (Rowe et al., 2007), nanofibers (Wnek et al., 2003), nanoparticles (Rejinold et al., 2010), microfibers (Swartz et al., 2005), microtubes (Rajangam et al., 2012), and microspheres (Rajangam et al., 2011) have been developed for a variety of tissue engineering applications, particularly dental tissue engineering.

Silk fibroin: Silk fibroin (SF) is an insoluble peptide with large hydrophobic motifs released by silkworms, arachnids, and other insects that may be readily isolated and used to make sericin-free silk-derived

scaffolds (Kaplan et al., 1994). Because of its extremely adjustable material characteristics, great biocompatibility, and moderate foreign body reaction *in vivo*, SF has been employed as an outstanding framework. Because of its outstanding mechanical qualities, superior biocompatibility, predictable biodegradability, and low antigenicity, SF has been researched in biological and pharmacological disciplines. Bombyx mori silk fibroin (BSF) has recently been used widely as a tissue-engineered framework for the formation of bones (Yang et al., 2001).

Alginate: Alginate is a seaweed-derived poly-saccharide extracted from Phaeophyceae-brown algae. It comprises -(1–4)-d-mannuronic acid and -(1,4)-l-guluronic acid connected as repeated linear chains (Diekjürgen and Grainger, 2017; Ahadifar et al., 2020, 2021a, b; Sahoo and Biswal, 2021; Najahi Mohammadizadeh et al., 2023). Alginate shows biocompatibility, biodegradability, a simple production process, and tunable mechanical properties, leaping to join in developing scaffolds in cartilage tissue engineering (Farokhi et al., 2020). Moreover, alginate is hydrophilic and is used in wound dressing to absorb the pus to help healing. It is also used in cell growth scaffolds, supporting blood vessels' formation, healing bone injuries, cartilage regeneration, and drug delivery systems (Kurowiak et al., 2020). Alginate-based scaffolds are widely used in various tissues or organs, including skeletal muscles, pancreas, nerve, liver, and dental tissue engineering (Rosellini et al., 2020; Sahoo and Biswal, 2021).

Gelatin: Gelatin is a protein molecule obtained by the hydrolysis of collagen, constituting the Arg-Gly-Asp (RGD) peptide sequence, which helps in cell adhesion, proliferation, and differentiation (Purohit et al., 2020). The primary source of gelatin production is extracted from mammals, especially bovine hides and porcine skin (Abedinia et al., 2020). however, in recent years, it has been extracted from fish bone and scale. Scaffold coated with gelatin inhibits complement system and opsonization, thus, it reduces their immunogenicity (Ashwin et al., 2020). *In vitro* studies show that scaffolds based on gelatin can control cell differentiation and gene expression (Kimura et al., 2021). Dehghan et al. (2021) combined gelatin, polycaprolactone, and polydimethylsiloxane to produce a scaffold, and further investigations on tests regarding biocompatibility, biodegradability, and mechanical properties gave a positive result (Dehghan et al., 2021). Singh et al. (2020) used gelatin as a fabricating material for a cellulose-based scaffold produced from cotton to improve cell adhesion (Singh et al., 2020). A poly ("-caprolactone) and gelatin composite scaffold incorporated with acetylated cellulose nanofiber was suggested to be an ideal scaffold for soft tissue engineering (Goudarzi et al., 2021).

Chitosan: Chitosan is a partially deacetylated derivative of chitin, one of the most abundant polymers in nature found in the shells of crustaceans and walls fungi. It is composed of randomly distributed -(1-4)-linked D-glucosamine (glucosamine) and N-acetyl-Dglucosamine (N acetylglucosamine) structure units, structurally similar to glycosaminoglycan, a key component of the bone matrix and cell surface which modulates the bioavailability and activity of various osteoclastic and osteogenic factors (Tan et al., 2009; Mansouri et al., 2017; Islam et al., 2017). Deacetylation of chitin is almost never complete and the chitosan chain still contains amide groups to some extent (Tan et al., 2009). The degree of deacetylation (DD%) is defined as the molar fraction of glucosamine in the chitosan composed of N-acetylglucosamine and glucosamine structure units (Jiang et al., 2017).

Chitosan has poor solubility in physiological solvents (e.g., water) due to its strong intermolecular hydrogen bonding considering a strong base due to primary amino groups with a pKa value of 6.3 (Pillai et al., 2009; Tan et al., 2009). Chitosan solution can be obtained in acidic aqueous (pH<6) media, which protonate chitosan amino groups, rendering the polymer positively charged and thereby overcoming associative forces between chains (Chenite et al., 2001; Pillai et al., 2009; Tan et al., 2009). If the pH of the chitosan solution increases above 6, chitosan amino groups become deprotonated and the polymer

chain loses its charge, which leads to insolubility. The solubility is highly dependent on the degree of the deacetylation, the used deacetylation method, and molecular weight. The solubility of chitosan can be increased by chemical modifications possible at two hydroxyl functional groups in the polymer chain (Pillai et al., 2009). The detailed review paper by Upadhyaya et al. (2013) provides an overview of the water-soluble carboxymethyl chitosan as a modification of the non-soluble chitosan. The polycationic nature of the chitosan chain is essential for antibacterial activity (Yilmaz Atay, 2019).

Protein adsorption is the first step to take place upon implantation. Protein adsorption occurs within a few minutes or even seconds after scaffold implantation and the cells that reach the biomaterial surface no longer attach directly to the biomaterial but to the adsorbed protein layer. Through cell membrane-bound receptors or ligands, cells identify bioactive binding sites on the protein layer and behave according to the stimuli received (Felgueiras et al., 2018). As a natural positive-charged polysaccharide, protonable amino groups on the chitosan backbone electrostatically interact with the various negatively charged proteins (Pillai et al., 2009). Electrostatic interactions between biomaterial and proteins depend on the biomaterials' surface and protein charges, which are a function of pH and the solution ionic content. Usually, at low pH, proteins are positively charged, whereas at polymers high pH, are negatively charged (Felgueiras et al., 2018). Bovine serum albumin protein is often used as a model protein for biomaterial characterization regarding protein adsorption capacity, because of its high stability, availability at high purity, and water solubility (Phan et al., 2015). Interactions between BSA protein and chitosan chain depend on the pH and the interaction mechanism is highly complex. The protein adsorption capacity of scaffolds needs to be determined, as protein adsorption is the first and crucial step after biomaterial implantation.

Collagen: Collagen accounts for 30% of the total protein weight in the body. There are many types of collagens (Liu et al., 2015), in which type I constitutes 90% of total collagens and is widely spread in the ECM of the native tissues, including dentine, bone, skin, tendon, pancreatic, and cartilage (Kadler et al., 2007; Mansour et al., 2018; Abazari et al., 2020). Three polypeptide chains, two $\alpha 1$, and one $\alpha 2$, constitute the triple-helix structure of collagen (Jung et al., 2009). The $\alpha 2$ chain's chemical composition differs in various types of collagens (Chattopadhyay and Raines, 2014). The polypeptides are rich in glycine (Gly), proline (Pro), and 4-hydroxyproline (Hyp) in a repeating Gly-Pro- Hyp triplet (Fratzl, 2008). The three polypeptide chains are assembled in a triple helix structure called procollagen. The N- and C-terminal of the helix are cleaved by metalloproteinase enzymes generating mature collagen (Mouw et al., 2014). These collagens are self-assembled and generate microfibrils with 3-5 nm in diameter. Finally, the microfibrils self-assemble and form a 3D collagen fiber (Orgel et al., 2006; Olszta et al., 2007).

The formation of collagen fibers from monomers is largely affected by environmental factors including pH, ionic strength, and temperature (Kadler et al., 1996; Fratzl 2008). Collagen has ideal characteristics to be used as a biomaterial in tissue engineering (Mansour et al., 2018; Abazari et al., 2020). It is abundantly found in the native tissue of all vertebrates. It has high biocompatibility and low antigenicity and could be easily processed and combined with other biomaterials to improve its properties (Ferreira et al., 2012). It is degraded by metalloproteinases in the human body (Liu et al., 2015). Collagen has functional groups in its polypeptide backbone which could be coupled with growth factors, genes, and therapeutic biological molecules (Ferreira et al., 2012; Pawelec et al., 2016). Bovine, ovine, porcine, and equine are the major natural collagen sources (Albu et al., 2011). Collagen could be isolated and used in two ways: decellularized collagen matrix and isolated collagen molecules (Chattopadhyay and Raines, 2014). However, the risk of infectious diseases due to pathogen transmission is high. The expression of recombinant collagen protein (RCP) in mammals, insects, and yeast is another source of collagen. In this

case, it is possible to control the amino acid composition and molecular weight of RCP. They can be produced at a large scale and the risk of pathogen transmission is low (Olsen et al., 2003). However, as RCP lacks hydroxyproline residues, it has a low tendency to generate fibrils (Ramshaw, 2016).

Type I collagen is abundantly found in the bone ECM which is secreted by osteoblasts. It has a major role in matrix mineralization (Tomoaia and Pasca, 2015). Therefore, the use of collagen products could improve the biological integration of cells with surrounding tissues and increase the function of osteoblasts (Chattopadhyay and Raines, 2014). The potential of collagen-based scaffolds in bone engineering has been shown in several *in vitro* and *in vivo* studies. *In vitro*, collagen scaffolds improve the maintenance and function of cultured osteoblasts, increase matrix mineralization, and elevate the activity of the alkaline phosphatase (ALP) osteogenic enzyme (Aravamudhan et al., 2013). *In vivo*, the implanted scaffolds showed highly osteogenic properties in critical bone defects (Miguel et al., 2013). Collagen could be easily combined with other biomaterials. Combining hydroxyapatite (HA) with collagen increases the differentiation of MSCs into osteoblasts. Collagen-HA also has shown osteochondral tissue regeneration in a large cohort of patients. The use of a dexamethasone-loaded collagen scaffold promoted *in vitro* osteogenic differentiation of MSCs and increased ectopic bone regeneration after subcutaneous implantation in an animal model (Chen et al., 2018). Letic-Gavrilovic et al. (2003) showed that the delivery of nerve growth factor β (NGF β) by a collagen/hydroxyapatite (Col/HA) composite increases new bone ingrowth in rats. It is also possible to combine viruses into the collagen matrix. Zhang et al. (2009) showed that introducing adenoviruses expressing bone morphogenetic protein 7 (BMP-7) and PDGF-B into chitosan/collagen scaffolds increases bone formation in animal models after 4 and 8 weeks of implantation. These studies indicated the great potential of collagen in bone regeneration. In clinical trial studies, collagen is a polymer of interest (among natural polymers) for bone tissue repair and regeneration. Jager et al. (2011) used a collagen sponge scaffold for bone deficiency patients. The radiographic images from the patients indicated new bone formation in all of them. Porous collagen scaffolds have been used in several clinical studies and they showed promising results in new bone formation. However, complications such as inflammation, hematoma hardware failure, wound secretions, and several other complications have been reported (Govender et al., 2002; Calori et al., 2008; Jäger et al., 2011).

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