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Review

Collagen for bone tissue regeneration

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ABSTRACT

In the last decades, increased knowledge about the organization, structure and properties of collagen (particularly concerning interactions between cells and collagen-based materials) has inspired scientists and engineers to design innovative collagen-based biomaterials and to develop novel tissue-engineering products. The design of resorbable collagen-based medical implants requires understanding the tissue/organ anatomy and biological function as well as the role of collagen's physicochemical properties and structure in tissue/organ regeneration. Bone is a complex tissue that plays a critical role in diverse metabolic processes mediated by calcium delivery as well as in hematopoiesis whilst maintaining skeleton strength. A wide variety of collagen-based scaffolds have been proposed for different tissue engineering applications. These scaffolds are designed to promote a biological response, such as cell interaction, and to work as artificial biomimetic extracellular matrices that guide tissue regeneration. This paper critically reviews the current understanding of the complex hierarchical structure and properties of native collagen molecules, and describes the scientific challenge of manufacturing collagen-based materials with suitable properties and shapes for specific biomedical applications, with special emphasis on bone tissue engineering. The analysis of the state of the art in the field reveals the presence of innovative techniques for scaffold and material manufacturing that are currently opening the way to the preparation of biomimetic substrates that modulate cell interaction for improved substitution, restoration, retention or enhancement of bone tissue function.

1. Introduction

In mammals, collagen is the most abundant protein, constituting more than one-third by weight of body protein tissue [1]. Around 28 types of collagen [2] have so far been identified and, among these, type I collagen is the most prevalent type found in the extracellular matrix (ECM), especially in tissues such as tendon and bone [2,3]. The ECM plays an important role in the morphogenesis and cellular metabolism of new tissues, conferring mechanical and biochemical properties [2]. Collagen has potential as a biomaterial for bone tissue engineering due to its abundance, biocompatibility, high porosity, facility for combination with other materials, easy processing, hydrophilicity, low antigenicity, absorbability in the body, etc. [4,5].

1.1. Collagen structure

Collagen protein has a complex hierarchical conformation divided in four structures: primary structure (amino acid triplet), secondary structure (the α-helix), tertiary structure (triple helix) and quaternary structure (fibrils) [2].

1.1.1. Primary structure: amino acid triplet

Collagen protein is recognized by the characteristic domain of proline-rich Gly-X-Y polypeptide (Fig. 1) with two unique features: (i) Gly is found every third residue with the strict repeating –(Gly-X-Y-)$_n$– tripeptide sequence along the entire length of the ~1000 amino acid chain. However, a single substitution of a Gly with an Ala residue has been found in the crystal structure of a triple-helical molecule after 10 repeating Pro-Hyp-Gly units [6]. (ii) A high proportion of residues (~20%) in the tripeptide sequences is frequently comprised of proline (X) and hydroxyproline (Y). Hydroxyproline is not commonly found in other proteins, while in collagen it constitutes more than 50% of the total amino acid content [7,8].

1.1.2. Secondary structure: α-helix

The α-chains are formed by repetitions of the tripeptide –(Gly-X-Y-)$_n$– and are linked to each other, building the characteristic triple helix of type I, II and III collagen [9]. The non-helical domains are at the end of the α-chains, where the C-terminus is involved in the initiation of triple-helix formation and the N-terminus is

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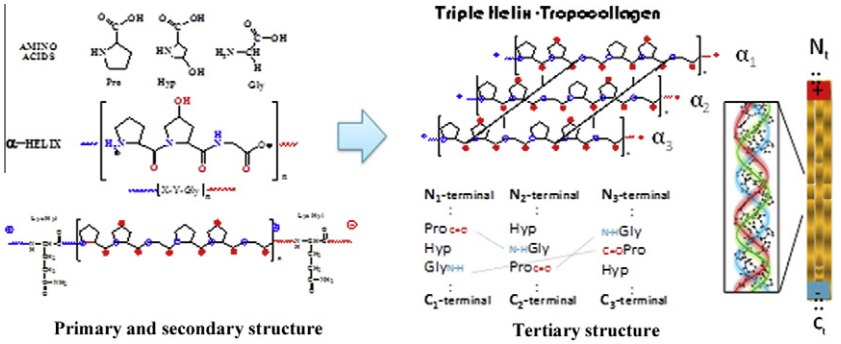

Fig.1. Schematic drawing of the hierarchical structure of collagen: primary, secondary and tertiary structure.

involved in the regulation of primary fibril diameters. The short non-helical telopeptides of collagen are linked by covalent cross-links which form between the collagen molecules and/or between collagen and other molecules present in the ECM [2,10].

1.1.3. Tertiary structure: triple helix

The triple helix, especially in collagen type I, is usually formed as a heterotrimer of two identical α_1(I)- and α_1(II)-chains and one α_2(I)-chain with about 1000 amino acids, and is approximately 300 nm in length (L) and 1.5 nm in diameter [9,11]. The three α-chains form a left-handed, rod-like helix, where the glycine residues are located around a central axis, while larger amino acids belonging to the X and Y residues (usually proline and hydroxyproline) occupy outer positions [9] (Fig. 1). The α-chains are linked to each other by hydrogen bonds through the single interstrand N–H(Gly)...O = C(X) as well as Cα–H(Gly/Y)...O = C(X/Gly), which are the major stabilizing interactions of the α-triple helical and β-sheet protein structures [8,12,13]. Some studies of collagen molecule assembling have hypothesized that the C-terminal (COOH-terminal propeptide) globular domains of the α_2(I)-chain in the collagen type I play a crucial role in the initiation of the intermolecular assembly, chain association and stable collagen heterotrimer formation [14–16].

1.1.4. Quaternary structure: collagen fibrils

Collagen molecules are able to self-assemble into a supramolecular form via a quarter-stagger package pattern of five triple-helical collagen molecules highly oriented with D-periodic banding spaces, where D is ~67 nm (Fig. 2) [11,17]. The telopeptides, composed of non-helical regions about 20 amino acid residues in length, play an important role in the fibrillogenesis, contributing to the stabilization of the mature collagen molecules by cross-link formation [18]. In fact, collagen cross-links are divided into two types: enzymatic cross-links, mediated by lysine hydroxylase and lysyl oxidase; and non-enzymatic cross-links, commonly called glycation or oxidation induced Advanced glycation end products (AGE) cross-links [19]. Fig. 2 shows an example of enzymatic cross-linking mediated by lysyl oxidase. The two chemical forms of 3-hydroxypyridinum cross-linking, namely hydroxyl lysyl pyridinoline (HP) and lysyl pyridoline (LP) cross-links, are formed between the amine side group present in the lysine and hydroxy lysine residues in collagen telopeptides, which are converted into aldehydes by the lysyl oxidase enzyme, and the specific active binding sites present in neighboring triple helices [10,11].

Various non-collagen proteins and bound water fill the space between cells and fibers of the connective tissue defining the features of the tissue. These macromolecules can be grouped into two main classes: glycosaminoglycans (GAGs) and glycoproteins [20]. Proteoglycans are complex molecules that resemble the shape of a brush used to clean test tubes and comprise around 80 GAG chains bound covalently (with the exception of hyaluronic acid) to the central core of a protein. A large number of anionic charges, such as carboxyl and sulfate groups, are present in the GAGs and interact electrostatically with water molecules, regulating the hydration of the connective tissue, and with ECM proteins, such as collagen, forming an interlocked supramolecular matrix [20,21].

1.2. Applications of collagen

Historically, the industrial uses of collagen in the form of leather and gelatin are widespread, including photographic gelatin, cosmetics, food and pharmaceutical applications, enzyme production, etc. [22]. Collagen, as a fibrous protein, is the principal component of connective tissues in mammals. The fibrillar collagens are insoluble in their native structure but can be solubilized in aqueous solution if they are denatured to soluble procollagens [23]. The denaturation of collagen is an irreversible kinetic process [24] and it may be obtained by thermal treatment: once the helix–coil transition temperature (e.g. ~37 °C for bovine collagen) is exceeded, collagen is converted into a randomly coiled gelatin [25]. Other methods to produce gelatin include acid or alkaline chemical treatments [22].

For the past decade, collagen has been among the most widely used biomaterials for biomedical applications, due to its excellent biological features and physicochemical properties [26]. Collagen may be easily modified by reaction of its functional groups, introducing cross-links or grafting biological molecules to create a wide variety of materials with tailored mechanical or biological properties [5,27,28]. The main drawbacks of collagen include the high costs of manufacturing (due to the time-consuming and complex procedures required for isolation and purification), careful selection of processing conditions to avoid denaturation, and high swelling in vivo, due to collagen hydrophilicity [21–22].

In recent years, demand for the development of innovative products aimed at the replacement, correction and improvement of poorly functioning tissues in humans or animals has increased. Collagen can be easily modified into different physical forms such as powder/particles, fibers/tubing, gel/solution, films/membranes, sponges, blends (with other polymers) and composites (with ceramics). Collagen has found a wide variety of applications in the field of medicine including: sutures, hemostatic agents, tissue replacement and regeneration (bone, cartilage, skin, blood vessels, trachea, esophagus, etc.), cosmetic surgery (lips, skin), dental composites, skin regeneration templates, membrane oxygenators, contraceptives (barrier method), biodegradable matrices, protective wrapping of nerves, implants, corneal bandage, contact lens, drug delivery, etc. [22,25,28].

In particular, among the various collagen types, type I collagen is the most abundant component of the ECM and may be used as scaffolding material, promoting cell migration, wound healing

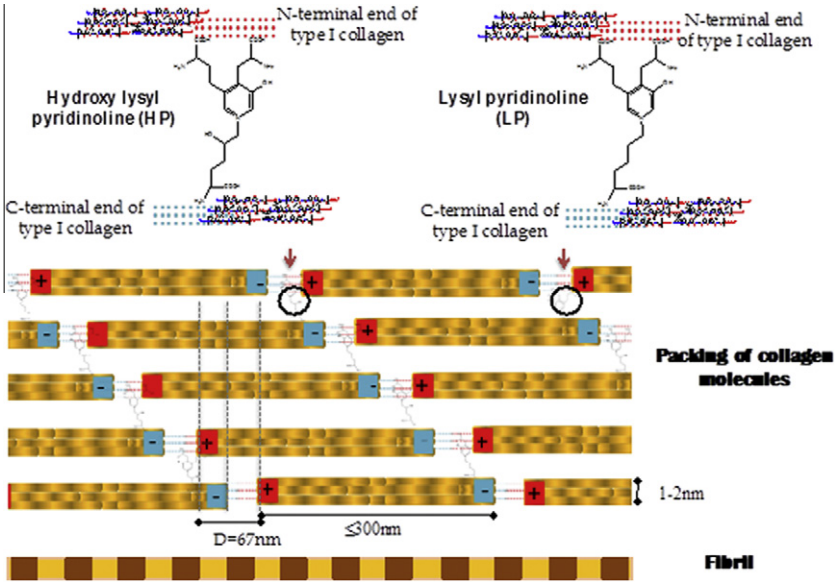

Fig. 2. Schematic drawing of the quaternary structure of collagen forming fibrils and the two chemical forms of 3-hydroxypyridinum cross-linking.

and tissue regeneration. As the bone ECM is very rich in type I collagen, it has found important applications in bone tissue engineering where a collagen-based scaffold provides the innate biological information required for cell adhesion, proliferation and orientation, and promotes the chemostatic response [29].

2. Bone tissue engineering

In the human body, bone belongs to a family of tissues with a complex structure organized hierarchically. Bone is composed of calcium phosphate (69–80 wt.%, mainly hydroxyapatite), collagen (17–20 wt.%) and other components (water, proteins, etc.) [30]. Natural bones are a complex assembly of parallel type I collagen nanofibrils and HA crystals precipitated on their surface [31].

Two types of cells play an important role in the formation of bone: osteoblasts (bone-forming) and osteoclasts (bone-resorbing). During the process of ossification, osteoblasts secrete type I collagen, in addition to many non-collagenous proteins such as osteocalcin, bone sialoprotein and osteopontin. Osteoblast-secreted ECM may initially be amorphous and non-crystalline, but it gradually transforms into more crystalline forms [32]. Mineralization is a process of bone formation promoted by osteoblasts and is thought to be initiated by the matrix vesicles that bud from the plasma membrane of osteoblasts to create an environment for the concentration of calcium and phosphate, allowing crystallization [33]. Collagen serves as a template and may also initiate and propagate mineralization independent of the matrix vesicles [34,35]. Eventually, some osteoblasts are surrounded by the bone matrix that they help to form; these are called osteocytes. Despite their location, osteocytes are not metabolically inactive; they dissolve and resorb some bone mineral though osteolysis [36]. Bone resorption is in fact the primary function of another bone cell, the osteoclast, which can also digest calcified cartilage and is then called the chondroclast. Formation by osteoblasts and resorption by osteoclasts maintains bone in a constant state of renewal as a dynamic tissue [37,38]. Moreover, the mineralization process has been credited to the work of osteoblasts, which are able to respond to mechanical and electrochemical stimuli produced by bone deformation [39]. In accordance with Wolff's law, bone remodeling may be influenced by electrical dipoles produced either by the piezoelectric effect due to bone microstructure orientation or to collagen anisotropy when tissue is subjected to mechanical stress [40–42]. In particular, the mineralization process of cortical bone collagen induced by the piezoelectric effect when the material is subjected to mechanical stress using a biomimetic approach in a cell-free system has been studied [43,44]. It was observed that bone collagen mineralization occurred mainly in the bone collagen side deformed under compression, suggesting that mineralization is strongly influenced by the piezoelectric effect induced in a sample immersed in simulated body fluid (SBF) [43]. Fig. 3 shows the initial steps of apatite growing on collagen fibers when deformed under compression after 3 weeks of immersion in SBF at physiological conditions [44]. This phenomenon was reported after 3 weeks of incubation and only in the compression side of the deformed samples (not in the tension side or the controls).

Bone tissue engineering is aimed at the development of suitable scaffolds, mimicking the native bone tissue microenviron-

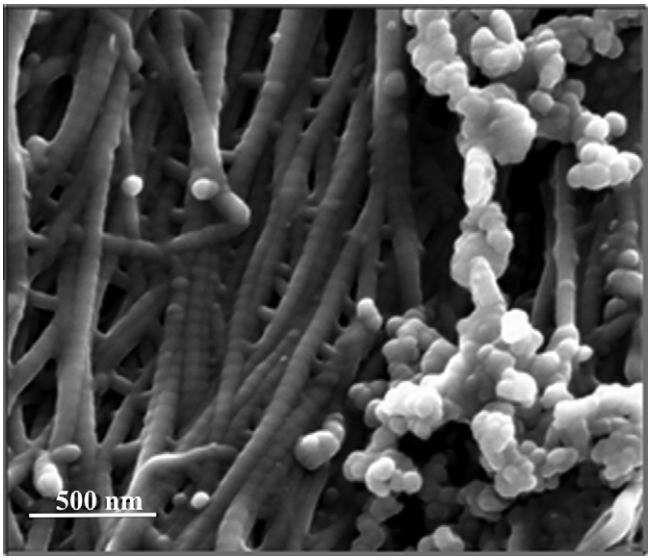

Fig. 3. SEM micrograph of mineral growing on collagen fibers induced by demineralized bone matrix deformation under compression forces and immersed in SBF for 3.5 weeks [44].

ment to exploit the natural biological response to tissue damage, and incorporating engineering and life science principles [45,46]. A synthetic bone scaffold should provide a temporary mechanical support and a porous architecture to promote bone cell migration and differentiation into the scaffold, encouraging osteoinduction and enhancing osteointegration with the host tissue. In addition it should be sterilizable without loss of bioactivity, release bioactive molecules/drugs and degrade in a controlled manner without producing toxic degradation products or eliciting a chronic inflammatory response [45,47]. One of the main challenges to bone tissue engineering is to develop scaffolds with optimal mechanical properties, biodegradability and architecture for cell colonization and organization, which can ensure the integration of the scaffold with the host tissue. The principal strategies for bone tissue regeneration include the introduction of morphogenetic, haptotactic and chemotactic signals into the scaffolds [48]. A wide variety of biomaterials has been studied for the preparation of bone scaffolds. They include polymers derived from natural sources, synthetic materials or hybrid materials the selection criteria for which depends on desired physicochemical properties for the scaffold and the required biological cues. In detail, suitable biological signals (i.e. ligands for specific cell integrins or natural polymers with a composition similar to natural ECM) may be included in the biomaterials [49,50]. Natural materials such as collagen, chitosan, fibrinogen, hyaluronic acid, alginates and glycosaminoglycans (GAGs) have attracted researchers' attention as biomimetic scaffolding materials due to their bioactivity and capability to interact with cells, as well as their biocompatibility and biodegradability. However, natural polymers have several disadvantages, such as poor mechanical properties, low reproducibility depending on the natural source, water solubility or high hydrophilicity (swelling), difficulties in processability, possible denaturation during processing, immunogenicity and, in some cases, potential risk of transmitting animal-originated pathogens [48,49,51].

Millions of people worldwide suffer from bone disorders, bone fractures/injuries and diverse musculoskeletal problems that are usually treated by drug therapies or surgeries, which generally include partial or total replacement of the diseased tissue. In Europe, 20–30% of adults are affected by musculoskeletal pain, which represents almost 25% of the total cost of illness (excluding trauma) [52,53]. Moreover, according to the American Academy of Orthopedic Surgeons, around 6.3 million fractures are registered each year in the United States. Musculoskeletal problems have a considerable socioeconomic impact related to the clinical care of patients that involves extensive stress, employment difficulties, reduced quality of life and large financial costs. In particular, the economic impact of these conditions is staggering: for example, in the United States the sum of direct expenditures on healthcare costs and the indirect expenditure of lost wages in 2004 was estimated to be $849 bn. The burden of musculoskeletal conditions is expected to grow for the next 10–20 years due to the aging of the population and an increasingly sedentary lifestyle [54].

In order to improve bone and joint health, bone tissue engineers are currently pursuing various solutions in this field, formulating innovative matrix/scaffolds by the synergistic combination of biomaterials and cell therapy. Once introduced into the body, optimal scaffolds should maintain appropriate mechanical properties, undergo degradation in a controlled manner without releasing toxic products, control temporally and spatially the release of encapsulated drugs or bioactive molecules, and guide cell behavior to reproduce the hierarchical architecture of native bone tissue [29,55].

2.1. Collagen manufacture for bone tissue engineering

Collagen is ubiquitous in the mammalian body, and tissues such as skin and tendon that are rich in fibrous collagen are therefore used as a source for collagen extraction. Various types of collagen can be obtained from different sources such as mammalian tissues, fishes [56–60], alligators [61], etc. Native collagen is a highly hydrophilic protein, insoluble in organic solvents. Collagen molecules are cross-linked by covalent bonds that help to preserve the quaternary structure and avoid the molecule dissociating from its fibrillar conformation. Depending on its maturity and the kind of tissue, the degree of native collagen cross-linking varies. The extraction conditions affect the quantity of dissociated collagen. Collagen can be isolated and purified as collagen molecules (soluble collagen) or collagen fibers (insoluble collagen). The fibrillar collagen matrix has a complex structure maintained by the intra- and intermolecular cross-links among the telopeptides. Fibrillar collagen is very resistant to proteolysis and, except for type I collagen, none of the other types can be isolated from adult tissues under non-denaturing conditions [62]. Fibrillar collagen can be extracted from tissues by neutral salt treatment (e.g. NaCl) to remove the non-collagenous molecules and collagen molecules that have not been bonded covalently to the collagen fibrils [63]. Otherwise, lipids can be removed using low-molecular-weight organic solvents. The small amounts of non-collagenous proteins not removed by neutral salt treatments (e.g. GAGs) can be extracted with acid or alkaline procedures, weakening the non-specific interaction between the proteins and collagen fibers [26].

Generally, collagen molecules can be extracted and purified from tissues by a variety of techniques such as acid treatments (commonly, dilute acetic acid), alkali treatments (usually using NaOH solutions) or proteolytic procedures, followed by treatments with neutral salts, dialysis, precipitation and centrifugation. However, the technique that offers higher yields and, consequently, is commonly applied for the isolation and purification of soluble collagen from native materials involves a proteolytic treatment in acidic environment (e.g. pepsin) to cleave the collagen cross-links and telopeptides which store the major antigenic determinants [64,65]. Collagen becomes soluble in aqueous solution at room temperatures and must be precipitated using neutral salts such as sodium chloride (NaCl) [28]. Purification of collagen material is important to minimize cytotoxicity and body reactions. The highly purified collagen can be self-assembled into fibrils of various polymorphisms, using phosphate buffers, at different solution temperatures and pH. However, the absence of telopeptides hampers the ability of fibril formation in comparison with the native intact molecules [26,28].

In the manufacture and processing of biomaterials, native collagen can be exploited in biomedical applications, improving in particular biological integration with the surrounding tissue in vivo. Designing resorbable collagen-based medical implants requires a thorough understanding of the structure and function of the tissue and organ to be repaired. Material degradation can result from biological processes such as enzymatic degradation or environmentally induced degradation [26]. In order to achieve some of the functions required for tissue regeneration in scaffold applications, collagen must therefore be treated (e.g. cross-linked or blended) depending on the specific tissue requirements. Collagen may be cross-linked by a variety of chemical agents or physical treatments to enhance the mechanical and chemical stability. Among the chemical agents, aldehydes such as glutaraldehyde, carbodiimides, polyepoxy, etc., are the most commonly used. However, glutaraldehyde and polyepoxy compounds are cytotoxic at concentrations of around 10^{-5} M, and thus their use has been limited due to resid-

ual cross-linking compounds remaining in the collagen implant [5]. Chemical cross-linkers may have toxic effects due to residual reagents or secondary products during implant degradation. Physical treatments such as dehydrothermal treatment, ultraviolet irradiation, gamma irradiation and microwave irradiation introduce cross-links efficiently. However, collagen could be degraded by too long an exposure to physical treatments. Non-conventional methods employing enzymes (e.g. transglutaminase) may be used as an alternative, with the advantage that mild conditions can be used [28].

2.2. Physical forms and applications based on collagen

Collagen can be processed in different physical forms as well as with a wide number of fabrication technologies. In this review, the physical forms of collagen for bone tissue applications have been divided into four groups: (i) injectable hydrogels, (ii) membranes and films, (iii) sponges and scaffolds, and (iv) micro- and nanospheres. This part of the review discusses the diverse processing techniques and applications in bone tissue engineering currently applied in the research field and commercially available. In Table 1, some of the methods currently employed for the different forms of collagen processing are summarized.

2.2.1. Injectable hydrogels

Several different types of scaffold materials have been used for tissue engineering applications, and hydrogels form one group of materials that have been used in a wide variety of applications [66]. A gel is defined as a three-dimensional network swollen by a solvent, and hydrogels are hydrophilic polymer networks that may absorb from 10–20%, up to thousands of times their dry weight in water; this property allows cells to adhere, proliferate and differentiate onto the hydrogels [67]. Hydrogels represent an important class of biomaterials in biotechnology and medicine because most of them exhibit excellent biocompatibility with minimal inflammatory responses and tissue damage, and thus many studies on bone tissue engineering applications have been undertaken [68–70]. Recently, minimally invasive treatments have been developed using injectable systems for bone tissue engineering. Several injectable gels have been used to carry cells in order to engineer bone.

A collagen hydrogel is an excellent candidate for cell encapsulation due to its swelling ability in water, suitable physical properties (e.g. mechanical properties, gelling ability), high water content facilitating the mass transport and diffusion, excellent biological properties, and susceptibility to enzymatic degradation [71]. As an example, hydrogels containing various weight ratios of chitosan and collagen have been fabricated by initiating gelation using β-glycerophosphate (β-GP), an osteogenic medium supplement and a weak base, and varying the temperature [72]. The presence of collagen in chitosan–collagen materials was associated with increased cell spreading and proliferation, as well as increased gel compaction and a resulting stiffer matrix. Adult human bone marrow-derived stem cells (hBMSCs) encapsulated in such hydrogels at different chitosan/collagen ratios exhibited high viability after the first day of encapsulation; however, DNA content dropped by about half over 12 days for pure chitosan materials, but increased 2-fold in materials containing collagen. Collagen-containing materials compacted more strongly and were significantly stiffer than pure chitosan gels. These chitosan–collagen composite hydrogel materials have potential applications in regenerative medicine, particularly in applications where injectable cell carriers are advantageous. Such materials can be used for cell encapsulation and delivery, or as in situ gel-forming materials for tissue regeneration.

Recently, to meet the challenges of designing injectable scaffolds able to regenerate bone, a biomimetic and injectable hydrogel scaffold based on nanohydroxyapatite (HA), collagen (Col) and chitosan (Chi) was prepared by Huang et al. [73]. The Chi/HA/Col solution rapidly formed a stable gel at body temperature, showing similar composition and microstructure to natural bone and representing a candidate for minimally invasive scaffolds with surface properties similar to those of physiological bone. To improve the osteoinductivity, recombinant human bone morphogenetic protein2 (rh-BMP2) was added to the injectable hydrogel. Sotome et al. [74] prepared an injectable HA/Col–alginate, which gelled in 30 min by ionic cross-linking (after incubation in 100 mM $CaCl_2$ solution for 30 min for cross-linking by Ca^{2+}ions), as a carrier of rh-BMP2. The HA/Col–alginate (20 μl) with the rh-BMP2 (100 μg ml^{-1}, 15 μl) showed bone formation throughout the implant, 5 weeks after implantation without obvious deformation of the material, whereas bone formation was observed only in a part of a squashed collagen sponge.

Collagen hydrogels have also been employed as hemostats. The US Food and Drug Administration (FDA) approved collagen gel for biomedical applications and it is currently available commercially. One practical example is VITAGEL™ (Orthovita, Inc. Pennsylvania, USA), which has been used in surgical procedures as an adjunct to hemostasis when conventional procedures for control of bleeding are ineffective or impractical. VITAGEL™ is combined with the patient's own plasma immediately prior to application to a bleeding site. A fibrin/collagen clot forms quickly to control bleeding and provides a three-dimensional matrix to facilitate healing.

2.2.2. Membranes and films

Resorbable collagen membranes have been utilized in guided tissue regeneration and guided bone regeneration procedures because of their proven biocompatibility and capability of promoting wound healing. Currently, in oral surgery, collagen barrier membranes for periodontal defect regeneration have been widely used because of their bioresorbability, which can avoid the need for a second surgery [75–77]. For guided tissue regeneration (GTR) procedures, collagen membranes prepared by different methods (freeze-drying, electrospinning, etc.) have been shown to be comparable to non-absorbable membranes (consisting of expanded polytetrafluoroethylene (e-PTFE)) with regard to probing depth reduction, clinical attachment gain and per cent bone filling [78]. Although these membranes are absorbable, collagen membranes

Table 1
Currently applied methods for fabricating various physical forms of collagen.

Physical form	Methods of preparation	Refs
Injectable hydrogel	Physical and chemical cross-linking (UV irradiation, freeze-drying, pH, enzymes, aldehydes, carbodiimide, multivalent ions, photopolymerization) and blends with other polymers	[55–66]
Membranes and films	Solvent casting, freeze-drying, phase separation, electrospinning	[67–74]
Sponges and scaffolds	Freeze-drying, phase separation, electrospinning, chemical modification, solid freedom modification	[75–80]
Micro- and nanospheres	Thermally induce phase separation, modified emulsification, high-voltage electrostatic field, desolvation	[81–84]

have been demonstrated to prevent epithelial down-growth along the root surfaces during the early phase of wound healing. The use of grafting material in combination with collagen membranes have been found to improve clinical outcomes for furcation but not for intrabone defects, compared to the use of membranes alone. Recently, collagen materials have also been applied in guided bone regeneration (GBR) and root coverage procedures with comparable success rates compared to non-absorbable ePTFE membranes and conventional subepithelial connective tissue grafts, respectively.

The drawbacks of collagen membranes for GTR and GBR applications are: (i) the loss of space-maintaining ability in humid conditions [79]; and (ii) the implantation of animal-derived collagen includes a potential risk of disease transmission from animal to human [80]. In contrast, collagen membranes have shown favorable regenerative results [81,82] due to their excellent cell affinity and biocompatibility. Even if collagen membranes showed excellent cell affinity and biocompatibility for tissue regeneration, however, the mechanical strength of the membranes was poor. Furthermore, the degradation rate of collagen membranes did not match the normal tissue-healing process. Other methods have been developed to meet the demands of degraded membrane in both mechanical properties and biocompatibility. Several collagen cross-linking techniques have been applied to prolong membrane reabsorption and increase membrane biodurability as described before.

The most important commercial collagen membrane is Bio-Gide® (GeistlichPharma AG, Wolhusen, Switzerland), which is composed of porcine type I and type III collagen fibers, without the use of any organic component. It comprises a bilayered structure composed of a "compact" layer and a "porous" layer. The compact layer of the membrane possesses a smooth and condensed surface to protect against connective tissue infiltration, while the porous layer permits cellular invasion. When used for GBR, the porous and compact layers may enable osteogenic cell migration and hinder connective tissue infiltration, respectively. In host animals, mesenchymal stem cells can differentiate into osteogenic cells under preferential circumstances [83]. Collagen fibers are the most abundant components in bone matrix [84], and may act as a reservoir of many local factors and in the cell–matrix attachment of osteogenic cells. Despite the absence of bone-specific proteins, collagen fibers of GBR-membranes may serve as a physical scaffold for osteogenic cells in bone defects and as a barrier against infiltration of surrounding connective tissues. Taguchi et al. [85] have investigated the histological changes of newly formed bone induced by GBR using a Bio-Gide® membrane inserted into artificial bone defects formed by drilling in 4 week old male Wistar rats, and verified whether collagen fibers of the resorbable membrane could affect its biological function in osteogenic cells. As expected, they found the following observations: (i) the collagenous membrane permitted the alveolar ridge of the newly formed bone to reach the same height as the pre-existing bone; (ii) alkaline phosphatase-positive cells and osteocalcin- and osteopontin-immunopositive bone matrices appeared at the second week post-implantation, suggesting osteoblastic differentiation in the porous layer of the membrane; (iii) the membrane-derived collagen fibers were incorporated into the matrix of the new bone neighboring the membrane; and (iv) the membrane-associated bone integrated with bone extended from the cavity. Moreover, they have found that the compact layer of Bio-Gide® prevented the invasion of undesirable connective tissue, and therefore preserved enough space for osteogenic cells to generate bone in the cavity. Analogous results were observed also by Zhao et al. [86] after Bio-Gide® membrane implantation into a subcutaneous pouch created by gentle blunt dissection with scissors into 24 week old male Wistar rats. Dissolution of the membrane material started only after 4 days after implantation with consequent body fluid penetration and subsequent appearance of a spongy structure in the membrane. Macrophages were detected starting to engulf the membrane material; by 21 days after implantation, they contained a bluish substance due to dissolution of the membrane. These histological findings fitted clinical signs in patients since an early reaction to Bio-Gide® often included major edema of the region.

To improve mechanical properties, composite membranes based on apatite crystals and collagen have received increasing attention due to their ability to preserve the structural and biological functions of the damaged hard tissues in a more efficient and biomimetic way [87]. These composites have been formulated to have adequate properties for applications in the field of bone repair, e.g. bioactivity, osteoconduction, osteoinduction and biocompatibility [88].

Examples of resorbable 3-D collagen products for use in orthopedic applications derived from highly purified type I collagen approved by the FDA are: DuraMatrix-Onlay™ Collagen Dura Substitute Membrane (Collagen Matrix, Inc., NJ, USA) indicated for use as an non-sutured substitute for the repair of the dura tissue in the contours of the brain and spine; TenoMend™ (Collagen Matrix, Inc.), a tendon wrap designed to manage and protect tendon injuries where there has been no substantial loss of tendon tissue; OssiPatch™ Collagen Bone Healing Protective Sheet (Collagen Matrix, Inc. New Jersey, USA) used to maintain the relative position of weak bony tissue such as bone grafts, bone graft substitutes or bone fragments from comminuted fractures; and Collatene™ Fibrillar Collagen Dental Dressing (Ace Surgical Supply Co., Inc., MA, USA), formulated as a dental dressing in a cohesive fibrillar form.

2.2.3. Sponges and scaffolds

Porous collagen scaffolds with ceramic particles have been formulated and studied by a number of authors [89–91] for bone tissue engineering purposes. Three-dimensional collagen scaffold materials have been designed to mimic one or more of the bone-forming components, in order to facilitate the growth of vasculature into the material, and to provide an ideal environment for bone formation. Scaffolds should possess open pores, fully interconnected geometry in a highly porous structure (allowing cell ingrowth and an accurate cell distribution throughout the porous structure) and be able to support neovascularization of the construct from the surrounding tissue (in vivo extrinsic vascularization) [92]. Pore size is a very important issue: if the scaffold pores are too small, pore occlusion by the cells may occur, preventing cellular penetration, ECM production and neovascularization of the inner areas of the scaffold. It is well accepted that for bone tissue engineering purposes, the pore size should be in the 200–900 μm range [93].

From a biological perspective, polymers and bioceramics have been combined to fabricate biomimetic scaffolds for bone tissue engineering, as native bone is a combination of naturally occurring polymers and biological apatite. Moreover, polymers and ceramics/glasses that have the ability to degrade in vivo are ideal candidates for composite scaffolds because they can gradually degrade while new tissue is formed. Certain inorganic/ceramic materials, such as hydroxyapatite or calcium phosphates, which have good osteoconductivity and have been studied for mineralized tissue engineering, show drawbacks such as poor processability into highly porous structures and brittleness. In contrast, polymers offer great design flexibility because their composition and structure can be tailored to specific needs, and therefore they have been extensively studied in various tissue engineering applications, including bone tissue engineering.

Over the years, a series of processing techniques, such as solvent casting [94–96], phase inversion [97,98], fiber bonding [99,100], melt-based technologies [101], high-pressure-based

methods [102], freeze-drying [103,104] and rapid prototyping techniques [105,106] have been developed with the aim of producing scaffolds with adequate properties for bone tissue engineering.

The mechanical properties of these porous scaffolds were generally poor, and therefore collagen was highly cross-linked for a better (biological) stability. However, this treatment could result in a decrease of biocompatibility when cross-linking agents such as glutaraldehyde are used [107]. Du et al. [108] used collagen I as matrix for CaP mineralization to obtain a collagen/CaP composite, by producing 60–70% CaP composites with a high tensile strength. In addition, three-dimensional porous biomimetic hydroxyapatite/collagen composites cross-linked by microbial transglutaminase (mTGase) have been developed in our research group (Fig. 4). The enzyme has been used here with the main purpose of increasing the mechanical resistance of the organic matrix. The obtained composites have been found to support the adhesion, proliferation, viability and differentiation of MG63 osteoblast-like cells and human umbilical vein endothelial cells [109]. To improve the bioactivity of natural polymers, bioactive glass was introduced in different types of scaffolds.

Pohunkova and Adam reported an in vitro biocompatibility study of Bioglass® particle–collagen hydrogel composite [110] in which silica–collagen composites exhibited in vitro osteoconductivity properties, whereas the individual components alone did not. This synergistic effect was attributed to the ability of the protein to bind calcium ions, which can further associate with silicic acid to form a bioactive layer. Andrade et al. [111] produced collagen fibers coated with a bioactive glass obtained through a sol–gel process. This coating exhibited in vitro bioactivity, improving the calcium and phosphate precipitation on the collagen surface when immersed into SBF solution. Coated samples immersed in SBF solution stimulated higher levels of alkaline phospahatase (ALP) production by osteoblasts compared to uncoated collagen, and were found to be promising for collagen secretion by osteoblasts, which is an important factor for bone healing. The combination of bioactive glass nanofibers, produced by the electrospinning method, with collagen to produce a hybridized nanocomposite was developed by Kim et al. [112] for use as a bone regeneration matrix. In particular, the bioactive glassfiber–collagen nanocomposite was produced both in the form of a thin membrane and a macroporous scaffold and exhibited an active induction of apatite minerals on its surface in contact with SBF, showing excellent bioactivity in vitro. Human osteoblastic cells grew favorably on the nanocomposite and expressed significantly higher ALP levels than those on collagen alone.

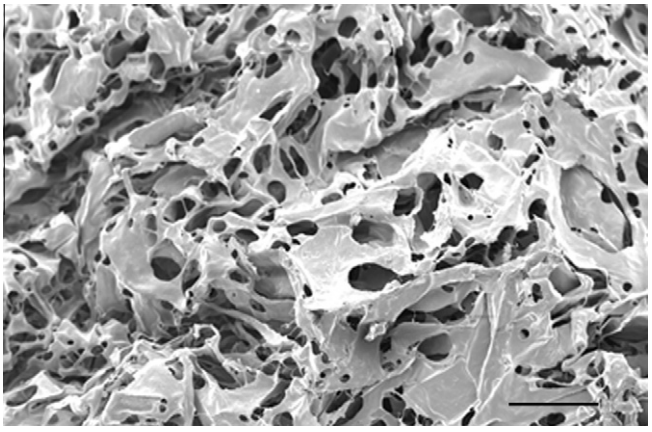

Fig. 4. SEM micrograph of porous collagen scaffold cross-linked with mTGase and treated by freeze-drying technique (bar = 100 μm).

The FDA in 1993 approved the first collagen-based implant for bone, called Collagraft™ (Collagen Corp., USA), which combines hydroxyapatite/tricalcium phosphate with bovine collagen mixed with the patient's bone marrow. In 1998, Dr. Jay Lieberman (University of California at Los Angeles, USA) began to include bone-morphogenetic protein (BMP) into an allograft and tested this material in clinical trials to treat osteonecrosis (bone death) of the hip. In fact, different bone graft substitutes products approved by the FDA are avalilable based on on highly purified type I collagen and BMP. As an example, INFUSE® Bone Graft (Medtronic Sofamor Danek, Inc., USA) is a recombinant human bone morphogenetic protein-2 (rhBMP-2) applied to an absorbable collagen sponge carrier for certain lumbar spine fusion, tibial fracture repair procedures, oral maxillofacial and dental regenerative bone grafting procedures. Other resorbable products such as OssiMend™ Bone Graft Matrix (Collagen Matrix, Inc. New Jersey, USA) combine porous bone mineral with collagen for use in orthopaedic and spinal surgery. The mineral particles are incorporated within a porous matrix consisting of about 55% bone mineral and 45% collagen. Bone Mineral–Collagen Composite Block is a combination of cancellous bone mineral granules and about 5% bovine collagen fibers.

2.2.4. Microspheres and nanospheres

Protein microspheres are commonly formed by phase separation in a non-solvent followed by solvent removal by extraction or evaporation. In particular, collagen microspheres 3–40 μm in diameter have in general been prepared by emulsifying methods using an aqueous solution of collagen in organic solvents (water-in-oil emulsions) and cross-linking native collagen molecules [113,114]. Collagen microspheres can be formed in a range of sizes for multiple applications, in particular as carriers for drug/protein delivery [115,116]. Particle size is highly controlled by the molecular weight of the collagen type employed [113]. Thus, collagen denaturation to a gelatin structure can allow for production of smaller spheres with diameter of about 0.1 μm with higher stability, and then permit their sterilization [113,117]. The surface charge of particles has a substantial influence on the stability of suspensions, on the interaction of microparticles with charged substances, as well as on the adherence of drug-delivery systems onto biological surfaces [114]. Nanosized particles from collagen type I and II were prepared exploiting a high-voltage electrostatic field system [118]. The authors described the temperature influence on collagen type II nanosphere shape and diameter, demonstrating that below 37 °C collagen II was able to form spherical nanoparticles. Above this temperature, collagen particles began to lose the spherical shape and formed a fibrous structure. Additionally, collagen type I nanospheres exhibited smaller size but poorer sphericity than collagen II particles [119]. Collagen nanospheres were also added directly into bone marrow stromal cells (BMSCs) in in vitro culture; bone nodules were formed with a increased mineralization, suggesting that collagen I nanospheres may be suitable for the cultivation of BMSCs for clinical applications [120].

In the last decade, the idea of using collagen as a drug-delivery carrier has attracted researchers due to this material's (i) high biocompatibility, (ii) biodegradability at neutral pH into non-cytotoxic monomers, (iii) ability to be dispersed in an aqueous medium as clear colloidal solution, and (iv) capacity to provide a natural extracellular environment potentiating the activity of the drugs and anti-microbial agents delivered in situ [27,114,121]. Degradation triggered by naturally occurring enzymes is advantageous for time-controlled delivery of incorporated proteins or drugs, as the degradation rate of the microspheres can be tuned by chemical cross-linking or other chemical modifications [25].

Collagen microspheres have potential uses in clinical applications, as carriers for drug delivery in tissue-engineering strategies.

Microspheres, liposomes or vesicles based on collagen or collagen/polymers have been used to support the growth of cells into scaffolds [121–124]. In particular, in bone tissue regeneration, collagen microparticles have demonstrated excellent characteristics as carriers for the delivery of antibacterial drugs [115,125] and growth factors (e.g. BMP [126], VEGF [127] and glucocorticosteroids [114]). In bone tissue engineering, collagen microparticles have been generally introduced into scaffolds based on synthetic polymers and/or ceramics (such as hydroxyapatite) with the aim of enhancing osteoblast cell growth within bone-filling materials [124,128].

3. Conclusions and future remarks

Collagen is a fibrous protein comprising the natural ECM of tissues, from which it can be extracted by a variety of techniques. A proteolytic treatment of animal tissues in acidic environment (e.g. using pepsin) is the most widely used collagen extraction procedure: it cleaves collagen cross-links as well as telopeptides, making collagen non-immunogenic. As native collagen contains amino acidic sequences (GFOGER, RGD, etc.) for cell bio-recognition, it has been widely used as a material for the preparation of scaffolds for tissue-engineering applications, in particular for bone regeneration. Collagen biomaterial has poor mechanical properties and swells readily when implanted in vivo due to its high hydrophilicity. Therefore collagen is commonly modified, cross-linked or mixed with other components (polymers or ceramics) in order to tailor the physicochemical and mechanical properties of the scaffold to the requirements of the final application. Fibrous collagens are abundant in nature and are used to obtain hydrogels, porous scaffolds, membranes, nano/microparticles for bioactive agent delivery; they are readily available in various forms (e.g. sheets, sponges or tubes) from commercial sources. Scientists, in their eagerness of imitate the ECM enviroment, are pursuing synthetic collagen-biomimetic approaches. By mimicking the structural or functional characteristics of natural collagens it becomes possible to understand the physicochemical factors responsible for functional ECM assembly [129] and also to overcome the biological drawbacks of collagen—risk of infection, inflammatory response, insufficient bioactivity, etc. Collagen is able to enhance cell activity and osteogenesis due to the hexapeptide sequence, GFOGER, present in its triple helical structure. One of the approaches to mimicking the biological properties of collagen consists of immobilizing this collagen peptide sequence to mediate specific cell interactions between integrin cell receptors, e.g. the $\alpha_2\beta_1$ integrin receptor, and the ECM protein ligand. As an example, in one of the recent works exploiting this strategy, GFOGER-coated polycaprolactone scaffolds were implanted into femoral rat defects, and a significantly accelerated and increased bone formation in the femoral defects was observed compared with the non-coated scaffolds and empty defects [130]. However, some biochemical and homeostatic processes associated with the ECM remodeling mediated by a specialized set of matrix-metalloproteins (MMPs) require an effective collagen triple-helical conformation [131]. More complex strategies for mimicking natural collagen's hierarchical structure and composition involve the synthesis of collagen-mimetic peptides (CMPs) that replicate collagen's characteristic repetitive unit glycine–proline–hydroxyproline and its hierarchical assembly with the characteristic triple-helical packing and length [132]. To elucidate the folding pathway of collagen triple-helical assemblies and the factors responsible for its stabilization, scientists have linked tailored CMPs on a template-assembled synthetic protein in order to modulate the collagen-like triple-helix stability to self-assemble into higher-order structures and to incorporate cell interactive sites. The folding of the peptide into its secondary or tertiary structures is very important to promote a proper cellular activity and response. An ideal CMP template should have three functional groups that can covalently connect three polyproline II-like helical chains and allow proper packing and stabilization of the three chains into the triple-helical structure by reducing the entropy loss involved in triple-helix formation. The incorporation of unnatural residue as peptoids in the collagen sequences, e.g. the peptoid residues N-isobutylglycine (Nleu) which has been succesfully incorporated into series of collagen mimetics composed of Gly-Pro-Nleu, Gly-Nleu-Pro and Gly-Nleu-Nleu, demonstrates potent and specific biological activity, enhancing metabolic stability against natural proteases and reducing racemization problems [133].

Despite the efforts of scientists to mimic the physical and the biochemical properties of native collagen, collagen-mimetics are not used as natural collagen substitutes because it is still necessary to have control over the high-order structures and biological functions of the fibrous natural collagen in the ECM [129,134]. However, research on collagen-mimetics has contributed much to understanding the collagen triple-helix structure, stability and biochemical interactions with other molecules [131]. The analysis presented in this review of the state of the art shows collagen to be promising biomaterial in bone tissue engineering, and outlines how the diversification of collagen products has been enhanced by the increase in scientific knowledge, resulting in specific biomimetic biomaterials able to properly interact with cells and other biomacromolecules. Advances in the control of collagen structure and properties by material manipulation and by refining processing techniques will allow highly biomimetic substrates to be obtained, which will contribute to advances in tissue engineering.

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Appendix A. Figures with essential colour discrimination

Certain figures in this article, particularly Figs. 1 and 2, are difficult to interpret in black and white. The full color images can be found in the on-line version, at http://dx.doi.org/10.1016/j.actbio.2012.06.014.

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