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Collagen- vs. Gelatine-Based Biomaterials and Their Biocompatibility: Review and Perspectives

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1. Introduction

Selection of a starting material, which will somehow mimic a naturally-existing one, is one of the most important points and crucial elements in biomaterials development. **Material biomimetism** is one of those approaches, where restoration of an organ's function is assumed to be obtained if the tissues themselves are imitated (Barrere et al., 2008). However, some of the biopolymers as e.g collagen can be selected from within a group of biomimetic materials, since they already exist, and have particular functions in the human body.

Collagen is one of the key structural proteins found in the extracellular matrices of many connective tissues in mammals, making up about 25% to 35% of the whole-body protein content (Friess, 2000; Muyonga et al., 2004). Collagen is mostly found in fibrous tissues such as tendons, ligaments and skin (about one half of total body collagen), and is also abundant in corneas, cartilages, bones, blood vessels, the gut, and intervertebral discs (Brinckmann et al., 2005). It constitutes 1% to 2% of muscle tissue, and accounts for 6% of strong, tendinous muscle-weight. Collagen is synthesized by fibroblasts, which originate from pluripotential adventitial cells or reticulum cells. Up to date 29 collagen types have been identified and described. Over 90% of the collagen in the body is of type I and is found in bones, skins, tendons, vascular, ligatures, and organs. However, in the human formation of scar tissue, as a result of age or injury, there is an alteration in the abundance of types I and III collagen, as well as their proportion to one another (Cheng et al., 2011).

Collagen is readily isolated and purified in large quantities, it has well-documented structural, physical, chemical and immunological properties, is biodegradable, biocompatible, non-cytotoxic, with an ability to support cellular growth, and can be processed into a variety of forms including cross-linked films, sheets, beads, meshes, fibres, and sponges (Sinha & Trehan, 2003). Hence, collagen has already found considerable usage in clinical medicine over the past few years, such as injectable collagen for the augmentation of tissue defects, haemostasis, burn and wound dressings, hernia repair, bioprosthetic heart valves, vascular grafts, a drug -delivery system, ocular surfaces, and nerve regeneration (Lee et al., 2001). However, certain properties of collagen have adversely influenced some of its usage: poor dimensional stability due to swelling in vivo; poor in vivo mechanical strength and low elasticity, the possibility of an antigenic response (Lynn et

al., 2004) causing tissue irritation due to residual aldehyde cross-linking agents, poor patient tolerance of inserts, variability in releasing kinetics, and ineffectiveness in the management of infected sites (Friess, 1998). In addition, there is the high-cost of pure type I collagen, variability in the enzymatic degradation rate when compared with hydrolytic degradation, variability of isolated collagen in cross-link density, fibre size, trace impurities, and side-effects, such as bovine spongiform encephalopathy (BSF) and mineralization. The above-mentioned disadvantages must be considered during collagen use in medical applications (Pannone, 2007).

In this review collagen will be presented and compared to its degradation product, gelatine, taking into account their molecular and submolecular structural properties, possibilities to overcome common problems related to their usage as biomaterial, i.e. the solubility and degradation rate mechanisms, as well as their applications in combination with other types of (bio)polymers.

2. Molecular and submolecular structure of collagen vs. gelatine

2.1 Collagen

The **collagen** rod-shape molecule (or *tropocollagen*) is a subunit of larger collagen fibril aggregates. The lengths of each subunit are approximately 300 nm and the diameter of the triple helix is ~1.5 nm. It is made up of three polypeptide α -chains, each possessing the conformation of a left-handed, polyproline II-type (PPII) helix (Fig. 1). These three left-handed helices are twisted together into a right-handed coiled coil, a triple-helix which represent a **quaternary structure** of collagen, being stabilized by numerous hydrogen bonds and intra-molecular van de Waals interactions (Brinckmann et al., 2005) as well as some covalent bonds (Harkness, 1966), and further associated into right-handed microfibrils (~40 nm in diameter) and fibrils (100-200 nm in diameter), being further assembled into collagen fibres (He et al., 2011) with unusual strength and stability.

The primary structure of collagen shows a strong sequence homology across genus and adjacent family line (Muyonga et a., 2004), thus a distinctive feature of collagen is the regular arrangement of amino acids in each of the three chains of collagen subunits. The sequence of amino acids is characterized by a repetitive unit of glycine (Gly)-proline (Pro)-X or Gly-X- hydroxyproline (Hyp), where Gly accounting for the 1/3 of the sequence, whilst X and Y may be any of various other amino acid residues. However, the X-position is occupied almost exclusively by Pro, whereas Hyp is found predominantly in the Y-position (Gorham, 1991), both constitute of about 1/6 of the total sequence. This kind of regular repetition and high Gly content is found in only a few other fibrous proteins, such as silk fibroin and elastin, but never in globular proteins. Thus the super-coil of collagen is stabilized by hydrogen bonds between Gly and Pro located in neighbouring chains and by an extensive water-network which can form hydrogen bonds between several carbonyl and hydroxyl peptide residues (Brinckmann et al., 2005). Furthermore, amino acids in the X- and Y-positions are able to participate in intermolecular stabilization, e.g. by hydrophobic interactions or interactions between charged residues, mostly coming from Pro and Hyp residues steric repulsion (Brinckmann et al., 2005). This helical part is further flanked by short non-helical domains (9-26 amino acids), the so called telopeptides, which play an important role in fibril formation and natural cross-linking. After spontaneous helix formation, cross-links between chains are formed within the region of the N-terminal telopeptides (globular tail portion of the chains), and then the telopeptides (containing the

cysteine (Cys) and tyrosine (Tyr) of pro-collagen) are shed leaving the rod-like ca. 3150 amino acid containing triple helix. These collagen rods assemble together with a quarter-stagger to form the collagen fibre and the fibres are stabilised by further cross-links.

Type I (Fig. 2) collagen, the predominant genetic type in the collagen family being the major component of tendons, bones and ligaments, is a heterotrimeric copolymer composed of two $\alpha 1$ (I) and one $\alpha 2$ (I) chains, containing approximately 1050 amino acids each. This collagen type contains one-third of Gly, contains no tryptophan (Trp) or Cys, and is very low in Tyr and histidine (His) (Muyonga et al., 2004). Its molecule consist of three domains: amino-terminal nontriple helical (N-telopeptide), central triple helical consisting of more than 300 repeat units and represent more than 95% of polypeptide, and carboxy-terminal nontriple helical (C-telopeptide) (Yamauchi & Shiiba, 2008). New data show that besides the telopeptides, tropocollagens still contain the N- and C-terminal propeptide sequences, called non-collagenous domains (Brinckmann et al., 2005), which are responsible for correct chain alignment and triple helix formation. The propeptides are removed before fibril formation and regulate the fibril formation process. Tropocollagens are staggered longitudinally and bilaterally by inter- and intra-molecular cross-links into microfibrils (4 to 8 tropocollagens) and further into fibrils. This periodic arrangement is characterized by a gap of 40 nm between succeeding collagen molecules and by a displacement of 67 nm. The fibrils organize into fibres which, in turn, can form large fibre bundles, being both stabilized by intermolecular cross-links (Friess, 1998).

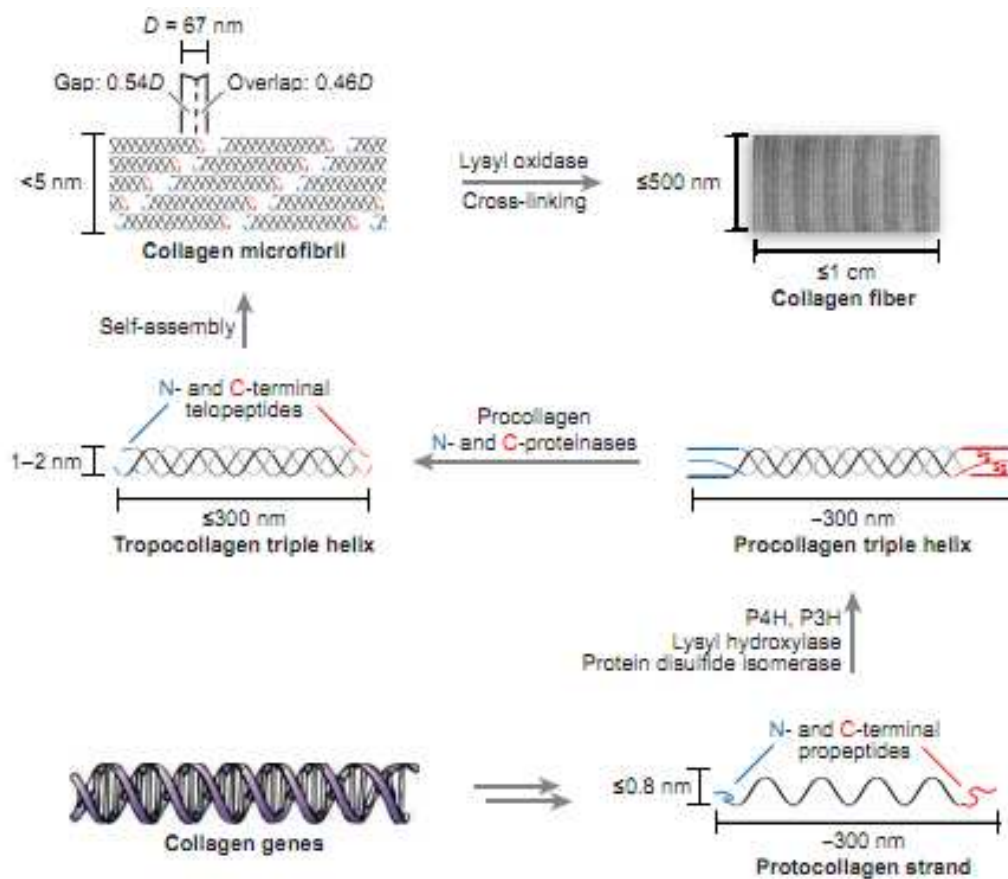


Fig. 1. Biosynthetic route of collagen fibers (Shoulders & Raines, 2009)

study of the structural and functional aspects of collagen (Chen et al., 1995) due its orderly arrangement of triple helix tropocollagen molecules, results in a formation of fibrils having a distinct periodicity. Thus this system also allows the studies of the stereochemistry of all the side-chain groups and specific atomic interactions, and further evaluation of its therapeutic effects on collagen related diseases.

2.1.1 Antigenicity of collagen

A chemical compound that stimulates an immune response is called an antigen, or an immunogen. A host's immune response is not directed toward the entire antigen molecule, but rather to specific chemical groups called epitopes, or antigenic determinants on the molecule, which are responsible for the immunogenic properties of the antigen. Two important characteristic of antigens are **immunogenicity** (specific immune response) and **reactivity** (ability to react with specific antigen) where "complete antigen" possess both characteristics, whilst, "incomplete antigen" do not show immunogenicity, but is able to bind with antibodies (Kokare, 2008). The status of **collagen as an animal-derived biomaterial** raise concerns regarding its potential to evoke immune response. Its ability to interact with secreted antibodies (**antigenicity**) and to induce an immune response-process that includes synthesis of the same antibodies (**immunogenicity**), are connected with macromolecular features of a protein, uncommon to the host species, such as collagen with animal origin. When compared with other proteins, collagens are weakly immunogenic, due to evidences of its ability to interact with antibodies (Gorham, 1991). Clinical observations indicate that 2-4 % of the total population posses an inherent immunity (allergy) to bovine type collagen (Cooperman & Michaeli, 1984).

According to Lynn (Lynn et al., 2004), antigenic determinants (epitopes, macromolecular features on an antigen molecule that interact with antibodies) of collagen can be classified into following categories (Fig. 4):

1. Helical- recognition by antibodies is dependent on 3D conformation (i.e., the presence of an intact triple helix).
2. Central- recognitions are located within the triple helical portion of native collagen, but recognition based solely on amino acid sequence and not on 3D conformation. They are often hidden, only interacting with antibodies when the triple helix has unwound, e.g. in denaturated state.
3. Terminal- recognitions are major antigenic determinants (Lee et al., 2001), located in the non-helical terminal regions (telopeptides), but can be eliminated by pepsine treatment leading to atelocollagen (Fig. 5) (Chevallay & Herbage, 2000; Hsu et al., 1999; Kikuchi et al., 2004). Telopeptide cleavage results in collagen whose triple-helical conformation is intact, yet as both the amino and carboxyl telopeptides play important roles in cross-linking and fibril formation, their complete removal results in an amorphous arrangement of collagen molecules and a consequent loss of the banded-fibril pattern in the reconstituted product, and significant increase in solubility (Lynn, 2004).

The possible use of recombinant human collagen (although more expensive) could be a way of removing concerns of species-to-species transmissible diseases (Olsen et al., 2003). However, complete **immunogenic purification** of non-human proteins is difficult, which may result in immune rejection if used in implants. Impure collagen has the potential for xenozoonoses, a microbial transmission from the animal tissue to the human recipient (Canceda et al., 2003). Anyhow, although collagen extracted from animal sources may

present a small degree of antigenicity, it is widely considered acceptable for tissue engineering on humans (Friess, 1998). Furthermore, the literature has yet to find any significant evidence on human immunological benefits of deficient-telopeptide collagens (Wahl & Czernuszka, 2006).

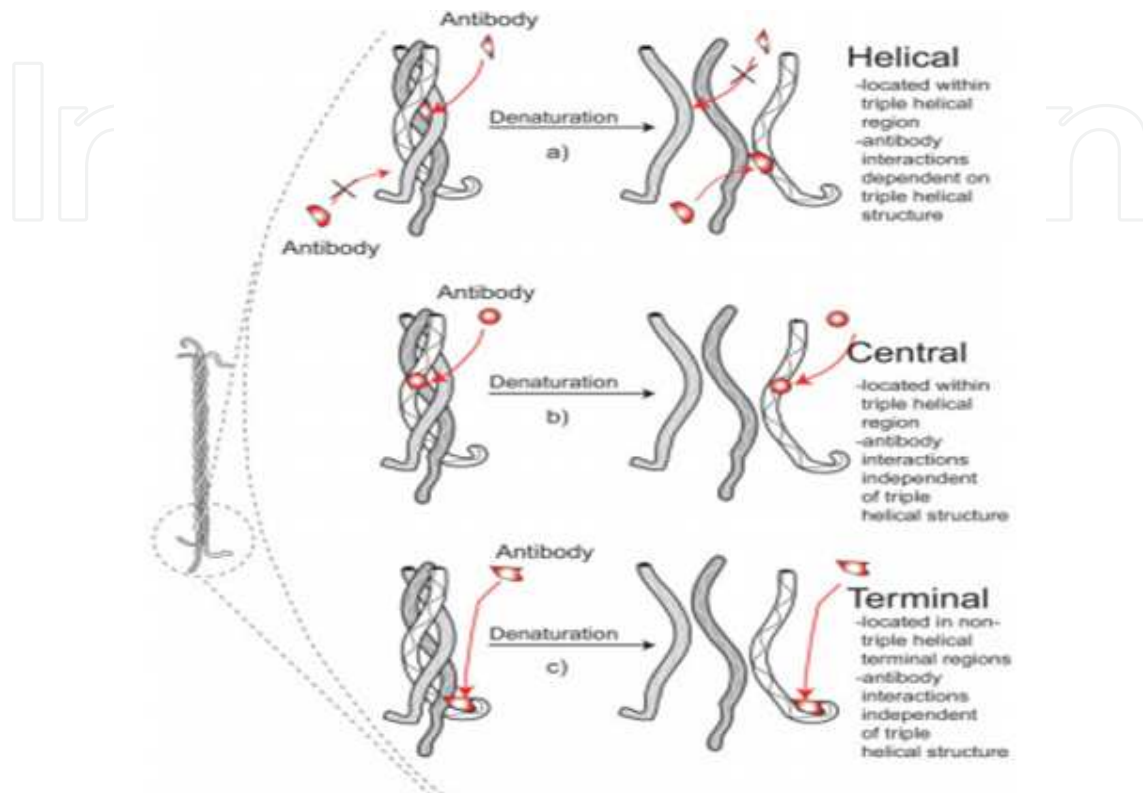


Fig. 4. Classes of antigenic determinants of collagen (Lynn et al., 2004).

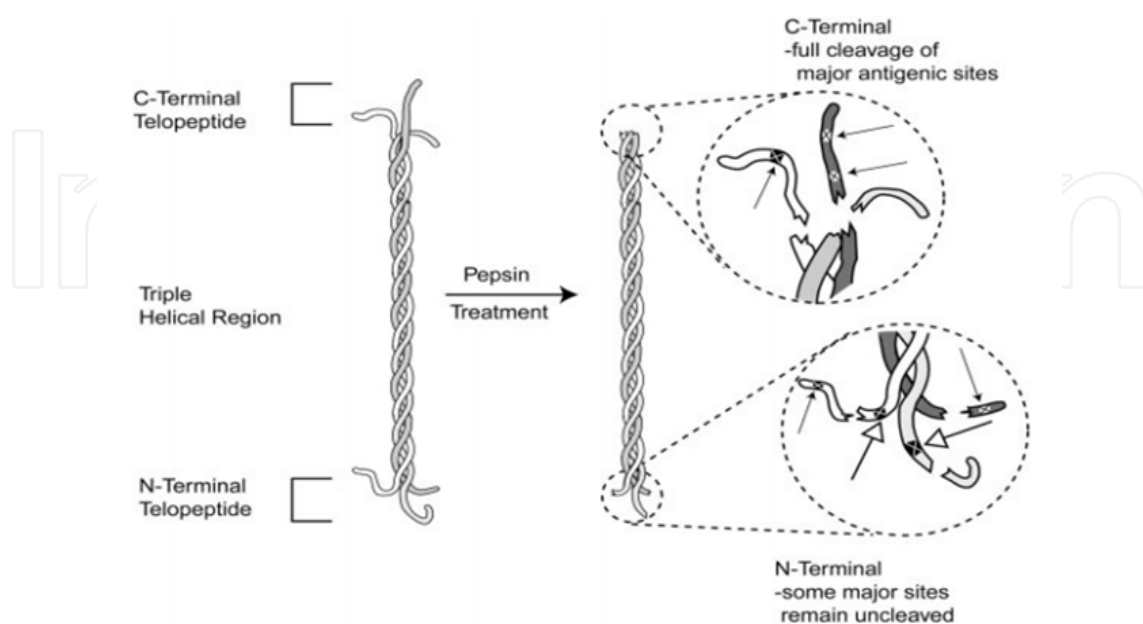


Fig. 5. Telopeptide removal via pepsin treatment (Lynn et al., 2004).

So, atelocollagen produced from type II collagen has demonstrated its potential as a drug carrier, especially for gene delivery (Lee et al., 2001). However, collagen type IV possesses a strong immunogenic character, even after pepsin treatment (Chevallay & Herbage, 2000).

Another approach for rendering the reduction of collagen antigenicity and the immune reaction, has been presented, where the amino and carboxyl side groups are blocked by glutaraldehyde cross-linking (Hardin-Young et al., 2000). However, data from studies using glutaraldehyde as the cross-linking agent are hard to interpret because glutaraldehyde treatment is also known to leave behind cytotoxic residues. It is, therefore, possible that the reduced antigenicity associated with glutaraldehyde cross-linking is due to nonspecific cytotoxicity rather than a specific effect on antigenic determinants.

2.2 Gelatine

Gelatine is the product of thermal denaturation or disintegration of insoluble collagen (Gomez-Gullien et al., 2009) with various molecular weights (MWs) and isoionic points (IEPs) depending on the source of collagen and the method of its manufacturing process of recovery from collagen. Collagen exists in many different forms, but gelatine is only derived from sources rich in **Type I collagen** that generally contains no Cys. Collagen used for gelatine manufacturing can be from different sources, among which anyhow bovine and porcine gelatines are more-widely used. Alternative sources of collagen for gelatine production have been studied in last decade, such as fish skins, bones and fins (Nagai & Suzuki, 2000), sea urchin (Robinson, 1997), jellyfish (Nagai et al., 2000) and bird feet from Encephalopat (Herpandi et al., 2011). However, the amino acid compositions are slightly different among all types of gelatine from different sources. Amino acids from pigskin gelatine and bone gelatines do not contain Cys, but fish scale and bone gelatine instead, which has less content of Gly in comparison with mammalian sources (Zhang et al., 2010). With the exception of gelatine from pigskin origin, all other gelatines do not contain aspartic acid (Asp) and glutamic acid (Glu).

During the **denaturation-hydrolysis process** (Fig. 6), collagen triple-helix organization is hydrolyzed at those sites where covalent cross-links join the three peptides, which in case of type B gelatine produced by partial alkaline hydrolysis of collagen, leads to polydisperse polypeptide mixture with average MW of 40-90 kDa, instead of MW ~ 100 kDa as related to collagen α -chains; the collagen denaturation in its passage to gelatine can be followed polarimetrically by reduction of specific optical rotation $[\alpha]_D$ of collagen (Cataldo et al., 2008).

As the collagen matures, the cross-links become stabilised, because ϵ -amino groups of lysine (Lys) become linked to arginine (Arg) by glucose molecules (Maillard reaction), forming extremely stable **pentosidine type cross-links**. During the **alkaline processing**, the alkali breaks one of the initial (pyridinoline) cross-links and as a result, on heating the collagen releases, mainly, denatured α -chains into solution. Once the pentosidine cross-links of the mature animal have formed in the collagen, the main process of denaturation has to be thermal hydrolysis of peptide bonds, resulting in protein fragments being from below 100 kDa to more than 700 kDa, and with IEP between 4,6 and 9. During the **acid process**, the collagen denaturation is limited to the thermal hydrolysis of peptide bonds, with a small amount of α -chain material from acid soluble collagen in evidence. Based on this, gelatine is divided into two main types: **Type A**, which is derived from collagen of pig skin by acid pre-treatment with IEP of 7 - 9, and **Type B**, which is derived from collagen of beef hides or bones by liming (alkaline process) with IEP of 4.6 - 5.4.

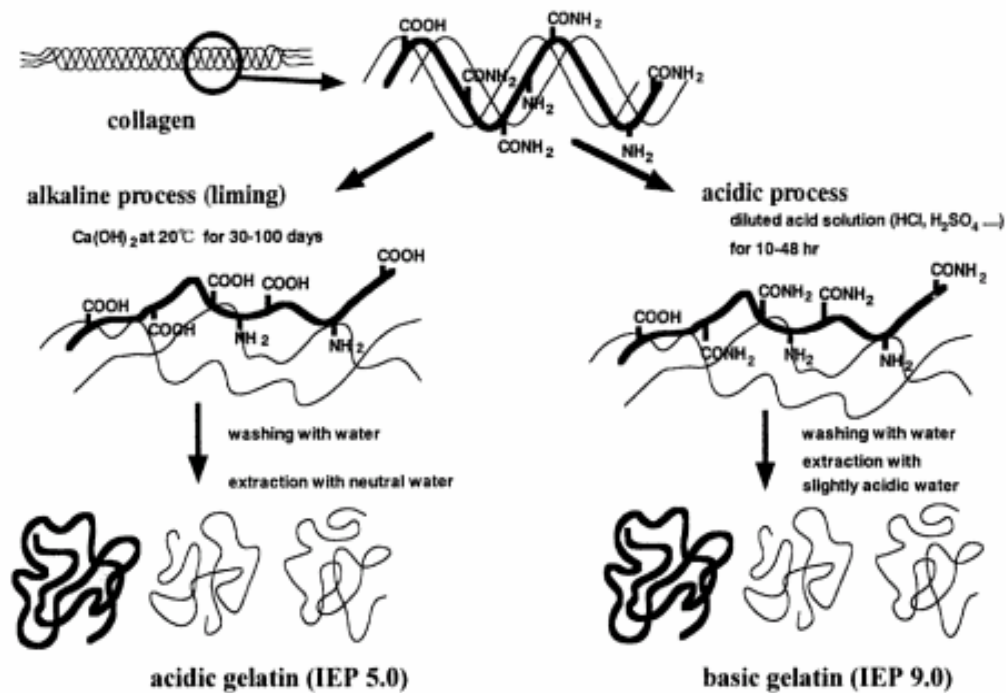


Fig. 6. Two methods for gelatine extraction from tissues containing collagen (Ikada, 2002).

Type A gelatine (dry and ash free) contains 18.5 % nitrogen, but due to the loss of amide groups, **Type B gelatine** contains only about 18% nitrogen. Amino acid analysis of gelatine is variable, particularly for the minor constituents, depending on the raw material and process used, but proximate values by weight are: Gly 21 %, Pro 12 %, Hyp 12 %, Glu 10 %, alanine (Ala) 9 %, Arg 8 %, Asp 6 %, Lys 4 %, serine (Ser) 4 %, leucine (Leu) 3 %, valine (Val) 2 %, phenylalanine (Phe) 2 %, threonine (Thr) 2 %, isoleucine (Ile) 1 %, hydroxylysine (Hyl) 1 %, methionine (Met), His < 1 % and Tyr < 0.5 %. It should be remembered that the peptide bond has considerable aromatic character; hence gelatine shows an absorption maximum at ca. 230 nm.

Collagen is resistant to most proteases and requires special collagenases for its enzyme hydrolysis. Gelatine, however, is susceptible to most proteases, but they do not break gelatine down into peptides containing much less than 20 amino acids (Cole, 2000).

Gelatine forms physical gels in hydrogen-bond friendly solvents above a concentration larger than the chain overlap concentration ($\sim 2\%$ w/v). The gelatine sol undergoes a first order thermo-reversible gelation transition at temperatures lower than T_g with is $\sim 30^\circ\text{C}$, during which gelatine molecules undergo an association-mediated conformational transition from random coil to triple helix. The sol has polydisperse random coils of gelatine molecules and aggregates, whereas in gel state there is propensity of triple helices stabilized through intermolecular hydrogen bonding, during which, three dimensional (3D) interconnected network connecting large fractions of the gelatine chains is formed (Mohanty & Bohidar, 2003, 2005).

On cooling, gelatine chains can rewind, but not within the correct register, and small triple-helical segments formed may further aggregate during gel formation. The lateral aggregation of gelatin triple helix that give rise to collagen fibrils in vivo, does not occur in gelatine gels (Chavez et al., 2006). Hydrogel formation, accompanied by a disorder-order rearrangement in which gelatine chains partially recover the triple helix collagen structure,

leads to forming of renaturated gelatine with amorphous main regions of randomly-coiled gelatine chains interconnected with domains of spatially-ordered microcrystallites, stabilized by hydrogen bonds between N-H of Gly and C=O from Pro. Stabilization of molecular conformation and inter-helix interactions are a consequence of the existence of a highly-ordered hydration shell with water bridges linking two groups within the same or different gelatine chains. Hydrogen bond formation is responsible for the increase in denaturation temperature of the fixed tissue; when compared to the pig-skin and bovine gelatines, which have ~30% Pro and Hyp, fish gelatines possess a lesser percentage of Pro and Hyp (~20 %), the impact of which is thermal stability and shifting by 5-10°C to lower gelling and melting temperatures (Farris et al., 2009) and gel strength (Herpandy et al., 2011).

Despite gelatine being one of the polymers recognized for millennia, questions about its structure and functionality are still being discussed today. The 3D network of gelatine has been defined by several authors using "fringed micelle" model in which there are microcrystallites interconnected with amorphous regions of randomly-coiled segments, whilst other authors propose the existence of local regions of protein quaternary structure, self-limiting in size, which can be triple-helical, only partially triple-helical or also include β -turn and β -sheet motifs (Pena et al., 2010).

2.2.1 Antigenicity of gelatine

Due to modern manufacturing sites and the use of highly advanced, controlled manufacturing processes with numerous purification steps (washing, filtration), heat-treatments including a final ultra-heat treatment (UHT) sterilization step followed by a drying of the gelatine solution, gelatine with highest quality can be prepared in regard to physical, chemical, bacteriological and virological safety.

During Bovine Spongiform Encephalopathy (BSE), all products of bovine origin were under suspicion as being possible transmitters of disease to humans. Thus several studies have been done to demonstrate the capability of certain steps during gelatine production to inactivate BSE infectivity, showing a reduction of SE infectivity for acid demineralization and lime-treatment of 10 and 100 times, respectively. The combined reduction has been found to be 1000 times.

The classical UHT sterilization used in gelatin manufacture should also reduce any residual infectivity 100 times, or more probably 1000 times (Taylor et al., 1994). Washing, filtration, ion exchange and other chemicals or treatments used in the manufacture of gelatine would reduce the SE activity even further (by an assumed ratio of 100 times).

However, it is also a known fact that gelatine is a non-immunogenic material, yet very little research has been done on this theme, thus most knowledge is based on early experiments (Hopkins & Wormall, 1933), where this gelatine property was described to be connected with the absence of aromatic ring. Gelatine non-antigenicity has attracted attention by (Starin, 1918) who, in particular, carried out an extensive investigation, using the precipitin, anaphylactic, complement fixation and meiotagmin reactions, and decided that the injection of gelatine into rabbits, guinea-pigs and dogs failed to produce antibodies by gelatine. This failure of gelatine to incite antibody production has been interpreted in several ways, but the view most commonly held suggests that the non-antigenicity, in this instance, is due to the absence of aromatic groupings, as gelatine is deficient in Tyr and Trp, and contains only a very small amount of Phe. A similar explanation for gelatin's non-immunogenic property was given by (Kokare, 2008), where is stated that gelatine is non-antigenic because of the absence of aromatic radicals.

3. Cross-linking of collagen vs. gelatine and their immuno response effect

Collagen isolation by pepsin digestion involves de-polymerization of collagen by removing amino and carboxyl- terminal telopeptides containing the intermolecular cross-links. The isolated collagen thus exhibits poor thermal stability, mechanical strength and water resistance, due to the destruction of natural cross-links and assembly structure by neutral salt, acid, alkali, or proteases during the extraction process (Sisson et al., 2009). In order to increase their strength and enzyme resistance, and to maintain their stability during implantation, especially for long term application, collagenous matrices are usually stabilized by cross-linking (Yannas, 1992; Tefft et al., 1997). In addition, cross-linking permits a reduction in the antigenicity of collagen and, in some forms, decreases its calcification (Damnik, 1996).

Method	Collagen	Gelatine
Chemical cross-linking		
Aldehydes e.g. glutaraldehyde (GTA)	Charulatha et al., 2003; Kikuchi et al., 2004; Mu et al., 2010, Ma et al., 2003	Sisson et al.; 2009, Farrist et al.; 2009
Dialdehyde starch (DAS)	Mu et al., 2010	Martucci & Ruseckaite, 2009
Acyl azide	Charulatha et al.; 2003, Friess; 1999,	
Diphenylphosphorylazide (DPPA)	Khor, 1997, Roche et al.; 2001	
Carbodiimides, e.g. 1-ethyl-3-(3 dimethylamino-propyl)(EDC)	Park et al.; 2002, Pieper et al.; 1999, Kim et al., 2001, Song et al., 2006	Barbetta et al.; 2010, Natu et al.; 2007, Chang et al.; 2007, Kuijpers et al.; 2000
Hexamethylene diisocyanate	Friess, 1999	
Ethylene glycol diglycidyl ether		Vargas et al.; 2008
Polyepoxy compounds	Friess, 1999; Khor, 1997, Zeeman et al., 1999	
Phenolic compounds	Han et al.; 2003; Jackson et al., 2010	Kim et al.; 2005; Zhang et al.; 2010; Pena et al.; 2010
Genipin	Ko et al.; 2007; Yan et al., 2010	Yao et al.; 2005, Lien et al.; 2010, Bigi et al., 2002; Chiono et al., 2008; Mi et al., 2005
Citric acid derivative (CAD)		Saito et al., 2004
Enzymatic crosslinking		
Transglutaminase	Jus et al.; 2011	Bertoni et al., 2006; Fuchs et al., 2010, Sztuka & Kolodziejaska, 2008
Tyrosinase	Jus et al.; 2011	Chen et al., 2003
Laccase	Jus et al.; 2011	
Physical crosslinking		
Dehydrothermal treatment (DHT)	Pieper et al., 1999; Tangsadthakun, et al., 2006	Dubruel et. al.; 2007
UV irradiation	Torikai & Shibata, 1999	Bhat & Karim, 2009
γ -radiation	Labout , 1972	Cataldo et al.; 2008

Table 1. Overview over cross-linking methods for collagen vs. gelatine materials

Different ways of collagen (as well as gelatine) cross-linking, **either chemical, enzymatic or physical**, have been carried out and often the method is prescribed by the target application (see Table 1).

Aldehydes have a long tradition as cross-linking reagents. Treatment with **glutaraldehyde (GTA)**, in particular, is intensively used. Besides its good efficiency, this cross-linking method is fast, inexpensive and mechanical properties are enhanced (Friess, 1999). Cross-linking reaction occurs between carboxyl groups on the Glu and amine groups of Lys, or Arg forming a Schiff-base as presented on Fig. 7. However, due to the polymerization of GTA, cross-linking is sometimes restricted to the surface of the device and a heterogeneous cross-linking structure can then occur (Cheung et al., 1984). Additionally, GTA is incorporated into the new linkage and unreacted GTA can cause local incompatibility, inflammation or calcification (Luyt et al., 1995), along with limited cell ingrowth (Jayakrishnan et al.; 1996) and cytotoxicity (Sisson et al., 2009) even at concentrations of 3.0 ppm after being released into the host as a result of collagen biodegradation. From other side, glutaraldehyde-based cross-linking is the current standard procedure for the production of heart valves, providing the prosthesis with low incidences of thromboembolism and satisfactory haemodynamic performance (Everaerts et al., 2007).

Reconstituted collagen membranes cross-linked with **3,3'-dithiobispropionimidate (DTBP)** and **diimidoesters-dimethyl suberimidate (DMS)** (Fig. 8) are shown to be more biocompatible than those treated with GTA (Charulatha & Rajaram, 2001).

Non-toxic, water soluble substances which only facilitate the reaction, without becoming part of the new linkage, are **acyl azides** and carbodiimides. **Carbodiimides**, e.g. EDC, couple carboxyl groups of Glu or Asp with amino groups of Lys or Hyl residues, thus forming stable amide bonds (Fig. 9). Reaction efficacy is increased by addition of **N-hydroxysuccinimide (NHS)** which prevents hydrolysis and rearrangement of the intermediate (Friess, 1999; Gorham, 1991; Olde Damink et al., 1996), thus causing the formation of a coarse structure instead of tougher microstructure, in its absence (Chang & Douglas, 2007). Because EDC can only couple groups within a distance of 1 nm, this treatment enhances intra- and interhelical linkages within or between tropocollagen molecules (Sung et al., 2003), without an inter-microfibrillar cross-links (Zeeman et al., 1999). EDC cross-linked collagens show reduced calcification, with no cytotoxicity and slow enzymatic degradation (Khor, 1997; Pieper et al., 1999).

Some natural non-toxic and biodegradable molecules with favourable biocompatibility have been exploited as protein cross-linkers, such as **D,L-glycceraldehyde** (Sisson et al., 2009), **oxidized alginate** (Balakrishnan & Jayakrishnan, 2005), **dialdehyde starch (DAS)** (Mu et al., 2010), Fig. 10) and **genipin**.

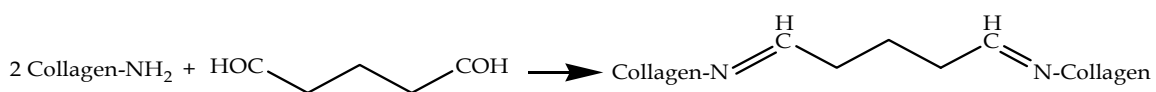


Fig. 7. Crosllinking of collagen with glutaraldehyde (GTA)

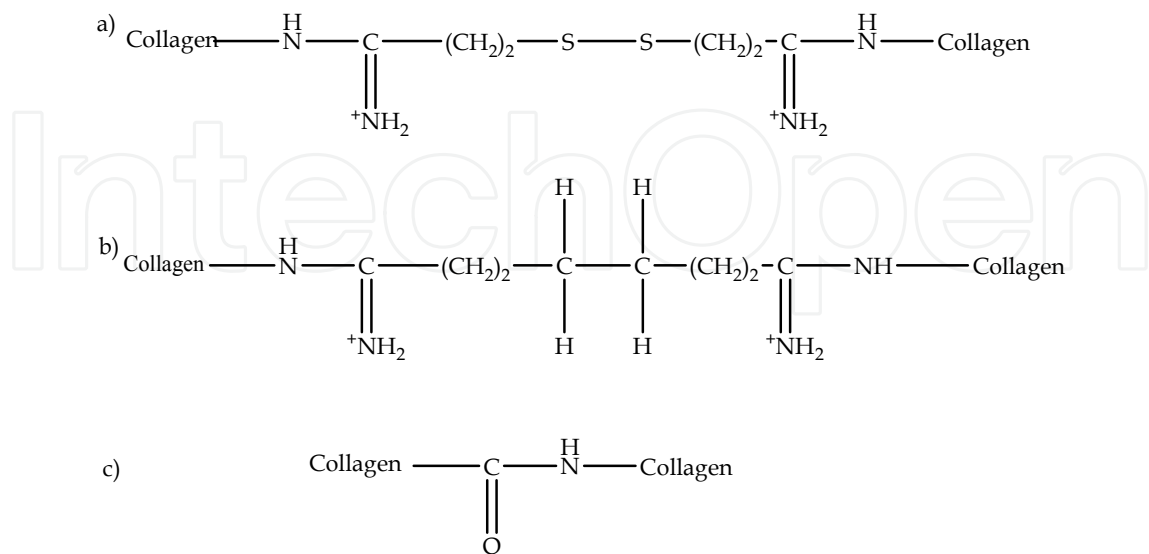


Fig. 8. Structure of cross-links obtained by (a) DTBP, (b) DMS and (c) acyl azide treatments (Charulatha & Rajaram, 2003).

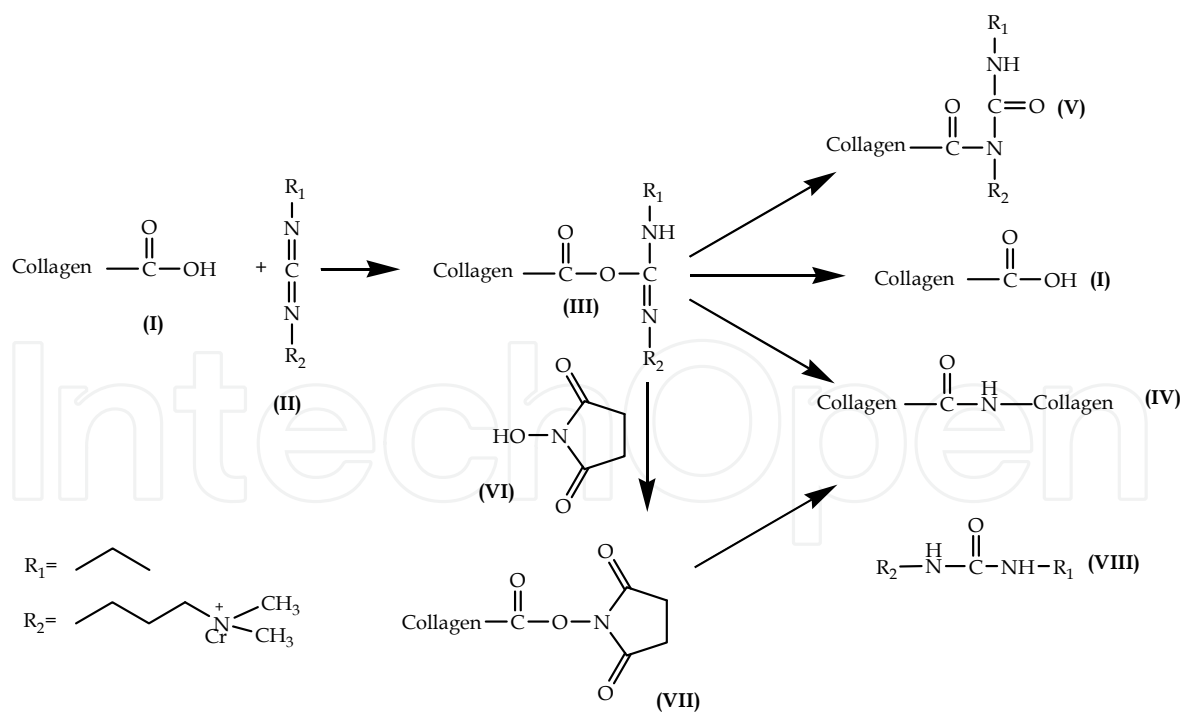


Fig. 9. Crosslinking of collagen with EDC and NHS: (I) collagen, (II) EDC, (III) O-acylurea intermediate, (IV) CO-NH bond formation, (V) N-acylurea intermediate (VI) NHS, (VII) NHS-activated carboxylic group in collagen and (VIII) substituted urea (Damnik et al., 1996).

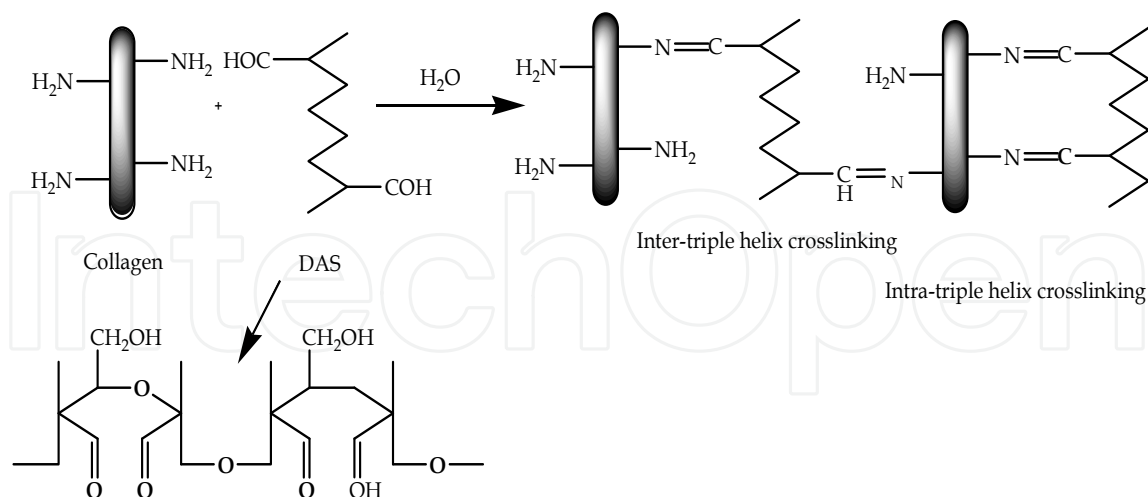


Fig. 10. Cross-linking of collagen with DAS involving Schiff's base formation between ϵ -amino groups from Lys or Hyl side-groups of collagen and aldehyde groups in DAS (Sisson et al., 2009)

Recently, **polyphenols**, such as procyanidin (He, 2011), proanthocyanidin (Kim et al., 2005; Han et al., 2003), caffeic and tannic acids (Zhang et al., 2010), epigallocatechin gallates (Jackson et al., 2010), and other tannins (Pena et al., 2010) have also been used for this purpose, additionally bringing antioxidant activity, pharmacological activity, and therapeutic potential to the biomaterial due their free-radical scavenging capacities. Not only the antioxidant activity of polyphenols, but also their other physiological properties, such as anti-allergenic, anti-inflammatory, antimicrobial, cardioprotective, and anti-thrombotic make these compounds very interesting raw materials for medical applications (Pena et al., 2010). It has been shown that some of them are able to stabilize collagen and protect the chains from collagenase degradation more effectively than glutaraldehyde and carbodiimides, thus extend the implanted material over a longer period, using very low concentration (Jackson et al., 2010). The interactions between protein and polyphenol can involve hydrogen bond, covalent linkage, ionic and hydrophobic bonding. The reaction mechanism involves an initial oxidation of phenolic structures to quinones, which can readily react with nucleophiles from reactive amino acid groups in protein: sulfhydryl group in Cys, amino group of Lys and Arg, amide group from Asp and Glu, indole ring of Trp and imidazole ring from His (Zhang et al., 2010). Nevertheless, the effect of polyphenol on the microstructure of collagen, i.e. from triple-helices to fibrils, remains largely unknown. In reaction mechanism between gelatin and tannin are involved hydrogen bonds between hydroxyl groups of tannin and polar groups of gelatin, and hydrophobic interactions between pyrrolidine ring of Pro and pentagalloyl glucose from tannin (Obrique-Slier et al., 2010; Pena et al., 2010).

Anti-inflammatory properties are added values during **genipin**-induced cross-linking, showing to be 10, 000 times less cytotoxic than glutaraldehyde which may produce weakly clastogenic responses in CHO-K1 cells (Tsai et al., 2000; Sisson et al., 2009). Moreover, the minimal calcium content of genipin-fixed tissue was detected (Chang, 2001). Genipin is a natural product, being obtained from an iridoid glucoside, geniposide abundantly present in *Genipa Americana* and *Gardenia jasminoides Ellis*. Although the cross-linking mechanism of

genipin with gelatine (or collagen) is insufficiently understood, it is known that genipin reacts with free amino groups of proteins (Fig. 11), such as Lys, Hyl and Arg, forming dark blue colour, thus acting as monomeric or oligomeric bridge which results in a comparable mechanical strength and resistance against enzymatic degradation as the glutaraldehyde-fixed tissues (Mi et al., 2005; Bigi, et al., 2002); the maximum cross-linking percentage when using genipin as a cross-linker enriched in gelatin films, is about 85% (Bigi et al., 2002). Touyama group (Touyama et al., 1994) proposed a mechanism for the reaction of genipin with a methylamine, where, reaction occurred through a nucleophilic attack of the primary amine on the C3 carbon of genipin, causing an opening of the dihydropyran ring. An attack then followed on the resulting aldehyde group by the secondary amine group. The final step in formation of the cross-linking material is believed to be dimerization produced by radical reactions, which indicate that genipin form intra- and intermolecular cross-links that have heterocyclic structure with primary amino group-containing proteins. During cross-linking reaction, genipin introduce intermicrofibrillar cross-links between adjacent collagen microfibrils, which affect the mechanical properties (Sung et al., 2003). The sizes of the interfibrillar cross-links can vary by pH variation, during cross-linking reaction, which is pH dependent: under basic conditions, genipin undergoes ring-opening polymerization, thus enlarging the spaces between fibrils, whilst under basic and neutral conditions, reaction with primary amines occur (Mi et al., 2005). Studies have been also have been conducted using material composed of genipin cross-linked gelatine and tricalcium phosphate, showing no inflammation and biocompatibility of such a composite (Yao et al., 2005). In addition, genipin cross-linking in certain polyelectrolyte multy-layer systems is shown to increase cell-adhesion and the spreading on polymeric films, thus improving tissue-implant interfaces (Hillberg et al., 2009).

Citric acid derivative (CAD) prepared by modification of citric acid carboxylic groups with NHS was introduced for cross-linking of gelatine through its amino groups leading to amide bonds formation (Saitoa et al., 2004).

Several components of **polyepoxy** family have been reported, between which **ethylene glycol diglycidyl ether**, with two epoxide functional groups located on both molecule's ends, most reactive due to the high energy is associated to the considerable strains that exist within the three-membered ring. For these type of cross-linking agent, the opening of the epoxide ring happen simultaneously to the occurrence of the cross-linking reaction, which can occur within acidic and basic media (Vargas et al., 2008).

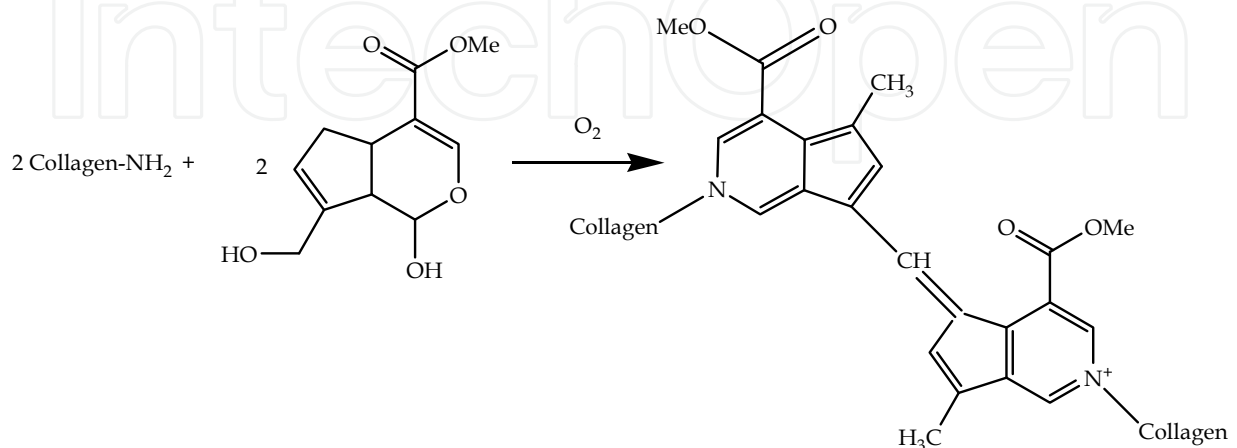


Fig. 11. Reaction between collagen and genipin proposed by (Mi, 2005).

Enzymatic cross-linking was introduced in an attempt to overcome some problems with traditional chemical approaches. The oxidative enzymes tyrosinase and laccase (Jus et al., 2011), as well as acyltransferase-transglutaminase, are capable of creating covalent cross-links in proteinaceous substrates (Fig. 12). Tyrosinases and laccases are capable of converting low-molecular weight phenols or accessible Tyr residues of proteins into quinones-reactive species capable for non-enzymatic reactions with nucleophiles, such as reactive amino groups of other amino acid residues, without disruption of gelatine's coil to helix transitions because of only 0,3% of Tyr residues in gelatine and their location outside of the Gly-X-Y tripeptide repeat region being responsible for gelatine's helix formation (Chen et al., 2003), and thus forming quiet weak gels because of the same reasons. Transglutaminase catalyses the cross-linking of gelatine by formation of isopeptide bonds between the γ -carbonyl group of a Glu residue and ϵ -amino group of Lys residue, and one molecule of ammonia per cross-link as by-product (Chen et al., 2003; Bertoni et al., 2006; Crescenzi et al., 2002). Presumably transglutaminase-catalyzed cross-linking occurs in the tripeptide repeat region that is responsive for gelatine's helix forming ability (Chen et al., 2003). The acyl-transfer enzyme catalyzes transamidation reactions that lead to the formation of N- ϵ -(γ -glutamyl)lysine cross-links in proteins (Crescenzi et al., 2002).

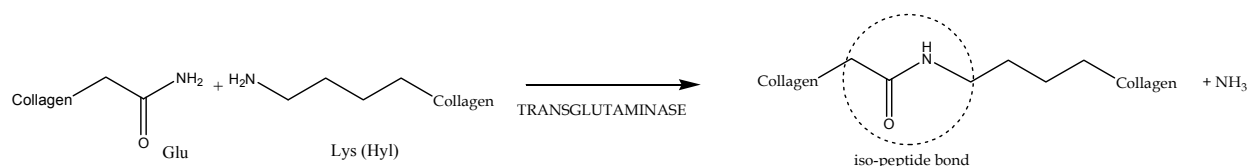


Fig. 12. Chemical cross-linking of Glu-carboxyl and Lys (Hyl)-amino groups in collagen and formation of iso-peptide bond, promote by transglutaminase (Crescenzi et al., 2002).

The treatment by **UV irradiation** only modifies the surface rather than the bulk of the collagen (Mu et al., 2010). Cross-linking of gelatine by UV-irradiation method involve pre-modification of gelatine amino groups (from Lys and Hyl side chains) (Dubruel et al., 2007), commonly by metacrylic-anhydride (Fig. 13) (Vlierberhe et al., 2009). In subsequent step, water-soluble gelatine-methacrylamide can be cross-linked not only by UV treatment, but, also by a number of suitable polymerization processes, such as redox, thermal, γ -irradiation or e-beam curing (Van Den Bulcke et al., 2000). Prolonged exposure to UV-rays can cause also the denaturation of molecule, which can be minimized by performing the irradiation in deaerated (oxygen-poor) solutions of the gelatine derivatives (Schacht, 2004). Cross-linkage by **electron beam and x-ray** irradiation additional perform sterilization of the substrate.

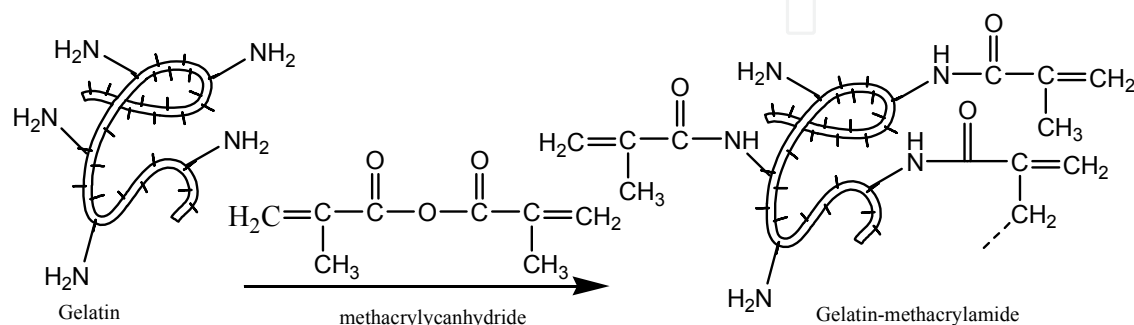


Fig. 13. Derivatization of gelatine amino groups with metacrylycanhydride (Schacht, 2004).

4. Collagen vs. gelatine as biomaterials

Collagen was first employed as a biomaterial in medical surgery in the late 19th century (Burke et al., 1983; Silver et al., 1997). Subsequently, it was used in many other medical applications, e.g. as wound dressings, hemostats or in cardiovascular, plastic or neurosurgery. Most commonly, collagen type I is used in medical devices (Silver et al.; 1997). Device production is uncomplicated and is performed in water without applying high temperatures, resulting in a variety of matrix forms, such as coatings, fibres, films, fleeces, implants, injectable solutions and dispersions, membranes, meshes, powders, sheets, sponges, tapes and tubes. Additionally, its properties can be adapted to desired requirements by additional cross-linking, although shape-instability due to swelling, poor mechanical strength, and low elasticity in vivo, may limit its unrestricted usage. Further limitations are possible antigenic responses, tissue irritations and variations in release kinetics (Sinha & Trehan, 2003). On the other hand, **gelatine** was employed as biomaterials more recently, i.e. tissue engineering from ~ 1970s and in recent years as a cell-interactive coating or micro-carrier embedded in other biomaterials (Dubruel et al., 2007). A non-exhaustive overview of the most recent publications, subdivided by application, for either collagen or gelatine alone or in a combination of other biopolymers is summarized in Table 2 which clearly indicates that gelatine has a wider- application range within the field of both soft and hard-tissue engineering.

Speciality	Collagen application	Gelatine application
Cardiology	heart valves (Everaerts et al., 2007; Taylor et al., 2006; Cox et al., 2010; Tedder et al., 2010)	heart valves- electrospun gelatine-chitosan- polyurethane (Wong et al., 2010) aortic valve -gelatine impregnated polyester graft (Langley et al., 1999) cardiac tissue engineering (Alperin et al., 2005)
Dermatology	soft tissue augmentation (Spira et al., 2004) skin replacement (Lee et al., 2001) artificial skin dermis (Harriger et al., 1998) skin tissue engineering (Ma et al., 2003; Tangsadthakun et al., 2006)	artificial skin (Choi et al., 1999; Lee et al., 2003) soft tissue adhesives (McDermott et al., 2004)
Surgery	hemostatic agent (Cameron, 1978; Browder & Litwin, 1986) plasma expander suture wound dressing and repair (Rao, 1995) skin replacement (artificial skin) nerve repair and conduits blood vessel prostheses (Auger et al., 1998; McGuigan et al., 2006, Amiel et al., 2006)	small intestine (Chiu et al., 2009) liver - chitosan/ gelatine scaffold (Jiankang et al., 2007) wound dressing (Tucci & Ricotti, 2001) nerve regeneration - chitosan/ gelatin scaffolds (Chiono et al., 2008) blod vesels(Mironov et al., 2005)

Orthopaedic	born, tendon and ligament repair cartilage reconstruction - collagen (Stone, 1997), composite of collagen type II/chondroitin/hyaluronan (Jančar et al., 2007) articular cartilage - collagen/chitosan (Yan et al., 2010)	bone substitute - gelatine/hydroxyapatite (Chang et al., 2007) hard tissue regeneration - gelatine/hydroxyapatite (Kim et al., 2005) cartilage (Lien et al., 2010) cartilage defects regeneration - chitosan/ gelatine (Guo et al., 2006), ceramic/ gelatine (Lien et al.; 2009) bone substitute - gelatine/tricalcium phosphate (Yao et al., 2005)
Ophthalmology	corneal graft (Lass et al., 1986) vitreous implants artificial tears (Kaufman et al., 1994) tape and retinal reattachment contact lenses eye disease treatment (http.....)	ocular inserts (Natu et al., 2007) carriers for intraocular delivery of cell/tissue sheets (Lai et al., 2010) contact lens - chitosan/gelatine (Yuan & Wei, 2004) eye disease treatment (Lai, 2010)
Urology	dialysis membrane hemodialysis (Kon et al., 2004) sphincter repair (Westney et al., 2005)	
Vascular	vascular graft (Yoshida et al., 1996) Vessel replacement, electrospin collagen (Li, 2005) angioplasty	
Others	biocoatings cell culture organ replacement skin test protein , drug and gene delivery (Mahoney & Anseth , 2007) vocal cord regeneration (Hahn et al., 2006) treatment of faecal incontinence (Kumar et al., 1998)	plasma substitutes (Kaur et al., 2002) drug delivery - gelatine/chondroitin sulphate (Kuijpers et al., 2000) adipose tissue engineering for soft tissue remodelling (Hong et al., 2005)

Table 2. Medical applications of collagen and gelatine

Different research groups have separately evaluated collagen/gelatine-based biomaterials that differ in the applied collagen/gelatine type, cross-linking agents, additives (in the case of composites), pore size, pore geometry, and pore distribution. Beside, only a limited number of cell types have been included in most studies, which makes a meaningful understanding of how one type of (collagen/gelatine) scaffold, with its specific properties, can be applied as a suitable substrate for a variety of cell types, rather difficult. In addition, since the collagen/gelatine-based biomaterial used as scaffolds for in vivo tissue engineering in the form of gels, sponges and woven meshes are required disappear by

resorption into the body after accomplishment of tissue regeneration, different tissues needs may demand biodegradable scaffolds with different physical and chemical characteristics.

4.1 Combination with other biopolymers

Fabrication of scaffolds from single-phase biomaterial with homogeneous and reproducible structures presents a challenge, due their generally-poor mechanical properties, which limit their use. Combination with different natural or synthetic polymers in composites or by introducing of e.g. ceramics is one of today's approaches for overcoming above mentioned limitations.

Along with **hydroxyapatite (HA)**, **collagen** is one of two major components of the bone, making up 89% of the organic matrix and 32% of the volumetric composition of bone (O'Brien, 2011). HA, being similar to bone mineral in physicochemical properties, is well known for its bioactivity and osteoconductivity in vitro and in vivo. Thus, gelatine/HA composite is a potential temporary biomaterial for hard tissue regeneration, in view of combining the bioactivity and osteoconductivity of HA with the flexibility and hydrogel characteristics of gelatine (Chang & Douglas, 2007; Kim et al., 2004; Narbat et al., 2006; Wahl & Czernuszka, 2006). Both, collagen and HA devices significantly inhibited the growth of bacterial pathogens, being the most frequent cause of prosthesis-related infection (Carlson et al., 2004).

Modification of the collagen/gelatine scaffold materials by **glycosaminoglycans (hyaluronan and chondroitin sulphate)** was introduced in order to enhance cells migration, adhesion, proliferation and differentiation, and to promote preservation of the differentiated states of the cells, as compared to collagen/gelatine alone (Jancar et al., 2007), as well as for control release of antibacterial agents (van Wachem et al., 2000). Hyaluronic acid is a component of the extracellular matrix of some tissue (cockscomb and vitreous humour) and possesses high capacity lubrication, water-sorption and water retention, whilst chondroitin sulphate is sulfated glycosaminoglycan and is important structural component of cartilage, which provides its resistance to compression (Baeurle et al., 2009).

The better collagen delivery systems, having an accurate release control, can be achieved by adjusting the structure of the collagen matrix or adding **other proteins, such as elastin or fibronectin** (Doillon & Silver, 1986). Thus, a combination of collagen with other polymers, such as collagen/liposome (Kaufman et al., 1994) and collagen/silicone (Suzuki et al., 2000), has been proposed in order to achieve the stability of a system, and the controlled release profiles of incorporated compounds.

The addition of collagen to a **ceramic** structure can provide many additional advantages to surgical applications: shape-control, spatial adaptation, increased particle and defect wall-adhesion, and the capability to favour clot-formation and stabilisation (Scabbia and Trombelli, 2004).

cross-linked **collagen/chitosan** (Kim et al., 2001; Ma et al., 2003; Wang et al., 2003; Chalonglarp et al., 2006) as well as **gelatine/chitosan** (Kim et al., 2005; Chiono et al., 2008) matrices were presented as a promising biomaterial for tissue engineering, to be used in several specific areas, such as drug delivery, wound dressings, sutures, nerve conduit, and matrix templates for tissue engineering. Human connective tissues do not contain chitosan, but it has structural similarity to glucosaminoglycan (GAG), mostly components of ECM. GAG attached to the core protein of proteoglycan consist of repeating disaccharide unit, usually includes an uronic acid component (e.g., D and L-gluconic acid) and a hexoamine component (e.g., N-acetyl-D-glucosamine, which, together with glucosamine build the

copolymer structure of chitosan and N-acetyl-D-galactosamine). Chitosan, because of its cationic nature, can promote cell adhesion, can act as modulator of cell morphology, differentiation, movements, synthesis and function. It is reported that chitosan induces fibroblasts to release interleukin-8, which is involved in migration and proliferation of fibroblasts and vascular endothelial cells, but, also promotes surface-induced thrombosis and embolization, which limits its application in blood-containing biomaterials (Wang et al., 2003). Chitosan addition enhances poor mechanical properties of gelatine and influence on more controllable biodegradation rate. Chitosan, with higher Degree of deacetylation (DD), modified with gelatine, possess more intensive cytocompatibility, enhance cell proliferation and decline cell apoptosis. From the other hand, a flexible gelatine complex with a rigid chitosan weakens the adhesion via neutralizing cationic sites of chitosan, with suitable negative-charges borne by the gelatine, and as a consequence, a gelatine/chitosan product shows improved cell mobility, migration and multiplication (Mao et al., 2004; Yuan et al., 2004). Thus, networks composed of gelatine and chitosan have been studied extensively due its excellent ability to be processed into porous scaffolds with good cytocompatibility and desirable cellular response (Mao et al., 2004; Wang et al., 2003).

The advantageous properties of collagen for supporting tissue growth have been used in conjunction with the superior mechanical properties of **synthetic biodegradable polymers** to make hybrid tissue scaffolds for bone and cartilage. Collagen has also been used to improve cells' interactions with electrospun nanofibers of poly (hydroxyl acids), such as poly(lactic acid), poly(glycolic acid), poly(ϵ -caprolactone), and their copolymers (Pachence et al., 2007).

Novel **gelatine/alginate** sponge serving as a drug carrier for silver sulfadiazine and gentamicin sulphate, used for wound healing (Choi et al., 1999). Alginate is known as a hydrophilic and biocompatible polysaccharide, and is commonly used in medical applications, such as wound dressing, scaffolds for hepatocyte culture, and surgical and dental materials. Its content in the above-mentioned sponge cause increasing in porosity, resulting in enhanced water uptake ability.

4.2 Mechanism of collagen degradation

4.2.1 In vitro degradation

Degradation of collagen requires water and enzyme penetration, and the digestion of linkages. Collagen swells to a certain extent by exposure to water, but due to its special sterical arrangement (triple helical conformation), native collagen can only be digested completely by specific collagenases and pepsin-cleaving enzymes being able to cleave collagen in its undenatured helical regions at physiological pH and temperature (Harrington, 1996; Sternlicht & Werb, 2001). Included are collagenases which cleave once across all three chains, such as tissue collagenases, as well as collagenases making multiple scissions per chain, such as collagenase from *Clostridium histolyticum* (CHC) (Seifter et al., 1971) whilst non-specific proteinases, such as pepsin, which can only attack the telopeptides or denatured helical regions of collagen (Weiss, 1976) are responsible for further degradation down to amino acids.

CHC types of collagenase are only present in tissue at very low- levels and tightly- bound to collagen (Woessner, 1991), while tissue collagenases cleave to all types of collagen, with no preference for a special collagen substrate (Welgus et al., 1983). Depending on the collagen type, about 150-200 cleaves per chain can be made (Seifter et al., 1971).

To date, seven forms of CHC are known (Mookhtiar et al., 1992). All seven enzymes contain zinc and calcium and consist of one polypeptide chain with one active site. The zinc (II) atom is located in the active site and is therefore essential for catalysis, whereas the calcium (II) atoms are required to stabilize the enzyme conformation and, consequently, the enzymatic activity (Bond et al., 1984). On the basis of their primary and secondary structures, their substrate specificities and their method of attack, CHCs can be divided into two classes. Class I contains α -, β -, γ -, and η -collagenase, and firstly attacks the collagen triple-helix near the ends. After cleavage at the C-terminal end, a cut near the N-terminus follows, before collagen is successively degraded into smaller fragments. Class II consists of δ -, ϵ - and ζ - collagenase and cleaves the tropocollagen in its centre, to producing two fragments. Further digestion of the bigger fragment follows (Mookhtiar et al., 1992). Consequently, class II CHC better resembles tissue collagenases, which cleaves collagen into TCA and a TCB fragment (Seifter et al., 1971; Welgus et al., 1980).

Collagen fibrils are degraded in a non-specific manner, with no preferential cleavage site in the interior or at the ends of fibrils (Paige et al.; 2002). It was concluded that collagenase is too large to penetrate into the fibrils, so digestion can only occur at the fibrils' surface (Okada et al., 1992; Paige et al., 2002). Hence, the degradation rate is directly correlated to those substrate molecules available on the surface. If collagen forms fibres and fibre-bundles, and the tropocollagens within becomes inaccessible, the degradation rate is reduced even more (Steven, 1976).

4.2.2 In vivo degradation

In vivo, degradation of collagen is more complex than in vitro. Collagen implants are infiltrated by various inflammatory cells, e.g. fibroblasts, macrophages or neutrophils, which cause contraction of the implant and secrete collagen-degrading enzymes, activators, inhibitors, and regulatory molecules. Infiltration depends on properties of the implant, such as collagen nature, shape, porosity and degree of cross-linking, implantation site and individual enzyme levels (Gorham, 1991). Collagen is degraded by endopeptidases from the four major classes (Table 3): metalloproteinases, serine proteases, cysteine proteases and aspartic proteases, although, non-enzymatic degradation mechanisms, e.g. hydrolysis, participate in collagen breakdown (Okada et al., 1992). Connective tissue, for example, is digested by the interplay between four different classes of proteinases, which are either stored within cells or released when required, while for degradation of the extracellular matrix, MMPs are mainly responsible. Cystein and aspartic proteinases (cathepsins) degrade connective tissue intracellularly at acidic pH (3-5) values, whereas serine and matrix metalloproteinases (MMP) act extracellularly at neutral pH values (Shingleton et al., 1996). Anyhow, cathepsins also play a major role in intracellular digestion of phagocytosed material, by cleaving telopeptide containing cross-links, and under certain conditions they can also act extracellularly by cleaving triple-helical regions, which is followed by denaturation of solubilised triple-helix and further degradation by proteases (such as gelatinases type MMP-2 and 9), due to susceptibility of individual α -chains (Baley, 2000).

MMP enzymes represent a family of structurally and functionally related zinc- and calcium-containing endopeptidases which degrade almost all extracellular matrix and basement membrane proteins (Wall et al., 2002; Bailey, 2000). To date, 24 different MMPs and 4 tissue inhibitors of metalloproteinases (TIMP) are characterized (Yoshizaki et al., 2002). According to their primary structure and substrate specificity, MMPs are divided into five sub-classes.

Five major MMPs have been identified in humans, namely fibroblast collagenase (MMP1), gelatinase A (MMP-2), gelatinase B (MMP-9), neutrophil collagenase (MMP-8) and stromelysin (MMP-3) (Netzel-Arnett et al., 1991). Besides collagenase 4MMP-8, which is stored in specific granules of neutrophils, and membrane-type MMPs (MT-MMP), which are integral membrane cell glycoproteins, all other MMPs are synthesized if required (Imai et al., 1998) and able to cleave native triple-helical fibrillar collagens.

Enzyme class	Cellular source	Substrate	Activator
Matrix metalloproteinases			
Collagenases			
• MMP-1	Connective tissue cells Monocytes/macrophages	Native triple helix	MMP-3 Plasmin
• MMP-8	Neutrophils	Native triple helix	MMP-3/NE
• MMP-13 (Rodent MMP-1)	as MMP-1	as MMP-q plus telopeptides	Plasmin MMP-2/3 MT-MMP
Gelatinases	Most cell types	Native type IV gelatin	MMP-1/2 MT-MMP
• MMP-2	Connective tissue cells		
• MMP-9	Neutrophils/monocytes	as MMP-2	Plasmin MMP-2/3
Stromelysins	Connective tissue cells		
• MMP-3	Macrophages	Collagen types III,IV and IX	Plasmin Cathepsin G
	Macrophages	Aggrecan	
• MMP-10			
Cystine proteinases			
• Cathepsins	Lysosomal		
• B,L,C,H,N and S			
• K		Telopeptide Bone/ triple helix plus telopeptides	Cathepsin D Low pH
Serine proteinases			
• Neutrophil elastase	Granulocytes		
• Cathepsin G		Telopeptides/triple helix	
Aspartic proteases			
• Cathepsin D		Telopeptide	
		Telopeptide	

Table 3. Major collagen degrading enzymes (Bailey, 2001).

The mechanism of collagen degradation by MMPs is not totally resolved. One of hypothesis is that collagen is actually unwound by MMPs (Chung et al., 2004). Collagenases bind and locally unwind the triple-helical structure before hydrolysing the peptide bonds. According

to these, MMP-1 preferentially interact with the Gly-Leu on $\alpha 2$ (I) chain residues and with Gly-Ileu on $\alpha 1$ chain and cleaves the three α chains in succession, generating two triple-helical fragments of $\frac{3}{4}$ and $\frac{1}{4}$ the molecule length, which show lower denaturation temperature than physiological one, and they both denature, producing random polypeptide gelatine chains (Baley, 2000), which are further degraded by gelatinases (MMP-2 and MMP-9) and other nonspecific enzymes as schematically presented on Fig. 14.

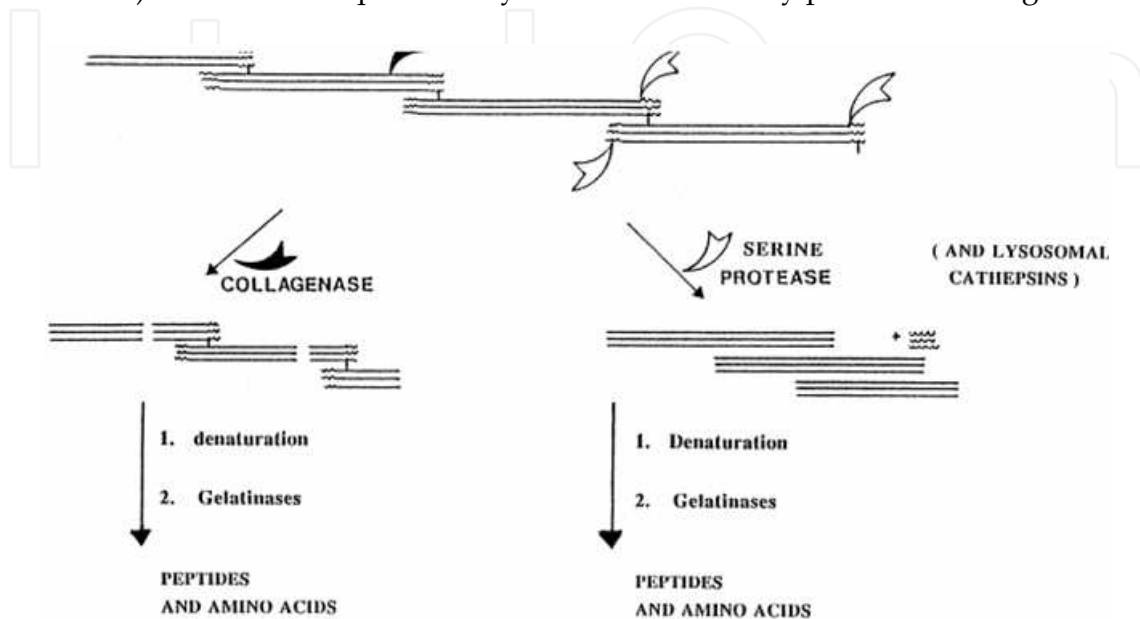


Fig. 14. Dual degradation mechanism of cross-linked fibers in the collagen implant. These mechanism include (left) neutral collagenase cleaving the three chains of the triple-helix and (right) the acid cathepsins and neutral serine proteases cleaving the nonhelical terminal regions (telopeptides) containing the intermolecular cross-links (Bailey, 2001).

Existence of locally unfolded states in collagen molecule has been also suggested (Escat, 2010), according to which, folded structure of collagen cannot fit into the catalytic site, since collagen triple-helix has a diameter of approximately 15 Å, while catalytic domain of MMPs has a catalytic site of only 5 Å wide. Besides, scissile bond, cleaved by collagenases is buried in collagen structure when collagen is exposed to solvent, which makes it inaccessible for scission. Local unfolding is collagen triple-helix property, which occurs without the presence of collagenases, but is necessary for collagen degradation in the presence of collagenases. In addition, immuno-poor regions in collagen are thought to carry biological information, such as cell recognition or protein binding sites (Brodsky & Persikov, 2005), but may also play an important role in collagen degradation (Fields, 1991).

MMP expression is induced by various cytokines, e.g. interleukin-1, and growth factors (Shingleton et al., 1996). MMPs are secreted as latent inactive pro-enzymes (zymogens) which have to be activated before they have complete proteolytic activity (Overall, 1991). Four amino acids (three His and one Cys) are coordinated to the zinc atom in the active center of zymogens (Birkedal-Hansen et al., 1993), being proposed by a "cysteine switch model". The linkage to the Cys residue is thought to be cleaved and a water molecule, which must be the fourth substituent in the active enzyme can bind (Nagase et al., 1999). In vivo, zymogens are activated by removal of a pro-peptide by proteinases, like plasmin or stromelysin, followed by a second activation step provoked by proteinases or autocatalysis

(Shingleton et al., 1996). Additionally, activation is controlled by TIMPs, which can prevent activation of zymogens and/or action of activated MMPs. In vitro, trypsin and organomercurials can be used for activation as well (Overall, 1991). Physical agents unfold the structure, the zinc-cysteine contact breaks and the propeptide is cleaved autocatalytically (Woessner, 1991).

Tissue collagenases cleave tropocollagen at one single site, producing TCA and TCB fragments about three-quarters and one-quarter of the original molecule size. After this initial cleavage, the helical fragments spontaneously denature at body temperature and are subsequently further digested by other proteinases (Mallya et al., 1992). This secondary degradation can take place extracellularly or intracellularly after phagocytosis (Harris, et al., 1974). Apart from collagenases, gelatinases play an important role in collagen degradation. Besides, with any further degradation of initially-cleaved collagen, gelatinases can degrade native collagen type I, IV, V and VII (Overall, 1991). Furthermore, levels of gelatinases are considered to be a good index whether inflammation is present or not, because high concentrations are only available when a normal remodeling process is disrupted (Tregrove et al., 1999).

4.2.3 Immunological response of collagen-based biomaterials

As already mentioned, the implantation of biomaterials often initiates acute inflammatory responses, which sometimes can cause chronic inflammatory response. Measuring the intensities and duration of the immune responses against implanted biomaterials is important for biocompatibility evaluation. The tissue response towards implanted biomaterials (also called the foreign body reaction) is influenced by morphology and composition of the biomaterial and the place where biomaterial is implanted (Ye et al., 2010; Jansen et al., 2008; Wang et al., 2008). Inflammation reaction is manifested by secretion of large amount of antibodies (secreting B cells and T cells with cytotoxic activity) and cytokines, in presence of foreign materials (as scaffolds) or pathogens. The microenvironment of the implant further changes, so, determination of immunological response after in vivo implantation is based of measuring the level of pro-inflammatory cytokine secretions and antibody secretions, and monitoring the population changes of immune cells (Song et al., 2006; Hardin-Young et al., 2000).

According to (Luttikhuisen et al., 2007), collagen-based scaffolds are mainly infiltrated by Giant cells that phagocytose and degrade the collagen bundles, until the material is completely disposed of. This is a chronic inflammatory reaction, which last until the material is completely degraded, after which the cells that are involved disappear.

As an alternative for collagens isolated from calf skin and bond, as a risk-carry materials of bovine spongiform encephalopathy and transmissible spongiform encephalopathy, novel **forms of acid-soluble collagen**, extracted from jellyfish was proposed (Song et al., 2006), because of their differences in amino acid composition: jellyfish collagen had higher content of Gln (glutamine) and Glu, lower Pro content, small Tyr content, comparing with bovine and also contains Cys, which is not common for bovine collagen.

4.2.4 New sources of gelatine

Currently gelatine for food and used by the pharmaceutical industry is derived almost exclusively from **animal products**. About 55,000 tons of animal-sourced gelatine is used each year. Recently, an advance toward turning corn plants into natural factories producing

high-grade gelatine in a safe and inexpensive manner has been introduced as an alternative, enabling the development of a variety of gelatines with specific MWs and properties tailored to suit various needs. Beside, **plant-derived recombinant gelatine** would address concerns about the possible presence of infectious agents in animal by-products and the lack of traceability of the source of the raw materials currently used to make gelatine. Resourcing plant materials to recover and purify recombinant gelatine has remained a challenge because only very low levels accumulate at the early stages of the development process. Furthermore, since recently, gelatines are also produced biotechnologically by the use of recombinant DNA technology, which opens the possibility to manipulate the amino acid sequence of gelatines, and thereby to functionalize them for specific purposes. The biotechnological production of recombinant gelatine also eliminates the risk of prion contaminations, which are possible, present in non-recombinant animal source gelatines (Sutter et al., 2007). Thus, many commercial recombinant collagens already exist on the market and are becoming commonly used in the development of medical soft and hard tissue repair applications (Pannone, 2007).

5. Conclusion

This review presents the characteristic properties of both fibrous proteins including biocompatibility, non-immunogenicity, their capacities for modification at the molecular level, thus rendering or tuning their functional (surface/interfacial, mechanical, topological and morphological) properties, characteristic gelation (sol-gel transition) and gel-forming abilities and, finally, their bio-absorbability and biodegradability. In addition, their expanding applications for biomaterials are compared, with emphasis on the importance of understanding their suitability, as defined biomaterials with specific properties, for certain cell types. Finally, new perspectives for further study and development indicated, providing satisfactory interaction and imitation of biological functions.

6. References

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These contribution books collect reviews and original articles from eminent experts working in the interdisciplinary arena of biomaterial development and use. From their direct and recent experience, the readers can achieve a wide vision on the new and ongoing potentialities of different synthetic and engineered biomaterials. Contributions were selected not based on a direct market or clinical interest, but on results coming from a very fundamental studies. This too will allow to gain a more general view of what and how the various biomaterials can do and work for, along with the methodologies necessary to design, develop and characterize them, without the restrictions necessary imposed by industrial or profit concerns. Biomaterial constructs and supramolecular assemblies have been studied, for example, as drug and protein carriers, tissue scaffolds, or to manage the interactions between artificial devices and the body. In this volume of the biomaterial series have been gathered in particular reviews and papers focusing on the application of new and known macromolecular compounds to nanotechnology and nanomedicine, along with their chemical and mechanical engineering aimed to fit specific biomedical purposes.

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