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Review article

Collagen – biomaterial for drug delivery[1]

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Abstract

The use of collagen as a biomaterial is currently undergoing a renaissance in the tissue engineering field. The biotechnological applications focus on the aspects of cellular growth or delivery of proteins capable of stimulating cellular response. However, basic knowledge about collagen biochemistry and the processing technology in combination with understanding of the physico-chemical properties is necessary for an adequate application of collagen for carrier systems. The purpose of this review article is to summarize information available on collagen dosage forms for drug delivery as well as to impart an overview of the chemical structures and the galenical properties including detailed description of the processing steps – extraction, purification, chemical crosslinking and sterilization. The most successful and stimulating applications are shields in ophthalmology, injectable dispersions for local tumor treatment, sponges carrying antibiotics and minipellets loaded with protein drugs. However, the scientific information about manipulating release properties or mechanistic studies is not as abundant as for some synthetic polymers. © 1998 Elsevier Science B.V.

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1. Introduction

In the 1970s and the 1980s expanding medical applications of biomaterials and connective tissue research challenged academically oriented scientists and commercial research laboratories to focus their studies on collagen [1–6]. At the same time, medical-grade collagen became easier to obtain, the processing technology improved, and new collagen products were successfully placed on the market [7]. In recent years the booming tissue engineering technology has given research in collagen material as scaffolds a new boost [8–12].

The use of collagen in the form of tendons as suture material goes back millennia and could hold its ground with catgut which is still representing a useful suture material in surgery [13–15]. Due to the long historic use of collagen materials generated from different sources by a variety of methods and because of the structural complexity of the protein, the term collagen is usually applied generically and may describe individual molecules, a native fibril in situ or in vitro, aggregates or bulk material of unspecified nature. The particular form of the collagen often has to be inferred from the context and in some instances the reader is left in the dark about the exact character to protect proprietary information.

The purpose of this review is to summarize information on the chemistry of collagen, its processing, characteristics of the dosage forms, application of collagen products in medicine and to discuss recent developments with a special focus on its use for drug delivery.

2. Collagen types

Collagen represents the chief structural protein accounting for approximately 30% of all vertebrate body protein.

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More than 90% of the extracellular protein in the tendon and bone and more than 50% in the skin consist of collagen [16]. Connective tissue derives prominent features such as mechanical strength and activation of the blood clotting cascade from the ubiquitous scleroprotein collagen and its architectural arrangement [17,18]

Although most of the scaffolding in mammals is composed of collagen, the collagenous spectrum ranges from Achilles tendons to the cornea. Hence, different collagen types are necessary to confer distinct biological features to the various types of connective tissues in the body. Thus, collagen comprises a family of genetically distinct molecules which have a unique triple-helix configuration of three polypeptide subunits known as α-chains in common. Currently at least 13 types have been isolated which vary in the length of the helix and the nature and size of the non-helical portions (Table 1) [19,20].

Type I collagen is predominant in higher order animals especially in the skin, tendon, and bone where extreme forces are transmitted. It is a compound of three chains, two of which are identical, termed α1(I), and one α2(I) chain with different amino acid composition or it can rarely represent a trimer built of three α1(I) chains. Type II collagen is essentially unique to hyaline cartilage and the α1(II) subunit is believed to be similar to α1(I). Type III is found in limited quantities (approximately 10%) in association with type I. Thus, type III can be a minor contaminant of type I collagen prepared from skin [16]. In addition, blood vessels predominantly contain type III. Collagen types I, II, and III have large sections of homologous ssequences, independent of the species [21]. Type IV is a highly specialized form found only as a loose fibrillar network in the basement membrane. For the other interstitial collagen types which occur in small quantities and are associated with specific biological structures the author refers to pertinent connective tissue research literature [19,22].

Most synthetic polymers represent mixtures of chains with variable length composed of repeating units. They show physical and chemical properties that are, to a considerable extent, determined by polymerization steps [23]. In contrast, collagen, as a protein has a specific amino acid sequence, size, and structure, which define its basic qualities as a biomaterial suitable for medical products. Therefore, knowledge about the native chemistry and structure is necessary to understand the properties of isolated collagen material and the effects achieved by potential modifications. Here the discussion will be limited to collagen type I which is by far the most abundant and the majority of collagen materials for biomedical applications are based on this type.

3. Collagen type I

3.1. Biochemistry

For details on the biosynthesis see Refs. [18,24,25].

3.1.1. Sequential structure

Early analytical work on the chemistry of collagen was driven by commercial interests in glue, gelatin and leather. The basic collagen molecule contains three polypeptide α-chains, each consisting of more than 1000 amino acids. The composition of the α1(I) and α2(I) chains of calf-skin collagen are given in Table 2 [16]. There are only minor differences between the collagen from different vertebrate species [21]. The amino acids are arranged in a unique triple-helix forming sequence. Glycine has the smallest side group and its repetition at every third position on the sequence allows close package of the chains into a helix which leaves little space for residues in the core. About 35% of the non-glycine positions in the repeating unit Gly-X-Y are occupied by proline, found almost exclusively in the X-position, and 4-hydroxyproline, predominantly in the Y-position (Fig. 1a). Hydroxyproline is derived from

Table 1

Chain composition and body distribution of collagen types, modified from [19]

Collagen type	Chain composition	Tissue distribution
I	$(\alpha1(I))_2\alpha2(I)$, trimer $(\alpha1(I))_3$	Skin, tendon, bone, cornea, dentin, fibrocartilage, large vessels, intestine, uterus, dentin, dermis, tendon
II	$(\alpha1(II))_3$	Hyaline cartilage, vitreous, nucleus pulposus, notochord
III	$(\alpha1(III))_3$	Large vessels, uterine wall, dermis, intestine, heart valve, gingiva (usually coexists with type I except in bone, tendon, cornea)
IV	$(\alpha1(IV))_2\alpha2(IV)$	Basement membranes
V	$\alpha1(V)\alpha2(V)(3(V)$ or $(\alpha1(V))_2\alpha2(V)$ or $(\alpha1(V))_3$	Cornea, placental membranes, bone, large vessels, hyaline cartilage, gingiva
VI	$\alpha1(VI)\alpha2(VI)\alpha3(VI)$	Descemet's membrane, skin, nucleus pulposus, heart muscle
VII	$(\alpha1(VII))_3$	Skin, placenta, lung, cartilage, cornea
VIII	$\alpha1(VIII)$ $\alpha2(VIII)$ chain organization of helix unknown	Produced by endothelial cells, Descemet's membrane
IX	$\alpha1(IX)\alpha2(IX)\alpha3(IX)$	Cartilage
X	$(\alpha1(X))_3$	Hypertrophic and mineralizing cartilage
XI	$1\alpha2\alpha3\alpha_1$ or $\alpha1(XI)\alpha2(XI)\alpha3(XI)$	Cartilage, intervertebral disc, vitreous humour
XII	$(\alpha1(XII))_3$	Chicken embryo tendon, bovine periodontal ligament
XIII	Unknown	Cetal skin, bone, intestinal mucosa

Table 2

Amino acid composition of type I collagen from calf-skin (the values in parentheses are the residues contributed by the non-helical telopeptide regions) [16]

Amino acid	α1(I)-chain	α2(I)-chain
Alanine	124 (2)	111 (3)
Arginine	53 (2)	56 (1)
Asparagine	13	23
Aspartic acid	33 (3)	24 (2)
Glutamic acid	52 (2)	46 (2)
Glutamine	27(3)	24 (1)
Glycine	345 (6)	346 (6)
Histidine	3 (1)	8
Hydroxylysine	4	9
Hydroxyproline	114	99
Isoleucine	9 (1)	18
Leucine	22 (3)	33
Lysine	34 (2)	21 (1)
Methionine	7	4
Phenylalanine	13 (1)	15 (3)
Proline	127 (4)	108 (1)
Serine	37 (5)	35 (1)
Threonine	17 (1)	20
Tyrosine	5 (5)	4 (3)
Valine	17 (1)	34
Total	1056 (42)	1038 (24)

proline by post-translational hydroxylation mediated by prolylhydroxylase [24]. It comprises approximately 10% in the amino acid composition of collagen and offers ways to quantify collagen or its degradation products in the presence of other proteins [26]. Collagen also contains the unusual amino acid hydroxylysine. Similar to the formation of hydroxyproline from proline, hydroxylysine is formed from lysine in the endoplasmic reticulum via enzymatic hydroxylation by lysyl hydroxylase. Formation of hydroxylysyl residues allows the attachment of sugar components, an unalterable requirement for the collagen molecule to form a triple-helical structure [25]. Both imino acids (approximately 23% of the residues) stabilize the triple helix. Due to their alicyclic nature, they stiffen the α-chain and form hydrogen-bonds limiting rotation [25].

Collagen type I is a glycoprotein with a carbohydrate content of less than 1%. The sugar components are either a single galactose unit or a disaccharide of galactose and glucose *O*-glycosidically attached via hydroxylysine residues [16].

3.1.2. Higher structures

The unique physiological and biomaterial characteristics of collagen compared with most synthetic polymers derive from the structural complexity of the collagen molecule. The various levels of order observed in collagen are illustrated in Fig. 1. The α-chains combine to form left-handed helices with 3.3 residues per turn and a pitch of 0.87 nm as identified by X-ray analysis (Fig. 1b) [19,27].

The tertiary structure refers to the fundamental unit originally known as tropocollagen: three polypeptide chains intertwined to form a right-handed triple-helix with a pitch of approximately 8.6 nm. The rod-shaped triple-helix has an average molecular weight of approximately 300 kDa, a length of 300 nm with a diameter of 1.5 nm (Fig. 1c) [18]. This extreme ratio of the dimensions gives rise to high viscosity in solutions and high mobility in electrical fields [28]. In addition, there are regions of 9–26 amino acids at the amino and carboxyl terminal chain ends of the molecule that are not incorporated into the helical structure. These non-helical regions are denoted as telopeptides.

On the fourth level of order, the triple-helical molecules stagger longitudinally and bilaterally into fibrils with distinct periodicity. The collagen molecules aggregate through fibrillogenesis into microfibrils consisting of four to eight collagen molecules and further into fibrils. Those fibrils reach from 10 to 500 nm in diameter depending on tissue type and stage of development [18]. The triple-helices are staggered by 67 nm with an additional gap of 40 nm between succeeding molecules. These collagen fibrils organize into fibers, which on their part can form even larger fiber bundles.

3.1.3. Natural crosslinks

The systematic packaging of the triple-helices lends strength and resilience to the collagen fibers. Additional mechanical and chemical stability derives from intra- and intermolecular crosslinks. Initially, the formation of crosslinks is mediated by lysyl oxidase during fibril formation [29]. The enzymatic activity is limited to the non-helical telopeptide regions and leads to the conversion of selective lysyl and hydroxylysyl residues to the corresponding aldehydes allysine and hydroxyallysine (Fig. 2). While the fibrils associate, the aldehydes can spontaneously react. Intramolecular crosslinks form between two α-chains in the non-helical section of the same molecule by aldol con-

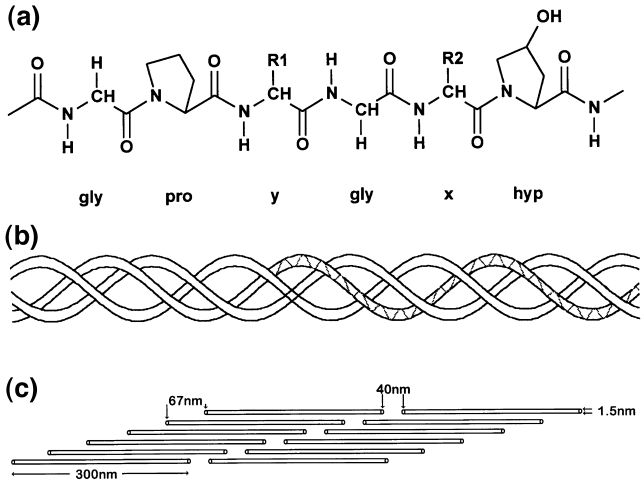

Fig. 1. Chemical structure of collagen type I. (a) Primary amino acid sequence, (b) secondary left handed helix and tertiary right handed triple-helix structure and (c) staggered quaternary structure.

densation of two aldehydes [29,30]. Intermolecular crosslinks occur between the telopeptide region of one collagen molecule and the helical region of a quarterly staggered, adjacent molecule. These bridges between two different tropocollagen molecules result from aldimine formation (non-, mono- or dihydroxylated dehydrolysinonorleucine (Δ-HLNL)) between aldehyde residues and ϵ-amino groups presented by lysine and hydroxylysine (Fig. 2) [30]. The interchain bifunctional crosslinks are still reactive and continue to form polyfunctional crosslinks through multiple condensations with histidine, lysine, or hydroxylysine residues [31]. Two major products originate from reactions of aldol condensation products with histidine residues and from condensation of dehydrodihydroxylysinonorleucine with hydroxy-lysine (Fig. 2) [29,32,33]. Besides the formation of enzymatic crosslinks there is a group of crosslinks derived from glycated lysine and hydroxylysine residues which occur more adventitiously and are important to pathobiological processes [30]. Reiser reviewed the current status with emphasis on structure, location on the collagen molecule, analytics, and pathophysiology of collagen crosslinks [30].

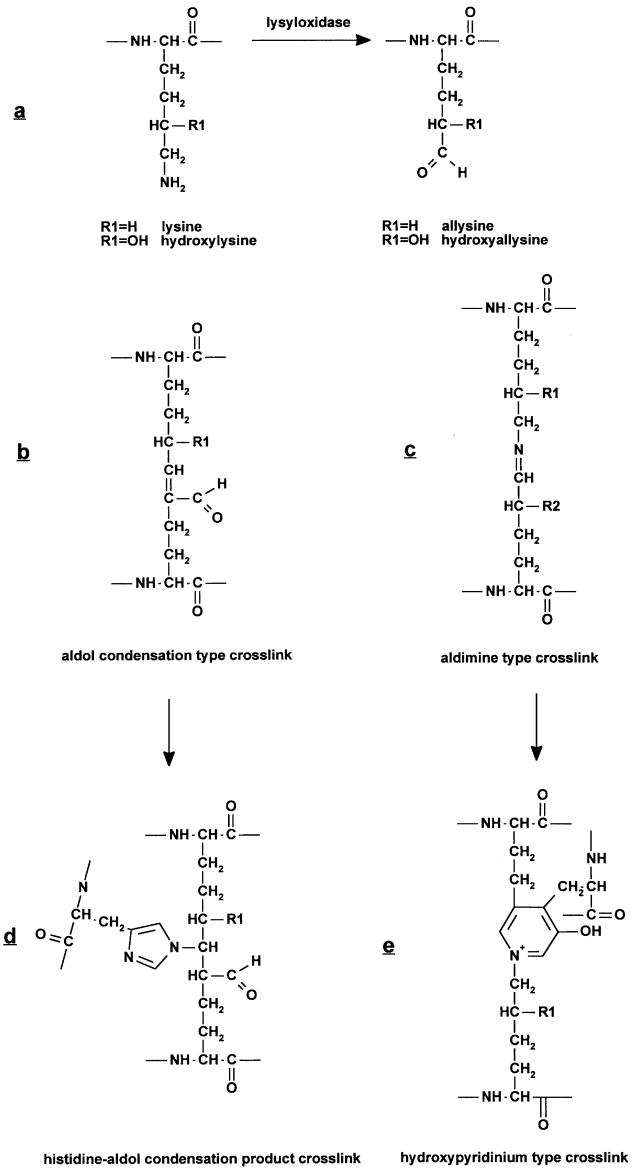

Fig. 2. Chemical structures of natural collagen crosslinks. (a) Oxidation of lysine and hydroxylysine via lysyloxidase, (b) intramolecular aldol condensation type crosslink, (c) intermolecular aldimine (Schiffs' base) type crosslink (R1, R2 = H, OH: dehydrolysinonorleucine, dehydrohydroxylysinonorleucine, dehydrodihydroxylysinonorleucine (may undergo Amadori rearrangement to from ketoamine), (d) condensation of aldol condensation type crosslink with hydroxyproline (can further condensate with lysyl residue to form histidinohydroxymerodesmosine) and (e) hydroxypyridinium type crosslink.

Hence, through specific self-aggregation and crosslinking, collagen can form fibers of unusual strength and stability, the primary reason for the usefulness of collagen in medical devices. The degree of crosslinking increases with age and stress [34] and consequently changes the properties of collagen material (see Section 3.3). The density of crosslinking is responsible for difficulties encountered in solubilizing collagen under mild conditions.

3.1.4. Degradation

Collagen is particularly resistant to attacks by neutral proteases, probably due to its function as the primary structural protein in the body. At neutral pH only specific collagenases cleave the native helix at a position, about three-quarters of the way from the N-terminus [35]. They are zinc containing metalloproteinases of approximately 40–50 kDa and generally require calcium as a co-factor for full activity [36]. Fibrils as aggregates of collagen molecules are degraded starting from the exterior. Collagenase binds tightly to triple-helices at or near the surface, whereas molecules in the interior become accessible to enzymes in the course of the progressive degradation from the outside [37]. The various types of collagen show different susceptibility to collagenolytic degradation [38]. After the triple-helix is cracked, further degradation of the collagen molecules is facilitated by enzymes such as gelatinases and non-specific proteinases which cleave the primary fragments into small peptides and amino acids. Elastases and cathepsins assist in proteolysis of collagen fibers with a higher degree of natural crosslinking based on their ability to digest non-helical telopeptide regions [39].

Administration of exogenous collagen activates a complex cellular response which depends on the type of collagen material. Collagen gels are penetrated by fibroblasts which cause contraction of the gel [40,41]. However, the infiltration of cells into collagen sponge systems depends on the composition and the physical attributes such as porosity. Collagen films which show a closed structure are penetrated by fibroblasts, macrophages and neutrophils [42–44]. Collagen films which are not additionally chemically crosslinked are degraded within 2–7 weeks [42]. Boon [44] came to the same result, but crosslinking the films prevented fibroblast ingrowth and the devices remain intact. Collagen degradation by all these cell types can occur either after

phagocytosis of partially cleaved fibers or by extracellular proteases acting at either neutral or acid pH.

Activation of macrophages has been shown to cause a pH decrease in the micro-environment of collagen implants to below pH 5 [45,46]. Together with the excretion of cathepsin from macrophages and neutrophils [47] this creates an acidic pathway for collagen breakdown. Thus, collagen material can be pretreated with acid in order to reduce the pH and to enhance non-specific proteolytic degradation [48]. Absorption of exogenous collagen in vivo is therefore a complex multi-enzyme process and it is not always clear which is the predominant route. In addition, deposition of newly formed collagen and remodeling of connective tissue has to be considered [49–51].

In vitro degradation is usually simulated by incubation with bacterial collagenase, cathepsin, pepsin or trypsin. These enzymatic tests allow relative comparison of similar materials, but a correlation with in vivo degradation rates is hard to achieve [52–55]. Even results obtained with bacterial collagenase have to be interpreted with care since the enzyme simply attacks the Gly-X-Y sequence and is capable of cleaving the triple-helix at multiple sites [39]. Moreover, commercially available bacterial collagenase represents a mixture of various enzymes with different activities [39].

Specific degradation rates are discussed in the context of the various applications. In general, absorption can be manipulated by additional chemical crosslinking (see Section 3.4) [40,44,56–61]. For drug loaded collagen systems, the effect of the active ingredient on the cellular response has to be taken into account. Whereas steroids can modify the immune response and slow down degradation [62], growth factors or immunostimulants stimulate cellular response leading to faster degradation [43].

3.2. Antigenicity and immunogenicity

With expanded medical use of collagen, questions were raised concerning the immunogenicity of the protein in humans. Collagen is known for its excellent biocompatibility due to low toxicity and poor immunogenic reactions, and until the 1950s collagen antigenicity was assumed to be non-existent [21,63]. Plausible explanations are attributed to the similarity in the amino acid sequence between species and the low content of aromatic amino acids [21]. Concerns are related to a massive immune response causing secondary effects such as damage of organs by immuno complexes or cross-reactions of antibodies to animal derived collagen with human collagen which might induce autoimmune diseases. A distinction needs to be made between immunogenicity, the induction of antibody response, and antigenicity, the existence of antibodies. Animals and humans can produce antibodies to three antigenic determinants in the collagen molecule: the terminal determinants are located in the non-helical telopeptide region (P-determinants), the central determinant presented by the amino acid sequence in the helical section and the tertiary triple-helix structure known as the helical (A-) determinants [64,65]. The importance of each determinant depends on the nature and the processing of the collagen products. For example, selective removal of the non-helical component from the collagen molecule suppresses its antigenicity [1,66]. Additional chemical crosslinking, e.g. with glutaraldehyde reduces the antigenicity but does not eliminate it completely [67,68]. Thus, the immunogenic response depends on the collagen source as well as the test technique and the species used for animal experiments [25,69]. Detailed results are described for the commercial product Zyderm® collagen implant (ZCI), a pepsin solubilized, bovine dermal collagen for tissue augmentation. In several extensive studies, the immunologic response was investigated [65,67,70–72]. In general, 3% of patients had elevated anti-implant antibody levels both against the helical conformation and the primary sequence after injection and experienced localized, inflammatory responses resulting in erythema, swelling, pruritus and induration, and histologically in granulomateous responses without any general systemic complaints. Another 2% showed reaction to a second injection. Despite theoretical concerns animal collagen in the form of sutures, hemostatic agents, and injectable collagen is considered safe [73] and only mildly antigenic [74], making it suitable for use as an implantable and injectable biomaterial.

3.3. Isolation and purification of collagen

Although collagen is ubiquitous in the mammalian body, those tissues rich in fibrous collagen such as skin and tendon are generally used as starting materials to generate collagen for use in implants, wound dressings, or drug delivery systems. In addition to bovine, porcine and sheep collagen varieties derived from numerous different sources including human placenta [75], marine sources [25,76,77], and recombinant human collagen from transgenic animals have been described [78]. Autologous collagen material offers [79] another gut alternative mucosa which is utilized in the manufacture of surgical sutures [80].

The major impediment to dissolution of collagen type I from tissue is the presence of covalent crosslinks between molecules. Collagen is insoluble in organic solvents. Water-soluble collagen represents only a small fraction of total collagen and the amount depends on the age of the animal and type of tissue extracted. In some tissues, notably skin from young animals, crosslinking is sufficiently low to extract a few percent under appropriate conditions. Furthermore, collagen molecules present within fibrillar aggregates, can be dissociated and brought into aqueous solution. However, the nature of the crosslinks prevalent in different tissues determines the particular solvent to be used and the corresponding yields.

3.3.1. Neutral salt soluble collagen

The most commonly used solvents are neutral salt solution (0.15–2 M NaCl) or dilute acetic acid [81]. Neutral salt

solutions will extract freshly synthesized and negligibly crosslinked collagen molecules present in the tissue. Modifications in temperature, shaking rate, and volume of extractant to tissue ratio will inevitably alter the composition of the collagen derived [81]. The extracted material is purified by dialysis, precipitation, and centrifugation. Most tissues have little or no salt-extractable collagen. In order to increase the yield for research purposes animals can be fed with β-aminopropionitrile an inhibitor of peptidyl lysyl oxidase, however this procedure is inadequate for larger commercial scale.

3.3.2. Acid soluble collagen

Dilute acidic solvents, e.g. 0.5 M acetic acid, citrate buffer, or hydrochloric acid pH 2–3 are more efficient than neutral salt solutions. The intermolecular crosslinks of the aldimine type are dissociated by the dilute acids and the repulsive repelling charges on the triple-helices lead to swelling of fibrillar structures [82]. Dilute acids will not disassociate less labile crosslinks such as keto-imine bonds. Therefore collagen from tissues containing higher percentages of keto-imine bonds, i.e. bone, cartilage, or material from older animals have a lower solubility in dilute acid solvents. In order to acid extract collagen, generally, tissue is ground in the cold, washed with neutral saline to remove soluble proteins and polysaccharides, and the collagen extracted with a low ionic strength, acidic solution [83].

It is possible to solubilize approximately 2% of the tissue collagen with dilute salt or acid solutions. These collagen molecules can be reconstituted into large fibrils with similar properties as native fibrils by adjusting the pH or temperature of the solution [25]. The remaining 98% is referred to as insoluble collagen although this dominant collagen material is not absolutely insoluble and can be further disintegrated without major damage to the triple-helical structures. The two most common approaches are the use of strong alkali or enzymes to cleave additional crosslinks and suspend or dissolve at first acid-insoluble structures.

3.3.3. Alkali- and enzyme-treated collagen

Additional collagen material can be solubilized by treating connective tissue with an aqueous solution comprised of alkali hydroxide and alkali sulfate, e.g. approximately 10% sodium hydroxide and 10% sodium sulfate for approximately 48 h [84,85]. Thus, fat associated with the insoluble collagen is saponified, non-helical telopeptide regions are truncated and the collagen fibers disintegrated. The size and molecular weight of the resulting collagen material depend on the time of treatment and alkali concentration [85]. The presence of alkali sulfate controls the swelling of the collagen structures and protects the native triple-helical characteristics. It has to be noted that similar to gelatin, the isoelectric point of the resulting material is shifted to lower pH as asparagine and glutamine are converted into aspartic and glutamic acid.

Much higher yields compared with acidic extraction can be achieved by taking advantage of the fact that the collagen triple-helix is relatively resistant to proteases, i.e. pronase®, ficin, pepsin or chymotrypsin below approximately 20°C [25]. The efficacy of enzymatic treatment arises from selective cleavage in the terminal non-helical regions breaking peptide bonds near crosslinks and releasing molecules which dissolve. Some crosslinks presumably remain, attaching small peptide remnants to the solubilized molecules [22]. Thus, the telopeptide ends of the polymer chains are dissected but under appropriate conditions the helices remain essentially intact. The resulting material, so-called atelocollagen, benefits from the removal of the antigenic P-determinant located on the non-helical protein sections and provokes milder immune response [1,66]. Pepsin at a 1:10 weight ratio of enzyme to dry weight tissue in dilute organic acid (0.5 M acetic acid) provides an propitious medium in which collagen can be swollen and solubilized [16].

Soluble collagen is purified mainly by precipitation after pH, salt concentration or temperature adjustment [86]. The high viscosity of even dilute solutions interferes with purification methods such as chromatography, electrophoresis and differential sedimentation. Collagen solutions contain varying proportions of monomer and higher molecular weight covalently linked aggregates, depending on the source and method of preparation. Truly monomeric solutions are difficult if not impossible to obtain [16]. Pepsin-solubilized collagen usually contains higher proportions of monomer than salt- or acid extracted material [25]. Soluble collagen can be stored frozen or lyophilized. In the course of drying, denaturation or non-specific crosslinking can occur and the degree of association upon reconstitution can change [87].

3.3.4. Insoluble collagen

Instead of disintegration and transfer into soluble material, extensively crosslinked collagen can be dispersed as opalescent, fine fibrillar suspensions by the use of mild denaturation agents and mechanical fragmentation usually at an acidic pH. Fibrilllar collagen is more resistant to proteolysis than most other non-collageneous tissue constituents which are removed during processing by selective proteolysis and washing [1,86,88].

In additional steps collagen material can be subjected to chemical modifications such as succinylation [89], acetylation [90,91], methylation [92] or attachment to other polymers (see Section 4.4) [93].

3.4. Crosslinking

Natural crosslinking gives high tensile strength and proteolytic resistance to collagen. Due to dissociation of crosslinks in the course of the above described isolation processes, reconstituted forms of collagen such as films, fibers, or sponges can lack sufficient strength and may disintegrate upon handling or collapse under the pressure from

surrounding tissue in vivo. Furthermore, the rate of biodegradation has to be customized based on the specific application. For example as a hemostat, collagen has accomplished its mission once the blood clot has formed, whereas for tissue augmentation an implant has to maintain its scaffolding properties while it is gradually replaced by host collagen [94,95]. Thus, it is often necessary to confer mechanical firmness and collagenase resistance by introduction of exogeneous crosslinking into the molecular structure.

3.4.1. Chromium tanning

Formation of ionic bonds is the basic principle of tanning. Trivalent metals such as chromium are used as crosslinking agents in absorbable medical products for instance catgut surgical sutures [1,74]. Chromium forms stable basic oligomeric complexes with acidic residues on the collagen molecule at a pH of 3–4. Aluminum and other polyvalent cations also form ionic bonds with collagen, but impart inferior stability [96]. The intramolecular mobility of collagen has been found to be significantly reduced by aluminum ions as demonstrated by thermally stimulated current spectroscopy [97].

3.4.2. Formaldehyde

Covalent crosslinks in collagen can be created in a number of ways [98]. The most utilized reagents are aldehydes with formaldehyde and glutaraldehyde being the most obvious examples. Formaldehyde reacts with the ϵ-amino groups of lysine and hydroxylysine residues to an intermediate imine which forms a crosslink either with tyrosine or with an amid group of asparagine or glutamine [1,98]. The degree of crosslinking can be inferred from the number of unmodified ϵ-amino groups [59,99]. Formaldehyde treatment prolongs the in vivo absorption of collagen-based material whereas it decreases the tensile strength [100]. A major advantage of formaldehyde is the possibility of crosslinking dry collagen material with reagent in the vapor phase instead of treatment in liquid, in particular aqueous environments [99,100]. Information about residual contents can be deduced from the British Pharmacopoeia [101] which limits water extractable formaldehyde in absorbable gelatin sponges to 0.2% w/w. Due to the brittleness of formaldehyde crosslinked products, potential toxicity and adverse reactions arising from residuals and reversible fixation, formaldehyde is no longer favored, albeit approved, for medical devices [74].

3.4.3. Glutaraldehyde

The most commonly used aldehyde is bifunctional glutaraldehyde (GTA) which was recently reviewed as a crosslinking agent [102]. Treatment with GTA is a commercially viable process, its efficiency has been optimized, it is inexpensive and completed in a relatively short period and provides more sustained long-term durability than crosslinking with formaldehyde [56,98]. The complex reaction events depend on pH, solvent, concentration, and purity of GTA [4,103–106]. Crosslinking is optimal at neutral pH and requires free non-protonated ϵ-amino groups to form imines [107]. GTA not only reacts with amino groups, but with carboxylic, amide and other groups of proteins as well [103]. Additional polymerization by aldol condensation results in covalent intermolecular crosslinks connecting distant collagen molecules [4,103]. Consequently, crosslinking can become less efficient with increasing GTA concentrations. Polymeric aldehyde chains can grow on the surface of collagen fibers which leads predominantly to intermolecular crosslinking and prevent further penetration of reagent into the fiber and fibril structures [74]. The degree of crosslinking can be measured via free amino group analysis, shrinkage temperature, resistance to enzymatic digestion and collagen melting temperature [106,108,109]. Variations in the GTA crosslinking procedure can be used to optimize collagen biomaterials. Diamino compounds such as lysine can be added to intensify intermolecular crosslinking compared with GTA alone [110]. Microwave irradiation has been used successfully to stimulate crosslinking of collagen with GTA and in vivo less inflammation was found around implants with microwave treatment compared with conventional glutaraldehyde treatment [111–113]. Furthermore, control of mechanical characteristics as well as degradation kinetics via GTA crosslinking can be used for composite materials based on collagen [57]. Several studies reported on the in vivo response to GTA-treated collagen materials in various animal models, as well as in cell culture [40,68,114–119] resulted in conflicting data dependent on the GTA concentration applied. Concerns about GTA treatment arise from an exacerbating effect on calcification of prosthesis materials [114], cytotoxicity due to post-implantation depolymerization and monomer release from the glutaraldehyde polymer [116–118,120,121]. However, treatment with glutaraldehyde has been shown to reduce the immunogenicity of collageneous material [68,115] while increasing its resistance to enzymatic degradation.

3.4.4. Hexamethylenediisocyanate

As an alternative to aldehyde treatment, strong and resistant collagen material can be obtained using hexamethylendiisocyanate (HDC) as a crosslinker [98]. HDC is solubilized in water using surfactants or dissolved in 2-propanol and forms crosslinks with two ϵ-amino groups via urea-type moieties, a principle which is also used in the plasma expanders based on crosslinked gelatin [74,122–126]. Van Luyn et al. [117,118] found less cytotoxic effects when compared with GTA treated material and evaluated the primary cytotoxicity due to release of extractables and secondary effects resulting from release of cytotoxic degradation products [117,118]. Both GTA and, to a lesser extent, HDC crosslinked material induced primary and secondary cytotoxic effects in contrast to non-crosslinked material which only led to low primary inhibition of cell proliferation. Whereas GTA crosslinked material showed strong

effects due to hydrolysis, the secondary effect of HDC was mild and due to enzymatic breakdown of the biomaterial.

3.4.5. Polyepoxy compounds

Several studies have recently focused on the use of epoxy (ethylene glycol diglycidyl ether, glycerol polyglycidyl ether, methylglycidyl ether) [127–134]. The epoxy functionality predominantly reacts with the amino groups on lysine, much like GTA. Anterior cruciate ligaments crosslinking with epoxy occurs a little bit slower than with GTA [131] but epoxy treatment rendered tissue more flexible and decreased the incidence of calcification in vivo [131,135]. The cytotoxicity of the polyepoxy components has also been shown to be acceptable [136].

3.4.6. Carbodiimides

Crosslinking with carbodiimides, especially 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC) offers the main advantage over aldehydes, HDC, or epoxy compounds in that these carbodiimides only facilitate the formation of amide bonds between carboxylic and amino groups on the collagen molecules without becoming part of the actual linkage. Thus, bifunctional crosslinking agents are obviated. Carbodiimides first couple to a carboxylic group to form *o*-isoacylurea structures. The resulting activated intermediate is attacked by a nucleophilic primary amino group to form an amide crosslink and the isoureaderivative of the applied carbodiimide is eliminated and can be washed out [60,74,103,137]. The optimum pH is approximately 5 [138]. The susceptibility of carbodiimide crosslinked material to enzymatic degradation can be controlled by varying the degree of cross-linking via the reaction conditions [58]. In vivo, dermal sheep collagen crosslinked with carbodiimide was slowly degraded and enabled collagen new-formation functioning as a guidance for muscle overgrowth in abdominal wall defects [94].

3.4.7. Acyl azide method

Acyl azide intermediate formation is an alternative method to the use of carbodiimides that results in induction of amide bonds [139]. Carboxylic groups are methylated and converted into hydrazides followed by reaction with sodium nitride to form the acyl azide or can be modified with diphenylphosphoryl azide. These azide functionalities couple with amino groups to give amide crosslinks. Improvements in thermal stability and resistance to collagenase digestion were found to be similar after the acyl azide procedure and glutaraldehyde treatment [139] with superior cytocompatibility for acyl azide treated material [140]. In vivo, a collagen–glycosaminoglycan sponge crosslinked via acyl azide was rapidly invaded by mononuclear cells with the formation of granulation tissue. Acyl azide treatment increased the persistence of a collagen–glycosaminoglycan sponge up to 3 months and inhibited calcification whereas glutaraldehyde-treated sponges were completely calcified after 15 days [61].

Van Wachem compared the four crosslinking methods GTA, acyl azide, HDC, and EDC with respect to biocompatibility and tissue regenerating capacity [50]. The most promising results were obtained with EDC crosslinked material which resulted in a mild cellular response after implantation with increased neutrophils and macrophage infiltration. In combination with the slow degradation rate it allowed replacement by newly formed collagen and tissue regeneration.

3.4.8. Physical treatment

A major handicap of chemical crosslinking agents is the potential toxic effect of residual molecules and/or compounds formed during in vivo degradation. Therefore, alternative physical methods are pursued, including dry heat, exposure to ultraviolet or γ-irradiation [98]. Both dehydrothermal treatment (DHT) and exposure to ultraviolet light at 254 nm increase the collagen shrinkage temperature, the resistance to collagenolytic degradation, and the durability under load in collagenase [141–148]. However, collagen becomes partially denatured by these physical treatments [147]. In order to keep degradation of the triple-helices to a minimum it is crucial for DHT treatment to reduce the water content via vacuum as thoroughly as possible prior to heating. Even small amounts of residual moisture can cause breakdown of the helical structures and proteolysis [141]. Severe dehydration itself already induces amide formation and esterification between carboxy and free amino and hydroxyl groups, respectively. But the effect is insignificant and typical DHT conditions are 110°C for several hours up to a few days [146,148]. The combination of degradation and crosslinking allows non-specific enzymes to attack and solubilize fragments of the crosslinked material. As Gorham et al. [143] showed, sensitivity of collagen material to trypsin was increased after DHT treatment, whereas degradation by pepsin and lysosomal cathepsins was reduced. In vivo there was little difference between control and heat-cured samples except for material treated at 120°C which biodegraded at a significantly faster rate.

Formation of crosslinks during UV-irradiation is thought to be initiated by free radicals formed on aromatic amino acid residues [147] which indicates a rather limited maximum degree of crosslinking due to the small number of tyrosine and phenylalanine residues in collagen. Exposure times can therefore be kept short since crosslinking density soon reaches its limits [147]. Nevertheless, UV-irradiation improves mechanical strength [146].

3.5. Sterilization

Ideally, sterilization procedures free collagen-based biomaterials of all viable microorganisms without inducing changes in the protein chemistry, the mechanical properties and the degradation behavior. However, all currently used methods will inherently damage and alter collagen in a way that might affect its in vivo absorption rate, mechanical

strength or performance in combination with drugs. As an alternative, expensive, complex aseptic processing is feasible and preparations of soluble collagen can be sterile filtered.

Moist heat cannot be used for sterilization of collagen products since autoclaving thermally denatures the hydrated protein. Higher temperatures could be applied after thorough removal of moisture. However, dry heat induces partial denaturation and crosslinking at the same time and is used to dehydrothermally crosslink collagen (see Section 3.2). Thus, although sterilization with dry heat may render material with acceptable tensile strength or other physical properties, the in vivo degradation may be substantially altered. Denatured portions of collagen can be more susceptible to non-specific proteases and absorption may be accelerated with a rapid loss in tensile strength [147].

3.5.1. Ethylene oxide

Ethylene oxide gas treatment is used to sterilize collagen reliably. This procedure requires humidification of the sterilization chamber and slightly elevated temperatures [149]. Under these conditions only a little denaturation occurs [1]. But amino acid analysis indicates intensive reaction of ethylene oxide with the amino-groups presented by collagen in the form of lysine and hydroxylysine residues and leads to an increase in pH [74,99,150]. Thus, helix stability becomes decreased, as indicated by a lower shrinkage and denaturation temperature of dermal sheep and bovine tendon collagen [99,150]. In addition, ethylene oxide sterilized dermal sheep collagen showed slower degradation by collagenase compared with non-sterilized material which was explained by reduced adsorption of collagenase onto the collagen matrix [150]. We could not find this effect of ethylene oxide on bovine collagen sponges in our tests [99]. Since collagen is altered permanently by ethylene oxide sterilization and both the physical and biological performance are affected by the process, consistency in the treatment is important. This specifically refers to the humidification process, the gas treatment as well as the subsequent aeration procedure which is critical to keep the concentration of residual ethylene oxide to a minimum in order to comply with regulatory limits and to avoid cytotoxic effects [151].

3.5.2. γ-irradiation

In recent years, γ-irradiation from a ^{60}Co source has become a method of choice to sterilize collagen biomaterials mainly for its high efficacy and accurately controlled dose. It is considered the most reliable sterilization method available [74]. A dose of 2.5 Mrad is considered to achieve complete sterilization [152]. Studies on the effect of γ-irradiation on collagen structure clearly indicate chain scission resulting in a fraction of lower molecular weight material [153]. This fragmentation may be compensated by the formation of additional crosslinks, depending on the moisture content in the product [150,154]. These molecular changes due to γ-sterilization reduce the mechanical strength of collagen [150]. At the same time the materials become more sensitive to enzymatic attack by collagenase [150] but more resistant to degradation by pronase® [153]. The enzymatic tests in vitro have to be interpreted carefully since they can not reflect the in vivo situation completely.

4. Applications

The attractiveness of collagen as a biomaterial rests largely on the view that it is a natural material of low immunogenicity and is therefore seen by the body as a normal constituent rather than foreign matter [25]. Collagen can be processed into a number of forms such as sheets, tubes, sponges, powders, fleeces, injectable solutions and dispersions, all of which have found use in medical practice [1,74,155]. Furthermore, attempts have been made to apply these systems for drug delivery in a variety of applications such as ophthalmology, wound and burn dressing, tumor treatment, and tissue engineering (Table 3).

4.1. Ophthalmology

Medical devices based on collagen have numerous applications in ophthalmology as grafts for corneal replacement, suture material, bandage lenses, punctual plugs, or viscous solutions for use as vitreous replacements or protectants during surgery [156]. One of the most widely studied drug carrier applications of collagen are inserts and shields for drug delivery to the corneal surface or to the cornea itself and delivered intraocularly.

4.1.1. Inserts

The concept of using ocular collagen inserts to provide prolonged delivery of medication to the eye was initiated in the early 1970s. The studies were focused on fabrication of drug-loaded inserts cut from films or as molded rods, prepared by air-drying aqueous mixtures of drug and collagen [157]. Such collagen films delayed the release of pilocarpine by over 25 min while the insert dissolved without provoking an inflammatory response in rabbit eyes [157]. Further prolonged release in vitro was described for devices crosslinked with glutaraldehyde, but the authors did not consider reactions of pilocarpine with the aldehyde and did not note the pilocarpine content of the devices [158]. Punch et al. [159] performed preliminary tests on inserts made from insoluble or soluble, succinylated collagen loaded with insoluble antibiotics penicillin–procaine, erythromycin, or erythromycin estolate. The most promising results were achieved with erythromycin estolate from succinylated, soluble collagen with effective concentrations released for more than 12 h. However, the physical properties were inappropriate and the systems failed when inserted into the conjunctival sac of cattle. Bloomfield et al. [3] compared delivery of gentamicin by ocular inserts made of succinylated collagen with

Table 3

Collagen drug delivery applications

Application form	Drug	Indications	References
Ophthalmology			
Inserts	Pilocarpine, erythromycin, gentamicin	Infection, glaucoma	[3,157–159]
Shields	Gentamicin, vancomycin, tobramycin, netilmycin, polymyxin B sulfate, trimethoprim, amphotericin B, 5-FU, pilocarpine, steroids, flurbiprofene	Infection, mycosis, glaucoma, inflammation	[160–165,167, 169–182,185,186]
	Model	N/A	[183,184]
Particles	Cyclosporine	Allograft implantation	[188,189]
Gels	Keterolac	Inflammation	[191]
Aqueous injection	Vinblastin, cisplatin, Tc, 5-FU, ^{111}In or ^{90}Y labeled monoclonal antibodies, TGF-β, fibroblast growth factor	Local cancer treatment, wound repair	[199–204,212]
	Insulin, growth hormone		[220]
Solid parenteral application			
Sponge	Gentamicin, cefotaxim, fusidic acid, clindamycin, all-*trans* retinoic acid	Infection, cervical dysplasia	[269–284]
	Growth factors, bone morphogenetic proteins	Tissue regeneration	[236–241,243,245–258]
Films	Medroxyprogesterone acetate, human growth hormone, immunostimulants, tetracycline, growth factors		[43,285,286,288–293]
Monolithic devices	Minocycline, lysozyme, interleukin-2, interferon		[222,223,294–298]
Microparticles	Retinol, tretinoin, tetracain, lidocain, ethacridine lactate,	Local anaesthesia, dermal	[304–307]

eyedrops, ointments, and subconjunctival injection. The collagen wafers gave higher drug levels in tear film, sclera and cornea. Succinylation was considered advantageous to enhance charge interaction between deprotonated carboxyl groups of succinylated collagen and positive amino groups of gentamicin [see Section 4.4]. In the late 1980s studies on inserts were superseded by research work on collagen shields which became commercially available in reproducible quality.

4.1.2. Shields

Collagen shields were developed as corneal bandages to promote wound healing after corneal transplantation, radial keratomy, in keratorefractive procedures, and for epithelial debridement procedures [160–163]. The thin collagen films conform to the shape of the cornea when applied to the eye, provide sufficient oxygen transmission to allow corneal metabolism and act as short term bandage lenses [164]. As the shields dissolve, they provide a layer of collagen solution that seems to lubricate the surface of the eye, minimize rubbing of the lids on the cornea, and foster epithelial healing [160,165]. Initially, the shields come in a dehydrated form and have to be soaked with liquid prior to application. It was recognized that these devices could be used in order to deliver ophthalmic medication when immersed in an aqueous drug solution (or suspension) immediately before placement in the eye. With virtually any preparation for ophthalmic or parenteral use the collagen matrix may (i) act as a reservoir, increasing the contact time between drug and cornea, (ii) reversibly bind drug molecules which are subsequently released in a delayed mode, and (iii) reduce the likelihood of systemic toxicity especially if dose reduction is possible [165,166]. The systems can either be used solely for drug delivery purposes or to combine this aspect with the beneficial effect of collagen on healing after surgery. Bio-Cor® (Bausch and Lomb, Rochester, NY) made of porcine scleral collagen (as is Kerashield®, Ioptex Research Inc. Irwindale, CA [167]) was the first shield commercially available and is the most widely tested. The shields are crosslinked using UV-irradiation during manufacturing to achieve 12, 24 or 72 h absorption time after insertion [164,165]. Other products on the market (MediLens®, Chiron, Irvine, CA and ProShield®, Alcon, Fort Worth, TX) are prepared from bovine corium tissue and last between 24 and 48 h on the cornea [168].

Drugs used in combination with collagen shields are often water-soluble antibiotics like gentamicin [169,170], vancomycin [171], tobramycin [160,172–177], netilmicin [178], polymyxin B sulfate [167], or trimethoprim [167] as well as amphotericin B as antimycotic [179,180], steroids [167,169,176,181,182], pilocarpine [176], or flurbiprofene sodium [176]. A soak time of 5–10 min is sufficient [171,175,182]. For example collagen shield immersed in tobramycin can enhance epithelial healing after surgery as well as provide antibiotic prophylaxis against infection [160,172–177]. Tobramycin delivery to the cornea was significantly improved with collagen shields over either combinations with hydrophilic soft contact lenses or eye drops as controls [172]. Unterman [173] demonstrated that collagen shields were effective in delivering sustained therapeutic concentrations of tobramycin to the cornea and aqueous humour of rabbits. The antibiotic level depended on the loaded dose. For treatment of bacterial keratitis, the application of tobramycin in the presence of a collagen shields was significantly more effective than application of drug alone. In addition, soaked shields (200 mg/ml tobramycin) produced a significantly higher concentration of antibiotic in the cornea 1 h after application than the sub-

conjunctival injection of 20 mg tobramycin. This effect helps to reduce the applied antibiotic dose and Hobden et al. [175] showed that collagen shields containing tobramycin were as effective as drop treatment every 30 min in experimental keratitis with the drug load in drops being an order of magnitude higher due to repeated application. In vivo studies with steroids showed that combination with collagen was superior to simple drop treatment [181]. An additional variation is the combination of collagen shield treatment with the repeated application of drops which resulted in higher drug levels than the use of drops alone [182]. Solubility of the drug and its concentration in the soak liquid determine the concentrations found in the tear film, cornea, and aqueous humour. In attempts to further improve the system, collagen shields were also impregnated with liposomes [183,184]. Grammer et al. [184] investigated collagen corneal shields soaked with liposomal formulations which were labeled either with a water soluble marker in the aqueous interior or with a lipophilic marker in the bilayers. The liposomes remained intact in the course of uptake into and release from the shields. The results indicated that surface charge and bilayer fluidity are of minor importance for the interaction of liposomes with collagen shields. However, since the release kinetics of liposome-encapsulated hydrophilic and lipophilic markers were similar to the release of a non-encapsulated drug, the combination may be useful with respect to encapsulation of drugs which do not penetrate the ocular surface as well as to prolong the corneal contact time of the liposomes.

For postoperative treatment after glaucoma filtering surgery collagen shields loaded with 5-fluorouracil (5-FU) have been tested in a subconjunctival implantation scenario. However, in vitro test results indicated complete release of 5-FU within 15 min and histology showed that collagen implants incited granulomatous inflammatory response in monkeys [185,186].

The application of collagen shields for drug delivery is limited by several disadvantages: (i) the shields are not fully transparent, reduce visual acuity, and cause slight discomfort, (ii) the insertion technique is complex, and (iii) they last only a brief period. Due to fibrillogenesis at neutral pH, clear acidic solutions convert to opaque dispersions when the pH is brought to neutrality for preparation of ophthalmological systems. However, most implant materials useful in ophthalmology have to be transparent and clear [187]. Collagen fibrillogenesis at neutral pH can be prevented by chemical modification of the molecule to change its pKa to either side of neutrality. The most widely used method is acylation with succinic or glutaric anhydride [156].

4.1.3. Particles

Collagen particles suspended in a viscous medium offer an alternative to overcome the difficulties associated with collagen shields. Gebhardt et al. [188,189] studied the effects of cyclosporine formulated into collagen shields or as a suspension with collagen particles in methylcellulose artificial tears and a solution in corn oil as a control. With either of the two collagen vehicles, peak concentrations were found in the cornea approximately 4 h after application. The corn oil vehicle yielded a significantly lower and earlier peak concentration. By 8 h, significant amounts of drug were still present in the corneas for collagen-treated animals, whereas drug levels in the corn oil treatment group had returned to baseline levels. Drug delivery profiles in the aqueous humour were similar, however drug concentrations were five times lower and peaked later. As a result, cyclosporine incorporated into collagen particles and collagen shields was more effective in preventing the onset of primary corneal allograft reaction and the loss of grafts by rejection, compared with cyclosporine in corn oil. As an alternative, collagen particles can be suspended in PVC sheets and dried to form a film which can be soaked in drug containing liquid to provide continuous delivery of tobramycin for approximately 6 h [190].

4.1.4. Gels

Collagen gels are primarily used for injectable systems (see Section 4.3). For ophthalmic use, formulations are patented which are initially liquids but gel after administration to the eye [191]. When applied, the gels will remain in place in the cul-de-sac of the eye substantially longer than liquid formulations and will allow for a sustained delivery of non-steroidal anti-inflammatory drugs or antibiotics.

4.2. Aqueous injectables

For most medical functions collagen is applied in a solid form designed for example as a sponge, suture, or ophthalmic lens. However, one of the most commercially thriving uses of collagen has been the subcutaneous injection of soluble collagen for the repair of dermatological defects [7]. For this purpose autologous, injectable dermal collagen has also been developed [79]. Similar collagen preparations have been injected with success in other medical applications like the treatment of vocal fold immobility [192] and urinary incontinence [193,194]. McPherson et al. [109] intensively studied GTA treated reconstituted pepsin-solubilized bovine corium collagen dispersions. Residual, extractable aldehyde content of such preparations was as low as 1–3 ppm. Corresponding to a decreased neutral solubility and sensitivity to proteases with glutaraldehyde treatment compared with non-crosslinked controls, the biologic response was found to be a function of the crosslinking degree [40]. At low glutaraldehyde concentrations the response was characterized by fibroblast invasion, neovascularization and little, if any, evidence of inflammation. Dispersions crosslinked with higher concentrations of glutaraldehyde elicited a foreign-body/giant cell reaction and varying degrees of erosion. Calcification was found in guinea pigs after 30 days, by 120 days certain areas of the injected material were observed to be undergoing ossification which identified a potential complication in various

treatment modalities. Rheological measurements demonstrated that such crosslinked suspensions were non-Newtonian, shear-thinning fluids, and that they were two- to threefold more viscous than untreated preparations [109,195, 196]. As a consequence of the increased viscosity the crosslinked formulation was more difficult to inject into tissue and did not spread uniformly, sometimes giving rise to palpable lumps or large masses evident in histological sections [40,196]. When hyaluronic acid in the range 0.3– 0.5% was blended with the crosslinked collagen the blend required lower forces to achieve deformation and flow and there was a significant improvement in ease of injection into tissue [196]. Particle size analysis of the blend revealed a reduction in fiber aggregate dimensions, compared with crosslinked collagen alone. A corresponding patent [197] additionally describes neutral polymers such as dextran and low molecular weight molecules such as maltose (as much as 40% of the formulation) as lubricants to improve intrusion into soft tissue. Rosenblatt et al. [198] studied the structure and mechanical properties of collagen fiber dispersions by dynamic rheological measurements and polarized microscopy over a pH range from 6 to 9 and over temperatures between 283 and 298°K. With increasing pH, the dispersions were found to possess more rigid fibers and stronger inter-fiber attractive forces resulting from changes in the ionization of amino acid side chains. Raising the temperature caused fibers to rigidify through enhanced hydrophobic attractive forces. Thus, collagen can be formulated so that it spans both fluid and solid domains controlled in part by the level of imposed stress and in part by temperature and pH.

4.2.1. Cytostatica delivery

The delivery of small molecules via injectable collagen preparations is pursued emphatically under the aspect of local cytostatica treatment [199–204]. In order to enhance the local drug retention, to minimize systemic side effects and potentially reduce the required dose, a collagen gel formulation containing epinephrin for local vasoconstriction was tested for cisplatin, vinblastine, ^{111}In or ^{90}Y-labeled monoclonal antibodies, and 5-FU. Subsequently to tumor removal, resection bed injection of this collagen matrix conveying cisplatin prevented tumor recurrence in all treated animals and led to a significant increase in resection bed drug levels of cisplatin at days 1,4, and 7 [200]. The collagen/epinephrine combination gel was also tested for treatment of existing tumors. The disappearance of 5-FU [203] or ^{195m}Pt–cisplatin [202] by diffusion after intratumoral injection in mice was delayed with the collagen/epinephrine gel. The system may also allow for improvement in the therapeutic index for radioimmunotherapy. As a study with ^{111}In or ^{90}Y attached to monoclonal antibodies demonstrated, the use of the collagen system markedly increased the retention of the radioisotope in tumors, enhanced the antitumor efficacy, and reduced systemic toxicity compared with systemic administration [201]. Forty-eight hours after the administration of the ^{111}In monoclonal antibody in the collagen gel intratumoral, in solution intratumoral and in solution intraperitoneal, the level of radioactivity remaining per gram tumor was 98, 49 and 16% of the injected dose, respectively.

4.2.2. Release mechanism

One of the first studies on the use of these preparations for prolonged delivery of drugs showed that the effectiveness of local anesthetics and central analgesics was extended 3–5 times when formulated as an injectable collagen system compared with control injections of the drug alone [205]. Chvapil et al. [206] speculated whether this effect is due to the viscosity of the collagen formulation simply slowing down drug diffusion into the systemic circulation or possible interactions between the drug and collagen [1]. Rosenblatt et al. [206] investigated both effects for protein drugs. In order to diffusionally control the release rate of a protein from a matrix it is necessary that the mesh size approaches the size of the protein molecule [206]. Fibrillar collagen matrices were capable of moderating the release rates of very large protein drugs only (such as fibrinogen) and a significant non-fibrillar content was necessary to modulate the diffusivity of smaller proteins such as chymotrypsinogen [206]. In order to modify the release of proteins from liquid collagen matrices, Rosenblatt and Singh studied the effect of charge interaction between collagen, modified collagen and model proteins [89,207,208]. The charge distribution on collagen was altered to yield negative net charges which exhibited binding interactions with positively charged lysozyme or polylysine. In studies, lysozyme release in a native collagen matrix was slower than theoretical profiles calculated on the basis of Fick's second law and the free solution diffusivity. The release rate was decreased further with succinylation of collagen [209, 210]. Lower apparent diffusivities from collagen matrices were due to a combined effect of both electrostatic and hydrophobic interactions. The effect of hydrophobic interactions between lysozyme and collagen explained the slower release of lysozyme compared with polylysine with a similar molecular weight [209]. Both increasing interaction strength and binding capacity retard the release rates for systems governed by binding and diffusion mechanisms.

The viability of adsorption/desorption and subsequent diffusion as a release mechanism was also evaluated for gentamicin [206,209]. Due to the small size and hence large diffusion coefficients of antibiotics a prolonged release cannot be obtained by simple Fickian diffusion. Whether the interactions between gentamicin and collagen which were demonstrated in vitro [209] can effectively prolong the release of the antibiotic in vivo remains to be tested. It is important to note that the source of collagen material plays an crucial role for the binding capacity of collagen due to differences in amino acid composition [211].

4.2.3. Tissue repair

Future applications in the delivery of growth factors for the tissue engineering field would benefit from the described positive effect of injectable collagen formulations on cellular regeneration and tissue repair. TGF-β and basic fibroblast growth factor formulated into a collagen dispersion have been tested successfully to enhance the breaking-load in intestinal wound repair [212]. Studies on collagen gels positioned between the stumps of a transected spinal cord demonstrated axons emerging from the interface with the spinal tissue and growing into the bioimplants after only one month [213]. Crosslinking or combination with chondroitin-6-sulfate increased the mechanical strength and the biological persistence of the matrix, modified the normal scarring process, and favored axonal regeneration [213] and fibroblast movement [214]. Joosten et al. [215] compared an injectable fluid collagen solution with implantation of a solid collagen gel. After application of a fluid collagen solution into the lesion area corticospinal tract axons can be visualized within the implant [215]. In addition, astroglial and reactive microglial cells invade the collagen matrix. With collagen implanted as an already self-assembled gel, no ingrowth of labeled CST axons nor astroglial/reactive microglial cells was observed. Saltzmann et al. [216–218] investigated the migration, proliferation, differentiation and function of cells in collagen gels to further understand the mechanism and kinetics of transport and motion [216,217] and the influence of growth factors, laminin, and fibronectin [218].

Prolongation of the release could also be achieved by encapsulation of proteins in plurilamellar vesicles sequestered in a collagen gel [219]. A maximum 3–5-day release for insulin with a more gradual change in insulin levels or a 14-day growth hormone release was observed. It was suggested that such a system could also be advantageous for topical, rectal or vaginal application. In addition, collagen interacted with vesicles and decreased lipid peroxidation and permeability of neutral or negatively charged liposomes dispersed in a gel formulation [220,221].

As seen in ophthalmology [188,189], collagen can also be used as an additive in oily suspensions to sustain the release of proteins when colyophilized, milled and dispersed in a lipophilic liquid [222,223].

4.3. Solid parenteral dosage forms

4.3.1. Sponges

Collagen sponges were originally developed as wound dressings and hemostyptics. They are generally prepared by freeze-drying aqueous acid or alkali-swollen collagen preparations of approximately 0.1–5% dry matter content. The porosity of the lyophilized collagen cake can be altered by varying the collagen concentration and the freezing rate [5,6,224,225]. In addition, collagen can be combined with other materials like elastin [226,227], fibronectin [228] or glycoaminoglycans [5,6,122,126,224,225,229–234] and the starting material or preferably the resulting fleece can be crosslinked for example with GTA or by DHT treatment in order to achieve highly resilient materials (see Section 3.4) [5,126,225]. These collagen sponges are invaluable in the treatment of severe burns and have found use as a dressing for many other types of wounds, such as pressure sores, donor sites, leg ulcers and decubitus ulcers [74,126] as well as for in vitro test systems [235]. Major benefits of collagen covers include their ability to easily absorb large quantities of tissue exudate, smooth adherence to the wet wound bed with preservation of this moist micro climate as well as its shielding against mechanical harm and prevention of secondary bacterial infection [7,225]. Besides these physical effects, collagen promotes cellular mobility and growth and inflammatory cells actively penetrate the porous scaffold [74,122,232]. This allows a highly vascularized granulation bed to form and encourages the formation of new granulation tissue and epithelium on the wound. Thus, collagen sponges can be considered active dressings which aid in the healing process. The team of Burke and Yannas [5,6,224,225] designed and constructed an artificial collagen/chondroitin sulfate composite matrix with a well-described porosity between 5 and 140 μm and a cross-linking density which optimized for cellular ingrowth while minimizing scar formation

Growth factor delivery. As a consequence of the scaffolding properties and the positive effects on cellular activity collagen based spongoid matrices play an important role in tissue engineering research and in combinations with growth factors [236–241]. By using recombinant platelet-derived growth factor together with a collagen scaffold, an enhanced infiltration of fibroblasts into sponges was apparent already 3 days after implantation and enhanced capillary formation was noticeable after one week [236]. Coating of a collagen sponge with basic fibroblast growth factor facilitates early dermal and epidermal wound healing as well [239]. Besides dermal wound healing the second major field for application of collagen are scaffolds in conjunction with bone morphogenetic proteins (BMPs). Depending on the application site, BMPs seem to influence both endochondral and intramembranous bone formation pathways by mesenchymal cell recruitment and differentiation [242–244]. In order to promote osseous regeneration, the osteoinductive BMPs are combined with osteoconductive, porous devices [245]. This scaffolding supports vascular ingress, cellular infiltration and attachment, cartilage formation, calcified tissue deposition, and governs and delineates the dimensions of the resulting bony tissue [243]. Favorite materials are based on calcium phosphate, collagen or combinations of both [95,245]. In one of the first studies a lyophilized mixture of human type I collagen and bone extracted BMP provided a suitable and easily manipulated delivery system for surgical implantation [246]. Bone formation by BMP/collagen matrices starts with progenitor cell chemotaxis, mesenchymal cell mitosis, chondrocyte differentiation and angiogenesis [243,245,247,248]. After 7–14

days the systems are surrounded and invaded by alkaline phosphatase-positive cells with new bone and cartilage at the periphery of the implant. Mineral deposits were seen not only on the new formed cartilage but also on the implanted collagen [248]. Subsequently, bone spreads to the center of the implant. Substantial trials showed that rhBMP-7 led to complete regeneration of clavarial defects in baboons as well as healing in large segmental defects of the tibia and ulna of African green monkeys [249–251]. Further promising data resulted in spinal fusion with rhBMP-2 combined with a bovine type I collagen matrix [252,253]. In periodontology, both rhBMP-2 and rhBMP-4 in combination with a collagen matrix induced pulp regeneration and osteodentine formation. [254,255]. Besides collagen type I, systems based on composites of tricalcium phosphate and collagen type IV have been successfully tested in vivo. Collagen type IV was described as advantageous due to more effective binding of BMPs than collagen type I which might enhance the performance [95,256]. Uludag et al. [257] studied the local levels of rhBMP-2 after implanting collagen sponges which were soaked with an aqueous protein formulation prior to implantation in rat ectopic sites. The results indicated that absorbing a solution of rhBMP-2 into a porous collagen device could localize high levels of rhBMP-2 in the implant. Approximately 70% of the applied rhBMP-2 could be recovered after 3 h. There was a gradual loss of implanted protein over at least a 14-day period characterized by two $t_{1/2}$ of approximately 0.1 and 2.5–4.9 days. Whereas binding of rhBMP-2 to collagen sponges in vitro was affected by sponge characteristics such as crosslinking, pH and collagen mass, significant differences in the in vivo kinetics could not be found [258].

Porous collagen scaffolds cannot only be combined with growth factors for tissue repair, but have also been seeded with cells to engineer tissue ex vivo which can be used either as test models or could be implanted for repair. The tissues created are cartilage using chondrocytes with or without fibroblast growth factor [259,260], abdominal wall originating from myoblasts [8], spinal cord developed from Schwann cells [261] and skin based on fibroblasts or keratinocytes [9,235,262–264]. The 3-dimensional matrices are seeded with cells and cultivated. The cells subsequently differentiate resulting in morphological structures which resemble the actual in vivo tissue. The collagen matrix has to direct the cell–cell interactions, serves as a scaffold to conduct tissue regeneration and supports cell transplantation [10].

Antibiotics delivery. Based on the tissue repair and hemostyptic properties of collagen sponges, combinations with antibiotics were developed for local delivery in the treatment and prophylaxis of soft tissue infections. Despite a reduction in the risk of contamination due to improved material, implant, and clean room technique as well as preoperative antibiotic prophylaxis, infections still remain a feared complication in orthopedic and traumatic surgery [265]. In the treatment of existing infections, parenteral therapy with high doses of antibiotics usually comes with the danger of side effects and sometimes even remains ineffective because of deficient blood flow or inadequate tissue penetration of antibiotics, for example, at bony sites. Thus, direct local therapy is indicated to inactivate seats which cannot be removed by the physician. In some cases antibiotics are delivered via bone cements or polymethylmethacrylate beads [266–268]. These chains, however, have to be removed in a second operation which poses additional risks of infection [269]. Here, collagen sponges proved to be advantageous [269–271]. After implantation of collagen–antibiotic combinations, high local levels and low serum levels are observed [270]. This therapy avoids the oto-, neuro- or nephrotoxic side effects of many antibiotics. The collagen material is resorbed after a few days and completed within a few weeks [270]. Comparing the gentamicin pharmacokinetics in the zone of subcutaneous implantation around polymethylmethacrylate beads, Firsov [272] observed a three-phase pattern for collagen sponges: (i) an initial high antibiotic peak which is in accordance with the microbiological demands of high doses for a short time; (ii) after a few hours, stabilization of the gentamicin concentration and achieves a practically constant level, above the minimal inhibitory concentration (MIC) of many pathogens for several days; (iii) finally, the antibiotic level in the tissue diminishes. The local drug levels very much depend on the application site [273]. Antibiotic concentrations that remain above the MIC for a few days can be achieved in areas of reduced blood circulation, for example, after vascular reconstruction [274], whereas at sites of adequate local blood flow the gentamicin levels are only effective for several hours [275,276]. The release of gentamicin can be further sustained when used as a salt with reduced solubility [277,278] or if the collagen sponge containing the antibiotic is additionally coated with resorbable polyester [279] or by incorporating the antibiotic into microparticles loaded into artificial skin [231,280]. As an alternative, the addition of anionic polymers such as alginic acid or pectin could result in aminoglycosid antibiotic derivatives with low solubility and charge interaction which hinders diffusion and reduces the release rates [281]. A study of collagen sponges soaked with solutions of various antibiotics including gentamicin, cefotaxim, fusidic acid, clindamycin, and vancomycin revealed that 99.9% were released in vitro under sink conditions after 2 days [282]. The authors infer that in cases when treatment in infected areas with high blood flow should ensure antibiotic cover for 5–10 days, the collagen systems are not suitable. The fleeces delivered a higher dose faster and with a shorter duration than polymethylmethacrylate beads in vitro. Because it deteriorates rapidly, collagen sponges may be unsatisfactory as an agent of antibiotic delivery in patients who have chronic osteomyelitis; however, they may be useful for patients who have acute trauma with highly contaminated bone or soft tissue, or during hemiarthroplasty revision, to deliver a high local concentration of antibiotic

for a short period of time [269,282]. The osteoconductivity of collagen may offer an additional positive effect in the treatment of bony infections.

Besides antibiotic treatment, collagen sponges have also been tested for example as a cervical cap to deliver all-*trans*-retinoic acid to patients with cervical dysplasia. The device was advantageous as it allowed high concentrations of all-*trans*-retinoic acid with a short elimination half-life to be delivered locally without undue systemic absorption [283,284].

4.3.2. Films

Collagen films of approximately 0.01–0.5 mm thickness are formed by air-drying a casted collagen preparation similar to ophthalmological shields. Their main biomaterial application is as a barrier membrane. For drug delivery implants, Bradley [285] used collagen films crosslinked by chromium tanning, formaldehyde or a combination of both, to sustain the release of medroxyprogesterone acetate. In another study, films made from collagen/polyvinylalcohol mixtures crosslinked with glutaraldehyde vapor have been tested as a depot formulation for recombinant human growth hormone [286]. In these investigations crosslinking was performed on films already loaded with drugs, endangering the chemical integrity of the active substance. This effect can be avoided with collagen materials which are crosslinked with glutaraldehyde prior to protein incorporation. Insoluble, bovine collagen was used in small matrices cut from films with a thickness of approximately 0.1 mm [108]. Release studies in the presence of bacterial collagenase demonstrated a prolonged release of proteins and polysaccharides by both diffusion-controlled and enzymatic degradation mechanisms [43,287].

Collagen films have also been used as a drug carrier for antibiotics [288–290]: after implantation into rabbits, tetracycline could be detected in the plasma for more than 7 days [288,289]. Antimicrobial activity was sustained for up to 10 days following periodontal implantation. The clinical indices of periodontal disease were significantly reduced at 4 and 7 weeks, with a corresponding decrease in the number of bacteria in the periodontal pockets. Tetracycline exhibits not only its antimicrobial activity but furthermore slows down proteolysis by collagenolytic enzymes [291,292]

European Patent Application 92305467.3 [293] covers single and multiple layer collagen films to improve the sustained release delivery of pharmaceuticals, specifically of growth factors. For multilayer preparation the films are attached together by evenly applying pressure. Platelet derived growth factor was released at constant rate for up to 100 h and improved wound healing in vivo.

4.3.3. Monolithic devices

Japanese groups [222,223,294–298] intensively studied collagen minipellets, little rods of approximately 1 mm in diameter and 15 mm length prepared from collagen solutions by molding, drying, and cutting. This cylindrical pellet can be administered via injection using a needle with a plunger. Takeuchi [294] showed that these systems are suitable for local delivery of minocycline and lysozyme to improve clinical symptoms in periodontitis. The system has also proven to be effective in vivo for the delivery of interleukin-2 [295]. Elimination half-life was 360 min for s.c. injected IL-2 minipellets, while it was 8 and 15 min, respectively for i.v and s.c. injections of an aqueous IL-2 preparation. Similar results were obtained for interferon [222,296]. The release of interferon varies with the processing conditions (collagen concentration, air- or ethanol drying, compression or extrusion molding compared with casted films) as explained by differences in the density of the collagen matrices [297].

4.3.4. Microparticles

The use of microparticulate collagen systems could simplify the use by injection compared with implantation and may broaden the fields of application. Despite successful studies on microspheres made from collagen descendent gelatin [299–302], a technology transfer to collagen is hindered by limitations in manufacturing temperature and use of solvents due to collagen denaturation and by the high viscosity of an aqueous collagen phase. Altankov [303] presented a spin-off from gelatin particles by coating the surface with collagen. This was achieved by utilizing the remainder of active groups from crosslinking gelatin spheres with GTA. These residual aldehyde functions were inactivated with collagen and thus collagen attached onto the surface. Precipitation from aqueous preparations by addition of non-solvents results in non-spherical, polydispers collagen fibrils and fibers. Kreuter et al. [304] transferred the classical emulsion techniques and dispersed a collagen dispersion in oils such as cotton seed oil supported by low polyoxyethylated sorbitan ester. The particles had to be stabilized by crosslinking with glutaraldehyde and were surface-loaded by adsorption of ethacridinelactate or chlorophyll. The technique was further improved and spherical particles ranging in size from 3–40 μm were obtained [305,306]. The drugs tested, retinol, tretinoin, tetracain, lidocain and steroids yielded loading rates up to 10% with lipophilic substances showing superior absorption over hydrophilic substances [307,308]. Embedded in a hydrogel matrix of hydroxyethylcellulose, the particles could reduce the tendency of retinol to crystallize and slightly enhanced retinol penetration in an in vitro skin model [306].

Gunkel [28] used a similar technique, emulsifying an aqueous isopropanol precipitated collagen preparation in methylenechloride, crosslinking with GTA and washing the particles. However, the emulsification technique leads to strong reduction in nativity. The particles were loaded by dissolving fluoresceinisothiocyanate-dextran in the aqueous collagen preparation prior to emulsification. The release profile was characterized by a drug burst followed by a second phase of extremely slow liberation over 7 days. In

conclusion it was speculated that enzymatic degradation could play a more important role. However, potential reactions of drugs with aldehyde have to be excluded in order to apply this technique.

As an alternative, spherical collagen particles can be achieved by spray-drying. A major obstacle is the high viscosity of collagen preparations. Therefore, dilute preparations have to be sprayed which leads to thin and fragile hollow spheres [28]. Using standard, elevated temperatures leads to denaturation and degradation of collagen [28]. In order to preserve the triple-helical structures, collagen preparations can be sprayed into liquid nitrogen [309,310]. The particles are subsequently frozen, tempered, lyophilized, crosslinked and sterilized.

4.4. Collagen conjugates

Besides the applications of collagen itself, composites with other biomaterials and succinylated, acetylated, or methylated collagen varieties have also been used. Furthermore, attempts to attach either drugs or polymeric structures to the collagen molecules are described in the literature. These efforts to either create a pro-drug or drastically modify the macromolecular properties of collagen have recently been reviewed [93].

Conjugates with drugs are of interest with respect to immobilization of therapeutic enzymes [93,311] or controlled drug delivery [93,158,312]. Boyce [313] describes the biotinylation of bovine skin collagen. After covalent addition of biotin, horseradish peroxidase as a model substance was bound either with avidin as a bridge or after avidinylation of the protease. The avidin bridge technique can also be used to attach peptide growth factors which retain their activity and could be used to modulate the response in wound treatment [314]. The feasibility of collagen conjugates for small molecule drugs has been investigated for kanamycin and pilocarpine [158,312]. Kanamycin was coupled to collagen using EDC, glutaraldehyde and periodiate-oxidized kanamycin [312], whereas pilocarpine was attached via collagen hydrazide [158]. As a copolymer poly(2-hydroxyethylmethylacrylate) attached to collagen was suggested for the preparation of hydrogels [93,315].

5. Bovine spongiform encephalopathy (BSE)

Cattle are the main source for collagen because of the reasonable costs for the material and the available knowledge about its use. However, one has to be aware of the active discussion on the inherent threat of BSE (bovine spongiform encephalopathy) or TSE (transmisssible spongiform encephalopathy). The risk of contamination has to be evaluated on a case by case basis. The factors which have to be considered for bovine collagen are [316]:

1. the country of origin and herd control; most collagen manufacturers carefully choose certified herds, preferably from North or South America or Oceania, and survey them;
2. the starting material; in most cases tendons are used which are considered to be of low infectivity [317,318]; tendons should not be exposed to high infectivity parts during slaughter;
3. risk reducing procedures during manufacturing; an important step of inactivation is the alkali treatment often performed in the course of collagen processing which is considered highly effective [319];
4. the amount of starting material necessary for the daily dose; in the case of collagen the material is not extracted from large quantities of tissue, but tendons consist almost entirely of collagen; thus small amounts are necessary, in most cases less than 1 g;
5. the number of applications per year; treatment with collagen containing devices is often a one time treatment or limited to a few administrations;
6. the route of administration; usually the most critical in the case of parenteral drug delivery.

Based on these considerations a calculation of the risk of contamination according to regulatory guidelines gives a probability of infection of less than one in a million [318]. However, with increasing knowledge about prions further tests have to be developed to ensure safety. By using starting material spiked with the infectious scrapie agent, inactivation during processing was shown for aprotinin from bovine lungs for example [320].

6. Conclusions and future perspectives

Despite various applications of collagen as a drug vehicle reported in the literature, it should be noted, however, that only a few collagen-based drug delivery products are going into clinical trials or are currently marketed. At the same time the amount of scientific information is not as abundant as for synthetic polymers such as polylactic acid derivatives. The major reasons are:

1. high cost of preparation of pure type I collagen;
2. variability of isolated collagen (e.g. in crosslink density, fiber size, trace impurities, etc.);
3. hydrophilicity which leads to swelling and more rapid release compared with synthetic polymers;
4. variability in enzymatic degradation rate as compared with hydrolytic degradation;
5. complex handling properties; and
6. BSE as a horrifying threat to collagen products based on bovine material.

The most successful uses of collagen for controlled drug release are shields in ophthalmology, injectable dispersions for cancer treatment, sponges carrying antibiotics, and

implantable minipellets loaded with protein drugs. With the exception of the last mentioned, these functions target local drug delivery. The applications profit from some of the major qualities of collagen:

1. good biocompatibility and well characterized low antigenicity;
2. degradation into well tolerated physiological compounds;
3. processing on aqueous base; and
4. enhancement of cellular penetration and wound repair.

These benefits will carry the future developments and uses as indicated by intensified studies to utilize collagen in the growing field of tissue engineering and for delivery of growth factors or cells.

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