

Recombinant collagen and gelatin for drug delivery

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Abstract

The tools of recombinant protein expression are now being used to provide recombinant sources of both collagen and gelatin. The primary focus of this review is to discuss alternatives to bovine collagen for biomedical applications. Several recombinant systems have been developed for production of human sequence collagens. Mammalian and insect cells were initially used, but were thought to be too costly for commercial production. Yeast have been engineered to express high levels of type I homotrimer and heterotrimer and type II and type III collagen. Co-expression of collagen genes and cDNAs encoding the subunits of prolyl hydroxylase has lead to the synthesis of completely hydroxylated, thermostable collagens. Human types I and III collagen homotrimers have been expressed in transgenic tobacco plants, while transgenic mice have been engineered to produce full-length type I procollagen homotrimer as well as a $\alpha 2$ (I) homotrimeric mini-collagen. Most recently, a transgenic silkworm system was used to produce a fusion protein containing a collagenous sequence. Each of these transgenic systems holds great promise for the cost-effective large-scale production of recombinant human collagens. As seen in other recombinant expression systems, transgenic silkworms, tobacco, and mice lack sufficient endogenous prolyl hydroxylase activity to produce fully hydroxylated collagen. In mice and tobacco, this was overcome by over-expression of prolyl hydroxylase, analogous to what has been done in yeast and insect cell culture. In addition to recombinant alternatives to bovine collagen, other sources such as fish and sponge collagen are discussed briefly. Recombinant gelatin has been expressed in *Pichia pastoris* and *Hansenula polymorpha* in both non-hydroxylated and hydroxylated forms. *Pichia* was shown to be a highly productive system for gelatin production. The recombinant gelatins produced in yeast are of defined molecular weight and physio-chemical properties and represent a new biomaterial not previously available from animal sources. Genetic engineering has made great progress in the areas of recombinant collagen and gelatin expression, and there are now several alternatives to bovine material that offer an enhanced safety profile, greater reproducibility and quality, and the ability of these materials to be tailored to enhance product performance.

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1. Introduction

Collagens and gelatins are widely used in drug delivery and pharmaceutical applications, as well as in many medical devices [1–3]. Today, these materials come from animal sources. In excess of 50,000 metric tons of collagen and gelatin are used in medical applications annually. These materials serve a variety of functions as implants, hemostats, device coatings, and stabilizers for biologics. There are increasing concerns with the continued use of animal-derived collagens and gelatins [4]. The concerns have several facets, including issues of biocompatibility, the ability of tissue-derived collagens and gelatins to transmit pathogenic vectors including prions, and finally, product homogeneity and the degree to which collagen and gelatins can be considered “well-characterized” biological materials is questionable [2,5]. These concerns have spurred interest in the development of alternate

materials including the development of new, non-animal sources of collagens and gelatins.

1.1. Characteristics of collagens

The collagen family has been well described (for a review, see Prockop and Kivirikko [6]). This section provides a brief discussion of collagens, focusing on their physical structure and properties. In humans, 25 distinct collagen types have been identified to date on the basis of protein and/or DNA sequence information. A list of collagen types and some tissues in which they are found is provided in Table 1.

Medical applications of collagens utilize preparations containing certain types of collagen in a ratio that reflects the composition of the tissue from which the collagen was isolated. For example, a commercial scale process has been developed for the extraction of collagen from bovine hides [7].

Table 1
Collagen types and tissues

Collagen type	Tissues
Type I	Most connective tissues, i.e., bones, skin, tendon, blood vessels, etc.
Type II	Cartilage and vitreous of the eye
Type III	Blood vessels
Type IV	Basement membranes in all organs
Type V	Tendons, cornea, and interstitial tissues
Type VI	Liver, kidney, and perichondrium
Type VII	Epidermal/dermal junction
Type VIII	Endothelial cells
Type IX	Cartilage
Type X	Hypertrophic and mineralizing cartilage
Type XI	Cartilage
Type XII	Tendons and fibril associated collagen
Type XIII	Epidermis, hair follicles, and nail root cells
Type XIV	Same as Type I
Type XV	Many tissues, homology to Type XVIII
Type XVI	Under study
Type XVII	Hemidesmosomes and skin
Type XVIII	Liver and kidney
Type XIX	Eyes, brain, testes, and embryonic tissues
Type XX–XXV	Unknown

This collagen preparation contains ~95% type I collagen and ~5% type III collagen, reflecting the ratio of collagen types found in skin as well as the inability to completely separate closely related collagen types from each other during the purification process. Thus, a preparation consisting of exclusively type I collagen is not available from non-recombinant sources. Type III collagen is the major collagen of the vasculature and cartilage is enriched in Type II collagen; such tissues represent sources for isolation of these specific collagen types [8]. However, the lack of large amounts of these tissues and the challenges associated with purifying these collagen types at commercial scale makes the availability of these materials very difficult. Purified preparations of type II and III collagen could be very useful for cartilage repair and as hemostats, respectively.

Collagens are subject to extensive post-translational modifications both prior to and after deposition in the extracellular matrix [8]. In particular, the fibrillar collagens are subjected to intra- and inter-molecular cross-linking that continues over the life of the molecule in the extracellular space. Thus, the amount of cross-linking present in collagens is influenced by, among other things, the age and physiology of the

tissue from which the collagen is harvested. These differences influence both the extractability of collagens from tissue and the biophysical characteristics of these collagens. As a result, collagens isolated from tissues exhibit significant lot-to-lot variability and, as bulk materials, are often analytically intractable.

Animal-derived collagens and gelatins can elicit immune responses in humans [7,9], even though collagens are relatively well-conserved across various species. Amino acid sequence homology between human and bovine type I collagen, for example, is 98% and 93% for the $\alpha 1$ and $\alpha 2$ chains, respectively [10–12]. Immune responses to non-human collagens occur at the rate of 3–5%, varying in type and severity, and can include anaphylaxis in some patients [7]. These responses have prompted a requirement for advanced screening for patient sensitivity in some applications and occasionally surgical retrieval of implants in others [13]. Immune responses to gelatins used as vaccine stabilizers have also been reported. In most cases, immunodominant epitopes of collagens have been identified on collagen $\alpha 2$ (I) chains [14,15]. Thus, there is the need for a production system that can deliver any human collagen type, in large quantities, in a pure form in order to make new innovative products.

1.2. Prolyl hydroxylase and collagen synthesis

The key to producing functional collagens in recombinant expression systems is the ability to effect appropriate post-translational processing of the recombinant collagen proteins. One the most important post-translational modifications is the hydroxylation of specific proline residues by the enzyme prolyl 4-hydroxylase (P4H; E.C. 1.14.11.2). Mammalian P4H is a multimeric protein comprising a $\alpha_2\beta_2$ structure [16]. The α subunit contains the catalytic and peptide–substrate binding domains. The β subunit is the multifunctional enzyme protein disulfide isomerase. This enzyme is retained and functions within the endoplasmic reticulum (ER), through a C-terminal ER retention signal, KDEL. During collagen biosynthesis, the procollagen α chains are co-translationally transported into the lumen of the ER where they are hydroxylated by P4H. Approximately 100 proline hydroxylation events occur per collagen α chain, each of these prolines occurring in the Y position of a Gly–

X–Y triplet. For example, in type I human collagen, the ratio of hydroxyproline residues to total proline residues plus hydroxyproline residues is approximately 0.46 [17]. In the absence of proline hydroxylation, the essential triple helical conformation of collagen is thermally unstable at well below physiological temperatures [18]. The production of thermally stable recombinant collagen for use in humans requires a system that enables high-level collagen expression along with sufficient P4H activity.

2. Recombinant collagens

Recombinant collagens have been produced by transfected mammalian cells [20–25], insect cells [26–30], yeast [31–36], *Escherichia coli* [37], transgenic tobacco [38–41], mice [42,43], and silkworms [44]. Of the various cell types evaluated, only mammalian cells transfected with a collagen gene and not P4H genes expressed hydroxylated full-length collagens [26–30]. The level of expression achieved in mammalian cells ranged from 0.6 to 20 mg/l, and up to 40 mg/l was achieved in insect cells. However, this level of expression is too low to make these systems viable for commercial manufacturing. Furthermore, the production cost of a cell culture process can be 5–10-fold higher than the fermentation process used for other cell types, such as yeast or *E. coli* [45]. In all other systems, a common problem encountered was the inability of the host cells to provide sufficient P4H activity to fully hydroxylate the expressed collagen. A multigene expression technology has been developed to co-express collagen and P4H and is described in US patent 5,593,859 [19]. The use of *Pichia pastoris* as a host for expression of recombinant collagen using the multigene expression technology has been described by Vuorela et al. [34] and others [35,36].

2.1. Expression in yeast

Any collagen type can be expressed using the multigene expression technology. For example, recombinant collagen types I–IV, VII, IX, XIII, XV, and XVIII have been expressed in *P. pastoris* with P4H [34–36] (unpublished data). *P. pastoris* strains for commercial production of recombinant human type I, II, and III collagens have been selected based

on the ability of this system to express high levels of fully hydroxylated recombinant human collagens. The initial report describing type III collagen expression in *P. pastoris* reported expression levels of 15 mg/l [34]. Expression levels were improved using a variety of approaches including genetic intervention, fermentation optimization, and process development. Today, yields in excess of 1 g/l have been achieved for certain collagen types (Table 2).

Procollagens expressed in *P. pastoris* are not secreted into the extracellular media but accumulate intracellularly [34–36,46]. This phenomenon is not unique to *P. pastoris* as it occurs when recombinant collagen is expressed in *Saccharomyces cerevisiae* and insect cells [26–33]. The procollagen is extracted from the cells and converted to collagen by treatment with a protease. In mammalian cells, specific extracellular metalloproteinases catalyze the removal of the N- and C-propeptides from procollagens [47]. This processing may be catalyzed by nonspecific proteases such as pepsin; this is routinely done during the isolation of collagen from tissues [48]. Pepsin treatment of yeast cell lysates has been used to process recombinant procollagen to its mature form. Currently, there is no readily available source of purified human pepsin, only animal-derived preparations. It is desirable to have a recombinant collagen production process that does not use any animal-derived materials. We cloned and expressed recombinant human pepsin for the purpose of commercial manufacturing of recombinant human collagens from *P. pastoris*.

Fermentation is conducted in a fed-batch mode using stirred tanks sparged with air supplemented with oxygen. *P. pastoris* can achieve very high cell densities (>500 g/l wet cell weight) when grown in a chemically defined, minimal media with a methanol feed [49]. Expression of recombinant procollagen and P4H is under the control of the alcohol oxidase promoter; thus, feeding methanol induces production while sustaining biomass accumulation. After maximization of cell density and procollagen accumula-

Table 2
Recombinant collagen expression levels in *P. pastoris*

Collagen type	Expression level (g/l)
Type I collagen	1.1
Type II collagen	0.7
Type III collagen	1.5

tion, the fermentor is harvested. The cells are washed free of media salts and lysed by physical disruption. The recombinant procollagen is converted to collagen by treatment with recombinant human pepsin.

Soluble recombinant collagen is separated from cellular debris by sedimentation or filtration. Additional purification steps remove soluble *P. pastoris* components and any collagen and procollagen fragments. The final bulk product is a solution of highly purified, sterile filtered recombinant collagen in 0.01 M HCl.

2.1.1. Characterization of recombinant collagen from *P. pastoris*

Co-expression of various procollagen cDNAs together with prolyl 4-hydroxylase leads to the synthesis of triple helical hydroxylated recombinant collagen. Fig. 1 shows SDS–PAGE analysis of recombinant human type I, II, and III collagens produced in *P. pastoris* using multigene expression technology.

The majority of the recombinant collagen molecules expressed in *Pichia* exist as monomers, about 5–10% of the material is covalently cross-linked dimers, depending on the collagen type (Fig. 2). A minor amount of trimeric material can be seen in the type III collagen preparation by SDS–PAGE (Fig. 1). These data also highlight one of the striking differences between the recombinant collagen and tissue-derived material. Native collagens derived from tissue contain significantly higher proportions of dimers, trimers and other higher molecular oligomers. For example, bovine type I collagen isolated from skin (Vitrogen; Cohesion Technologies, Palo Alto, CA) was analyzed in parallel with *Pichia*-derived recombinant material, approximately 54% of the α chains were present as covalently cross-linked dimers, trimers, and higher-order molecular aggregates. Thus, the collagen produced by *P. pastoris* provides a much more homogeneous preparation of collagen than material obtained from tissues.

The hydroxyproline content of the *Pichia*-produced recombinant collagens, expressed as the ratio of hydroxyproline to proline plus hydroxyproline, is 0.47 and 0.56 for types I and III collagens, respectively. These values are similar to the hydroxyproline content of the corresponding tissue-derived collagens, indicating complete hydroxylation of the α chains by the co-expressed P4H [17,50]. The helical content and

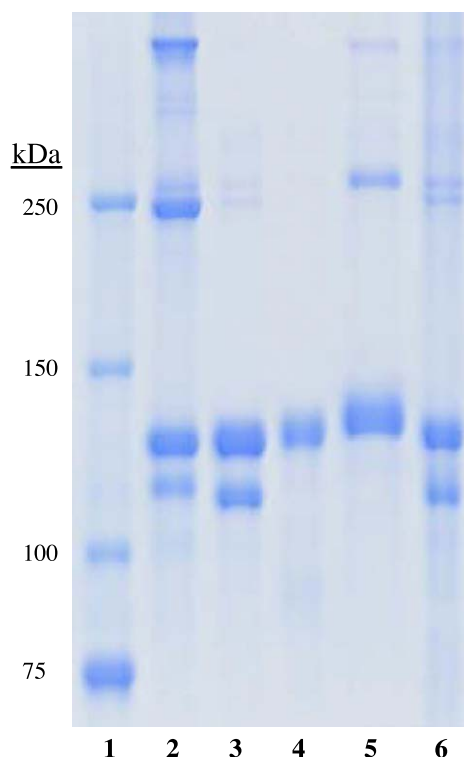
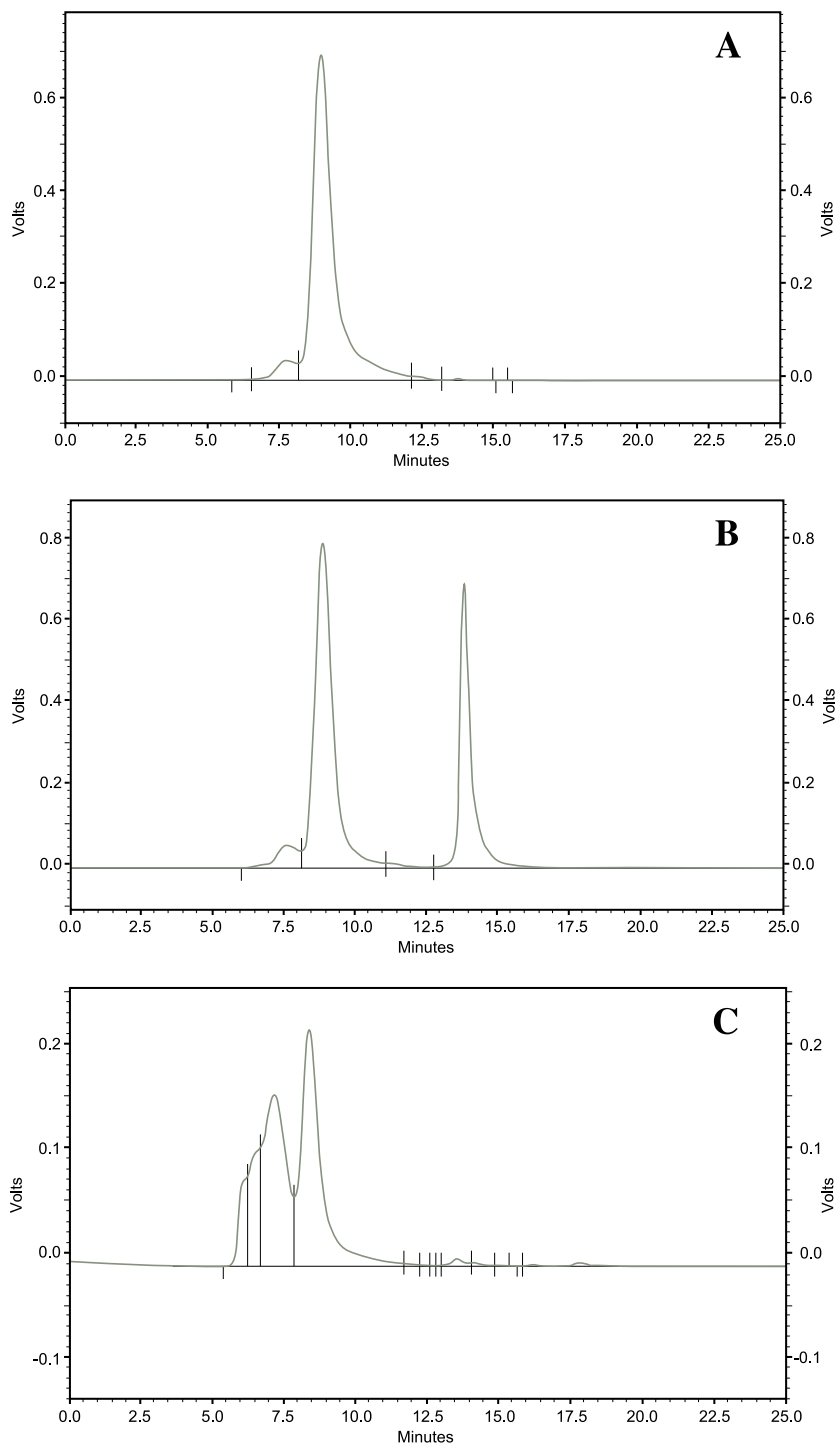


Fig. 1. Analysis of recombinant human collagens expressed in *P. pastoris*. Recombinant human types I, II, and III collagens (3 μ g each in lanes 3, 4, and 5, respectively) were analyzed by SDS–PAGE on a 6% Tris–glycine gel. Proteins were visualized by staining with Gelcode blue. Type III collagen was reduced with β ME prior to electrophoresis. Lanes 2 and 6 contain 3 μ g of bovine skin type I collagen and human placental type I collagen, respectively.

melting temperature (T_m) of these collagens was measured by circular dichroism (Fig. 3). The CD spectra for the *Pichia*-derived recombinant types I and III collagens were characteristic of tissue-derived triple helical collagens showing a negative ellipticity at 197 nm and a positive ellipticity at 221 nm [17]. The T_m determined for the *Pichia*-derived types I and III collagens and bovine type I collagen was 40.5, 40.3, and 42.6 $^{\circ}$ C, respectively. Additional analytical data regarding recombinant type I collagen is shown in Table 3.

Purified collagens are capable of undergoing spontaneous alignment to form fibrils that have defined features characteristic of collagen fibers formed in vivo [51,52]. The recombinant collagen expressed in yeast formed these fibrillar structures at neutral pH in



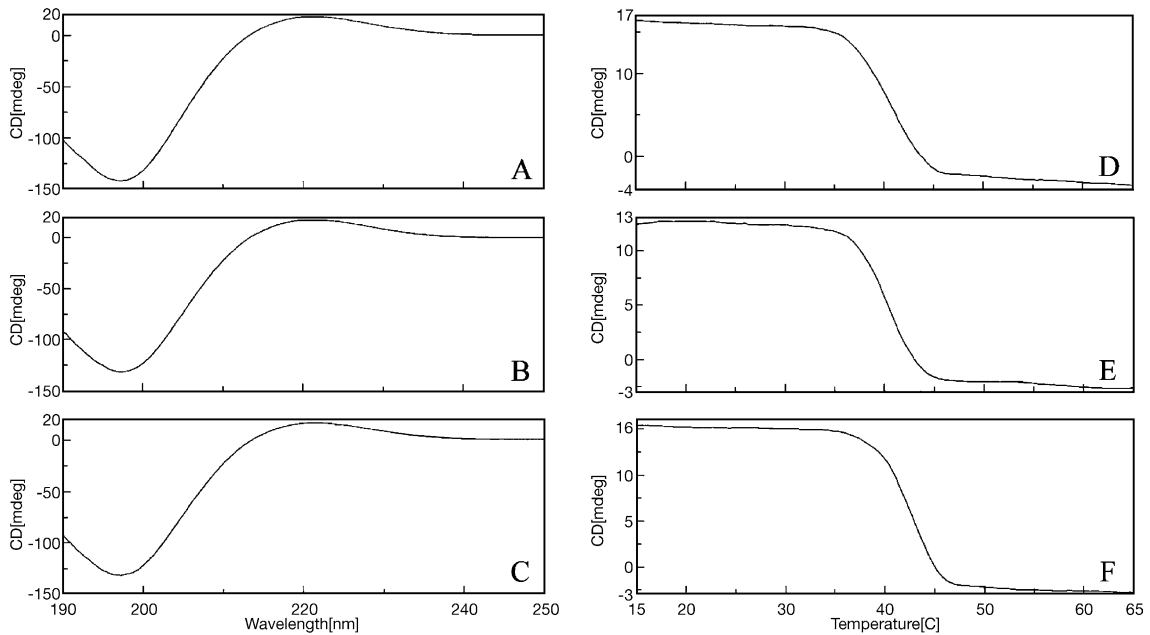


Fig. 3. Analysis of triple helical structure and melting temperature by circular dichroism spectropolarimetry. Purified samples of recombinant type I collagen (A, D), recombinant type III collagen (B, E), and bovine type I collagen (C, F) were diluted to 200 $\mu\text{g}/\text{ml}$ with 200 mM sodium phosphate, pH 7.0. Aliquots of 200 μl were analyzed in 1-mm quartz cuvettes using a Jasco Model J-715 CD spectropolarimeter with a Peltier controlled sample holder (Easton, MD). Samples were scanned over the wavelength range 190–250 nm, plotting molar ellipticity as a function of wavelength (left side). Five scans were averaged for each sample. Melting temperature (T_m) analysis was performed by scanning from 15 to 65 $^{\circ}\text{C}$ at a scan rate of 1 $^{\circ}\text{C}/\text{min}$ and monitoring at 221 nm. The T_m was taken from the midpoints of the sigmoidal curves as determined using Jasco Spectra Manager™ software (right side).

phosphate buffer. Evaluation by transmission electron microscopy (TEM) shows that the fibrils closely resemble those formed with tissue-derived collagen. These fibrils were made from recombinant type I collagen and show the characteristic banded pattern (Fig. 4).

2.2. Transgenic systems for expression of recombinant collagen

Three transgenic systems, tobacco, mice, and silkworms, have been investigated as potential large-scale cost-effective methods to manufacture

recombinant collagen. During the last 10 years, the use of genetically modified animals and plants for the production of recombinant proteins has become a reality [41–43,53–58]. Plants are capable of producing and correctly assembling complex mammalian proteins such as secretory antibodies containing five subunits [59] and viral particles [60]. Using proven techniques of agricultural biotechnology, recombinant proteins can be produced efficiently from relatively small acreages or from a few animals combining conventional agronomic practices with traditional pharmaceutical manufacturing [61,62].

Fig. 2. Size exclusion chromatography analysis of collagen. Approximately 50 μg of recombinant human type I collagen (Panel A), recombinant human type III collagen (Panel B), and bovine type I collagen (Panel C) were fractionated on a Bio-Sil SEC 400-5 column (300 $\times$ 7.8 mm; BioRad, Hercules, CA) in 2 M guanidine–HCl at a flow rate of 1 ml/min and absorbance was monitored at 220 nm. Peaks areas were integrated to determine the percent monomer (retention time 8.8 min) and oligomer (retention time 7.8 min or greater). The peak in Panel B eluting at 13.8 min corresponds to the included volume and contains the βME used to reduce the disulfide bonds in type III collagen.

Table 3
Specifications for recombinant human type I collagen

Analytical assays	Specifications
Appearance (visual)/solubility	Clear, colorless solution with no visible particles
pH	2.0–2.4
SDS–PAGE with reference standard	Matches rh collagen reference standard
Western blot	Matches rh collagen reference standard
Protein concentration	2.5–5.5 g/l
Fibrillogenesis	≤ 30% unincorporated
Hydroxyproline	9–10%
Purity	≥ 95% total collagen, no single impurity ≥ 0.5%
Host cell protein	≤ 2.0 ppm (L.O.D)
Host cell DNA	≤ 10 pg/mg
Bioburden	≤ 1 cfu/ml
Endotoxin by LAL	≤ 0.1 EU/mg collagen
Heavy metals	≤ 10 ppm total

2.2.1. Expression in tobacco

Plants are known to synthesize hydroxyproline-containing proteins [63]. However, the prolyl hydroxylase that is responsible for synthesis of hydroxyproline in plant cells does not exhibit the same substrate sequence specificity as mammalian P4H [64,65]. Thus, production of collagen containing hydroxyproline only in the Y position of Gly–X–Y triplets requires the co-expression of collagen and P4H genes. Expression of recombinant collagen types I and III homotrimers in plants has been reported by two groups [38–41]. Tobacco cells were shown to express hydroxylated type III collagen following transformation with a full-length cDNA under the control of the constitutive promoter from the cauliflower mosaic virus 35S gene [38]. This promoter was also used to drive expression of transfected human P4H α and β subunit cDNAs. Nuclear transformation via a gene gun was carried out, and the cell lines expressing recombinant collagen and P4H were selected by Northern and Western blotting (Fig. 5). The selected tobacco cells, which expressed recombinant human type III collagen and human P4H, were grown in shake flasks. Amino acid analysis of the purified recombinant human type III collagen indicated that significant levels of hydroxyproline were present (Fig. 5), confirming the expression of active P4H in the selected cell line. This recombinant tobacco-derived type III collagen preparation contained 8.1% hydroxy-

proline, or about 75% of the levels found in collagen purified from tissue [17].

Transgenic tobacco plants transformed with a human pro $\alpha 1(I)$ or pC $\alpha 1(I)$ cDNA expressed and assembled full-length triple helical collagen molecules [39]. This collagen was underhydroxylated but was found to be processed from procollagen to mature collagen by naturally occurring plant proteases, thus eliminating the need for pepsin to proteolytically remove the N- and C-propeptides. This system for producing underhydroxylated collagen was used to study folding of the triple helix as well as the physical properties of the recombinant collagen [40]. In the absence of hydroxyproline, correct folding of the helix occurred, but at a markedly slower rate. The non-hydroxylated collagen molecules were found to be more flexible, had a melting temperature of 30 °C, and different conditions were required for the formation of striated collagen fibrils. This material was also used to demonstrate that efficient binding of type I collagen to $\alpha 1\beta 1$ and platelet GPVI receptors requires prolyl hydroxylation while binding to $\alpha 2\beta 1$ does not [66]. Transgenic tobacco plants produced hydroxylated type I collagen homotrimers by co-expression of cDNAs encoding pC $\alpha 1(I)$ with a *Caenorhabditis elegans*/mouse hybrid P4H [41]. The collagen expressed and purified from tobacco plants co-expressing this hybrid enzyme contained 8.4% hydroxyproline compared to the 10% hydroxyproline content of *P. pastoris* expressed collagen and native human collagen [17,34,35,41].



Fig. 4. Transmission electron micrograph of recombinant human type I collagen fibril. Purified recombinant human type I collagen in 10 mM HCl was dialyzed against 20 mM Na_2HPO_4 pH 7.2 at room temperature for ~18 h. Aliquots of the reconstituted fibril slurry were transferred to carbon-coated copper grids. The fibrils were positively stained with 2% uranyl acetate and examined by transmission electron microscopy (original magnification was 36,000).

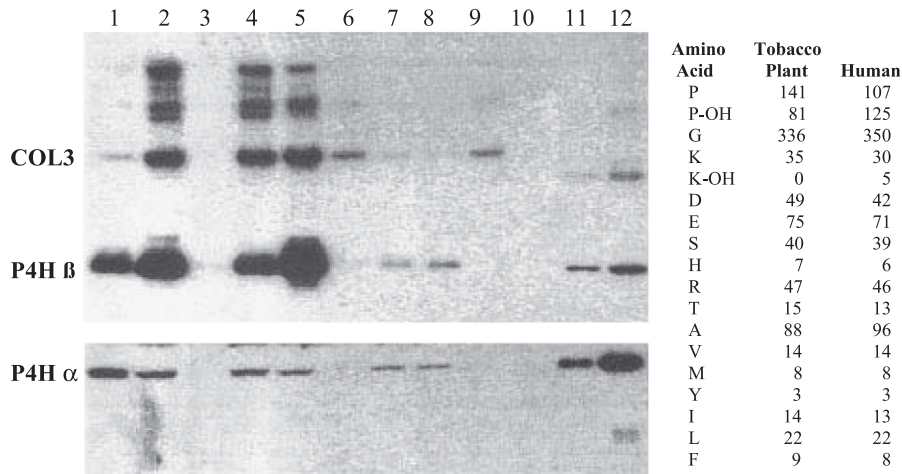


Fig. 5. Co-expression of type III collagen and prolyl 4-hydroxylase subunits in tobacco cells. The expression of recombinant type III collagen and the α and β subunits of prolyl 4-hydroxylase were demonstrated by Western blot analysis. Extracts from individual calli were run on 4–20% Tris–glycine gels, transferred to PVDF membrane, and blotted with rabbit anti-human type III procollagen (Chemicon, AB764), P4H β subunit and P4H α subunit antibodies (left panel). Recombinant type III collagen purified from cell extracts was subjected to amino acids analysis (right panel). The results are expressed as number of residues per 1000 amino acids. P–OH—hydroxyproline; K–OH—hydroxylysine.

2.2.2. Expression in mice

The feasibility of using the lactating mammary gland of transgenic mice to produce full-length type I procollagen homotrimers using a 37-kb genomic fragment and the α S1-casein mammary gland specific promoter has been demonstrated [43]. High levels (8 mg/ml) of soluble procollagen were produced in the milk and converted to homotrimeric type I collagen (α 1)₃ by pepsin treatment. However, when amino acid analysis was performed on the purified recombinant collagen from milk, less than 50% of the expected level of hydroxyproline was detected. The under-hydroxylated collagen had a melting temperature of 30–31 °C. The authors speculated that the levels of P4H in the mammary epithelium may be rate limiting for the expression of recombinant collagen and hypothesized that introduction of a second transgene, encoding P4H α subunit, could overcome this limitation. Generation of transgenic cattle co-expressing both collagen and P4H genes could in theory provide very large volumes of milk as a source of recombinant collagen. While this would be a source of recombinant human collagen, it would still have bovine origins and thus would still carry the concerns associated with animal-derived materials.

The second transgenic mouse report describes an engineered truncated collagen molecule lacking 852 amino acids of the helical domain and consisting of α 2 (I) chains exclusively [42]. Triple helical collagen molecules consisting of only α 2 (I) chains are not found in tissues and could not be produced recombinantly in transfected insect cells [27]. Surprisingly, this engineered collagen formed a triple helix in transgenic mice. This mini-collagen was co-expressed with prolyl hydroxylase in order to achieve complete hydroxylation. The engineered collagen gene expressed alone produced underhydroxylated collagen, similar to what was reported by Toman et al. [43].

2.2.3. Expression in silkworms

A new system that utilizes transfected silk worms was recently described for expression of recombinant collagen [44]. A fusion cDNA encoding *Bombyx mori* fibroin L-chain and a fragment of the human type III collagen helical domain was expressed under the control of the fibroin light chain promoter. Analysis of the extracted cocoon proteins by Western blot and collagenase digestion confirmed expression of the fusion protein. However, pepsin digestion of the extract indicated triple helical collagen molecules

were not assembled in the silk glands. The authors speculated this was due to low levels of endogenous P4H activity in gland cells. Theoretically, this problem could be solved by co-expression of collagen and P4H subunits, similar to what has been done in *P. pastoris*, insect cells, and transgenic mice.

The potential of this system for high-level economical expression is tremendous in that silk glands synthesize large amounts of protein. According to the authors, a single cocoon produces approximately 70 mg of total protein. The fusion protein containing the collagenous sequence was found to represent 3.6% of the total extracted protein. The authors calculated that a 300 m² factory containing 1.5 million silkworms could produce about 5 kg of recombinant protein.

The finding that over-expression of P4H is required for the production of fully hydroxylated collagen in such varied systems further highlights the importance of the multigene technology for making thermostable recombinant collagen.

2.3. Expression in *E. coli*

A novel approach to the production of hydroxylated collagen not involving P4H co-expression was reported [37]. This study demonstrated the co-translational incorporation of *trans*-4-hydroxyproline into a fragment of collagen, full-length $\alpha 1$ (I) and $\alpha 2$ (I) chains and other non-collagenous recombinant proteins, during over-expression in *E. coli*. This was achieved by supplementing the cultures with hydroxyproline while growing the cells in hyperosmotic media. Hydroxyproline was incorporated at all proline codons in a 192-amino-acid collagen fragment. It is also of significance to note that efficient synthesis of collagen could only be achieved when a synthetic collagen gene, optimized for *E. coli* codon usage, was expressed. This recombinant collagen is biochemically distinct from tissue-derived collagen in that hydroxyproline was present at both X- and Y-positions of the Gly-X-Y triplets. However, it was intriguing that the over-hydroxylated collagen fragment, consisting of 64 Gly-X-Y repeats, assembled into a triple helix, based on circular dichroism analysis. The authors further demonstrated that they could affect the level of hydroxyproline incorporation by supplementing the cultures with a mixture of hydroxyproline and proline. The

remaining challenge would be to affect the specificity of hydroxyproline incorporation and the authors speculated this may be possible using genetic approaches.

3. Formulations of collagen for drug delivery

Collagen molecules in physiological solutions will spontaneously self-assemble into higher-order structures [51,52]. The fibril-forming collagens form highly ordered thread-like aggregates solely on the basis of their biophysical properties. In these fibrils, the axis of the triple helix in the collagen monomer is parallel to the axis of the fibrils. In tissues, the size and organization of the fibrils varies according to the function of the tissue, associated proteins, degree of cross-linking, and other factors [67,68].

As described above, recombinant collagens self-assemble into ordered biological structures or fibrils (Fig. 4). Thus, any desired physical and structural forms (e.g., porous matrices, films, gels, or monofilaments) that can be fabricated from tissue-derived collagens can also be produced using recombinant collagens. Drugs may be incorporated into or added after the construction of the final forms. Among the advantages of using recombinant collagens in these applications is the opportunity to begin with uniform homogeneous monomolecular collagen. The subsequent processes may, therefore, be standardized for consistent formulation output.

3.1. Collagen sponges

Most commercially available collagen sponges are made of insoluble collagen derived from a variety of animal species, such as cows, horses, and pigs. Animal tissues are treated enzymatically and chemically to remove the majority of non-collagenous proteins. The insoluble collagen is then resuspended, in many cases, in a dilute organic acid, and lyophilized into sponges. To increase the mechanical integrity and handling properties of the material, the sponges are cross-linked with glutaraldehyde, formaldehyde vapor, diisocyanate, or thermal dehydration [69]. The resulting collagen sponges are porous and absorbable. These absorbable collagen sponges were developed primarily for use as hemostats.

SEM of RhCI Sponge and Instat

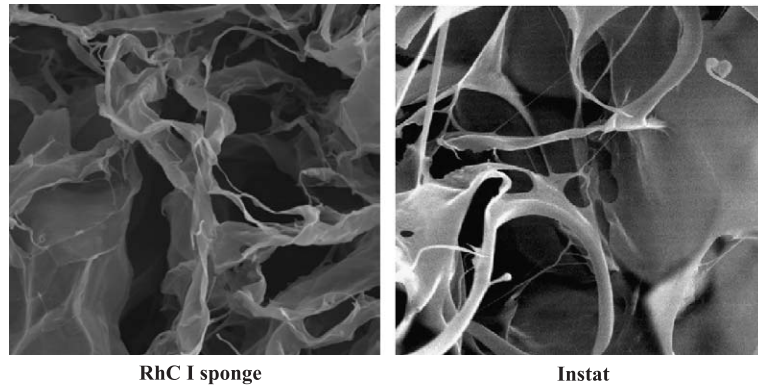


Fig. 6. Scanning electron micrograph (SEM) of recombinant human collagen type I (RhC I) sponge and bovine collagen I sponge (Instat, Ethicon, Johnson & Johnson).

Recombinant human collagen was formulated into a sponge using an in-mold fibrillogenesis/cross-linking process. This process, coupled with the homogeneity and unique structural characteristics of recombinant human collagens, results in a homogeneous 3D structure in a sponge format (Fig. 6). Analysis of recombinant human type I collagen sponges by scanning electron microscopy revealed porous microstructures interconnected by thin sheets of collagen fibrils. In contrast, a commercially avail-

able collagen sponge (Instat) consists of thick sheets and fibers. The sponges made from recombinant type I collagen induce a lower inflammatory response than animal-derived material in a subcutaneous implantation model (Fig. 7). In addition to the homogeneous nature and lower inflammatory responses seen, recombinant human collagen sponges made of specific types of collagen can be formulated for specialized applications. For example, sponges containing recombinant human collagen type II may be a natural choice as a carrier for chondrogenic growth factors for cartilage repair. Recombinant human collagen type III might be more suitable for wound management applications, due to the superior hemostatic properties of type III collagen [70].

Tissue Response to SQ Implanted Collagen Sponges

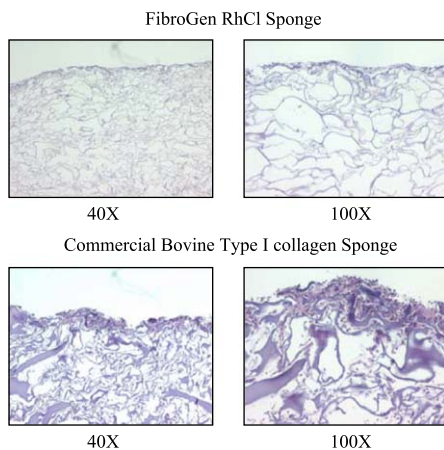


Fig. 7. Tissue response to subcutaneous-implanted (SQ) recombinant human collagen type I sponge and commercial bovine collagen I sponge (Instat). Sponges (1 × 1 cm) were implanted into Wistar rats subcutaneously. The implants were explanted 3 days after implantation and examined histologically by H&E staining.



Fig. 8. Photograph of type I collagen membrane made with recombinant collagen from *P. pastoris*.

3.2. Collagen membranes

Collagen membranes have been used for dural closures, wound dressings, reinforcement and support of weak tissues, guided tissue regeneration, and tissue engineering applications [71]. As is the case with sponges, all products currently on the market are formulated with animal-derived collagen. We have demonstrated the feasibility of fabricating a collagen membrane with recombinant type I collagen from *P. pastoris* (Fig. 8). Such devices can be used in applications such as tissue engineering and guided tissue regeneration following dental surgery.

3.3. Collagen stents and vascular graft coatings

Expandable intra-arterial stents are widely used for treating coronary artery diseases [72,73]. Biopolymer-coated stents may provide supplementary functions such as local drug delivery, gene transfer, reduction of operative blood loss, and facilitation of endothelial cell in-growth, in addition to the mechanical dilation [74–76]. As shown in Fig. 6, recombinant collagens form fibrils that interweave with each other to form a network structure. This superstructure may account for the mechanical strength of collagen fibril-based coatings. In a series of experiments, recombinant human type III collagen fibrils were used to coat knitted DACRON® vascular grafts. The grafts were analyzed by TEM, and the results show the coated surface served as a suitable substrate for human

umbilical vein endothelial cell (HUVEC) attachment and spreading (Fig. 9).

4. Non-recombinant alternatives to bovine collagen

In general, non-recombinant alternatives to bovine-derived collagens and gelatins can be divided into two major categories. First, collagen-rich tissues from animals other than cattle can serve as a source of collagens and gelatins; and, secondly, human tissue sources. Porcine and equine sources of collagens and gelatins have also been used for medical applications [1,3], but use of these materials is associated with concerns similar to those surrounding the use of bovine material. Fish and sponges represent other alternative sources of collagen and gelatin.

4.1. Fish collagen

Fish-derived collagen and gelatin hydrolysates are used widely in foods, in the manufacture of glues, and in several industrial applications [77]. Like recombinant and vertebrate collagens, fish collagens form the typical fibrillar structures but differ at the primary amino acid sequence level. The total imino acid content of fish collagen and gelatin is significantly lower; the amount of hydroxyproline is only 62% of that found in calf skin collagen [78]. As a result, fish collagen has a significantly lower melting temperature; this decreased thermal stability could affect

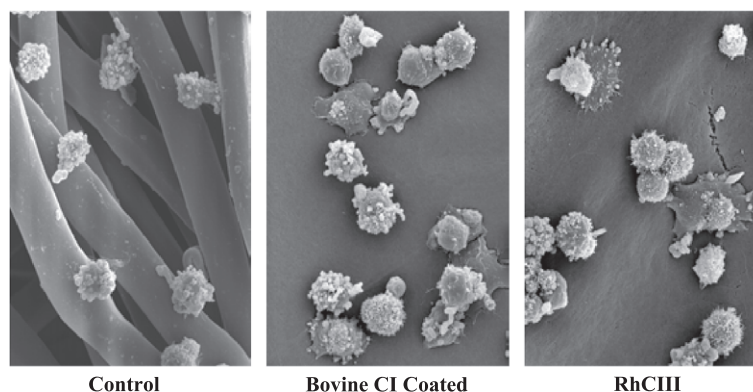


Fig. 9. HUVEC attachment on DACRON® vascular graft coated with bovine type I collagen, recombinant human type III collagen (RhCII) or no coating (control).

performance at body temperature. Another potential drawback for the use of fish collagen and gelatin in pharmaceutical preparations could be the occurrence of allergic reactions. Fish is one of the most common allergenic foods [79]. IgE antibodies to fish gelatin/type I collagen have been found in the sera of children with fish allergies [80].

4.2. *Sponge collagen*

Sponges represent another alternative source of collagen and gelatin. Sponges contain both fibrillar and non-fibrillar collagens, as is the case in vertebrates [81,82]. Collagen from sponges is biochemically distinct from vertebrate collagens in that it is highly insoluble and therefore difficult to manipulate [83]. Additionally, sponge collagen is highly glycosylated; most of the lysine residues are found in the consensus sequence for hydroxylation and are subsequently glycosylated [84]. An efficient process for extraction of collagen from the marine sponge *Chondrosia reniformis* Nardo has been reported [85]. Collagen yields of 30% (dry weight collagen to dry weight sponge) were obtained using a dilute basic extraction medium. The collagen was shown to have potential uses in cosmetic formulations, causing a slight increase in skin hydration as measured by sebumetry.

4.3. *Non-recombinant human collagen*

Efforts to provide non-recombinant human collagen for medical use have been reported as well. In one report, human cadaver tissue (i.e., specifically banked human skin) was used as a source of collagen and prepared for intradermal injection [86]. Concerns around safety and availability could be an issue with this preparation. In a second report, mammalian tissue culture was used to produce a source of human collagen intended for much the same purpose [87].

5. Characteristics of gelatins

Gelatin is denatured collagen and is typically isolated from bovine or porcine skin or bone by acid or base extraction [88]. Regardless of the process used, gelatin preparations consist of a distribution of

polypeptide fragments of different sizes, different isoelectric points (pI), and different gelling properties, and often exhibit lot-to-lot variability [88–90]. Furthermore the physiochemical properties of gelatin vary depending on method of extraction, amount of thermal denaturation employed [91], and electrolyte content of the resulting material [88–90]. The variable nature of gelatin preparations, therefore, presents a significant challenge to those who use these protein mixtures in the manufacture of other products. Gelatin hydrolysates represent a specialized preparation for use in situations where gel formation is undesirable. Gelatin hydrolysates are commonly used as stabilizers in liquid vaccine formulations [9]. This class of gelatin requires additional processing steps such as thermal hydrolysis or treatment with a protease [92].

The introduction of recombinant gelatins eliminates many of the variables and drawbacks associated with tissue-derived material. This technology allows the production of gelatins with defined molecular weights, pI, guaranteed lot-to-lot reproducibility, and the ability to tailor the molecule to match a specific application. There is clearly a need for reproducible, high quality preparations of gelatin since approximately 50,000 metric tons of gelatins are produced annually for medical use [93]. Most of this volume is consumed in some aspect of oral drug delivery. Additionally, thousands of metric tons are used annually for parenteral formulations and devices.

5.1. *Recombinant gelatins*

Recombinant gelatin, irrespective of the expression system, may be produced using two general approaches. In one approach, recombinant collagen is expressed, purified, and denatured (with or without chain fragmentation) to yield recombinant gelatin. Depending on the type of collagen and the extent of α chain fragmentation, a heterogeneous mixture of peptides may be generated. Such gelatins differ from animal tissue-derived gelatin in that the starting material is completely soluble and homogeneous. The downstream processes may therefore be standardized and the resultant product reproducible time after time. If chain fragmentation is not performed, the resulting preparation is a homogeneous recombinant gelatin consisting of α chains of specific and consistent length.

A second approach to recombinant gelatin production is to intentionally direct the recombinant system to generate a specified fragment of any selected collagen α chain. Werten et al. [94] discusses the

utility of *P. pastoris* as an expression system for recombinant gelatin. Fragments of rat type III and mouse type I collagen, ranging in size from 21 to 74 kDa, were expressed and secreted into the extracellu-

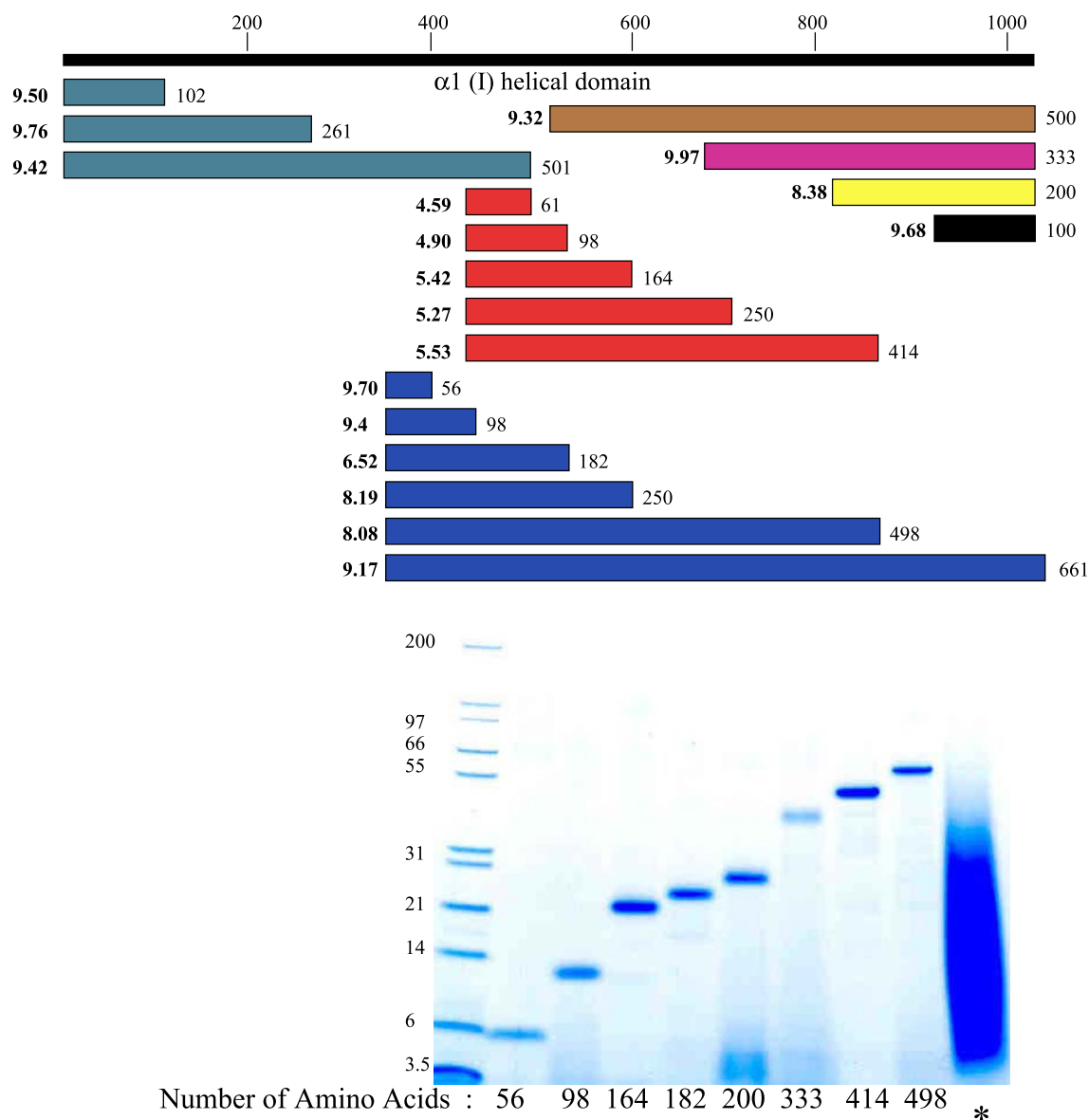


Fig. 10. Schematic representation of recombinant human gelatin fragments expressed in *P. pastoris* (Panel A) and analysis by SDS–PAGE (Panel B). Panel A: Bars representing different recombinant human gelatin fragments indicate the relative location of the fragment within the helical domain of the human $\alpha 1$ (I) chain, similarly colored bar share the same N-terminal sequence. The numbers to left of each bar represent the calculated isoelectric point, based on the amino acid sequence. The numbers to the right of each bar indicate the number of amino acids in the fragment. Panel B: Conditioned media from *Pichia* strains expressing various fragments were analyzed by SDS–PAGE on a 10–20% Tricine gel and stained with Gelcode blue. *Gelatin made by thermal hydrolysis from recombinant type I collagen expressed in *P. pastoris*.

lar media by transfected *P. pastoris* strains. A strain containing 15 copies of rat type III collagen cDNA expressed gelatin at concentrations as high as 14.8 g/l of clarified fermentation broth. *P. pastoris* does not contain an endogenous active prolyl hydroxylase [34]. The *Pichia* strains used to express these recombinant gelatins were not engineered to co-express P4H, and, thus, the gelatin was not hydroxylated. These expression levels are some of the highest reported for the expression of a recombinant protein in yeast. Expression of a non-hydroxylated procollagen fragment has also been reported in *H. polymorpha*, although expression levels were not nearly as high [95].

Expression of hydroxylated recombinant gelatin, without co-expression of P4H, was reported using *H. polymorpha*, [96]. In this study, a recombinant 28 kDa fragment of the mouse $\alpha 1$ (I) collagen was expressed and was found to be non-hydroxylated when fermentation was performed using a minimal salt media and methanol. However, if peptone was used to supplement the fermentation media, the recombinant gelatin was hydroxylated in the Y position of Gly–X–Y triplets, the expected site of hydroxylation for prolyl hydroxylase [8]. This represents the first report of an endogenous yeast prolyl hydroxylase activity. When the same fragment was expressed in *P. pastoris* in the presence of peptone, no hydroxylation was seen. This finding suggests that the P4H activity observed was specific to *H. polymorpha*.

The utility of *P. pastoris* for production of homogeneous, well-defined, recombinant human gelatin fragments possessing specific characteristics such as size and isoelectric point has been investigated further. A series of recombinant gelatin fragments has been developed by cloning and expressing defined segments of the human $\alpha 1$ (I) procollagen gene. Fragments of the human procollagen gene were fused in-frame to the yeast α factor prepro sequence to direct these fragments to the yeast secretory pathway [97]. Expression and secretion of a number of recombinant gelatin fragments, ranging in size from 56 to 1014 amino acids, was observed (Fig. 10). By expressing fragments of similar size, from different parts of the $\alpha 1$ (I) chain, gelatins with different isoelectric points and therefore divergent chemical properties have been produced. Additionally, recombinant gelatin fragments encoding sequences from human $\alpha 1$ (II) and $\alpha 1$ (III) chains have been expressed (data not shown).

The ability to express several different α chain sequences from different species demonstrates the *Pichia* system can be used to make any type of gelatin, of any desired size, from any species.

Most of our efforts have focused on the expression of a low molecular weight gelatin fragment of ~ 8.5 kDa (Fig. 10, Panel A, fragment shown in dark blue with pI of 9.4), to be used as a stabilizer for various biologics. A purification process involving a cell separation procedure following fermentation, precipitation, solvent extraction, filtration, and ion exchange chromatography steps has been established. Furthermore, a series of assays to assess product purity and to detect host and process contaminants have been developed. Table 4 lists the assays and specifications for this 8.5 kDa recombinant human gelatin.

P. pastoris has also been utilized to demonstrate the suitability of this system for the production of a custom-designed gelatin fragment. A gene was designed to produce a synthetic, hydrophilic gelatin with a pI similar to the gelatin obtained from tissue following lime extraction. Successful high-level expression and secretion was achieved, demonstrating the ability of *Pichia* to produce designed gelatins [98]. These results are in contrast to a previous study in which the synthesis of designed collagen-analog peptides was attempted in *E. coli* with only limited success [99]. Expression levels of the synthetic gene were low due to degradation and gene instability issues. More recently fragments of the bovine $\alpha 2$ (I) gene, ranging in size from 93 to 245 amino acids,

Table 4
Specifications for 8.5 kDa recombinant human gelatin

Analytical assays	Specifications
Appearance (visual)/solubility	White solid, no visible particles
SDS–PAGE with reference standard	Matches rh gelatin reference standard
Protein content	80.0–105.0%
Purity–SDS gels/densitometry	$\geq 95\%$ total gelatin, no single impurity $\geq 0.5\%$
Host cell protein	≤ 0.05 ppm (L.O.D)
Host cell DNA	≤ 5 pg/mg gelatin
Bioburden	20 cfu
Endotoxin by LAL	≤ 0.1 EU/mg gelatin
N-terminal sequence	Matches rh gelatin reference standard
Heavy metals	≤ 50 ppm total
Moisture	$\leq 20.0\%$

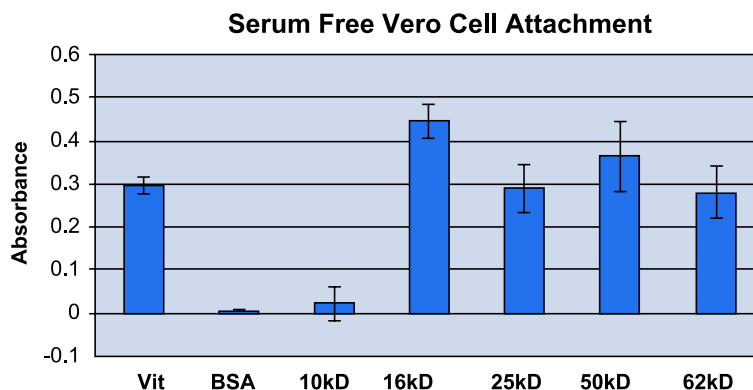


Fig. 11. Attachment of cultured Vero cells in vitro. Costar Maxi-adsorb 96-well plates were coated with 10 μ g of protein in carbonate buffer at pH 10. Cells were added at 4×10^4 per well and allowed to attach for 2 h at 37 °C. Plates were washed and incubated with WST-1 reagent (Roche), and cell numbers were estimated by measuring optical density at 450 nm. Vit—bovine type I collagen (Vitrogen; Cohesion); BSA—bovine serum albumin used as a negative control; 10, 16, 25, 50, 62 kDa—corresponds to the size of the gelatin fragment used to coat the wells.

were expressed in *E. coli* using a T7 promoter plasmid [100]. Each of the fragments was expressed as a fusion protein containing a 6 histidine tag, a portion of the phage T7 gene 10 leader, and the Xpress Epitope tag (Invitrogen). No information was provided on expression levels but enough material was produced for purification and testing for reactivity with anti-gelatin antibodies. Interestingly, the purpose of the study was to identify the major epitope on bovine gelatin used as a stabilizer in a combined

measles–mumps–rubella vaccine that has caused allergic reactions in children following immunization [9,15].

5.2. Production of gelatins in plants

The high volume demand for gelatin in the thousands of metric tons offers an excellent oppor-

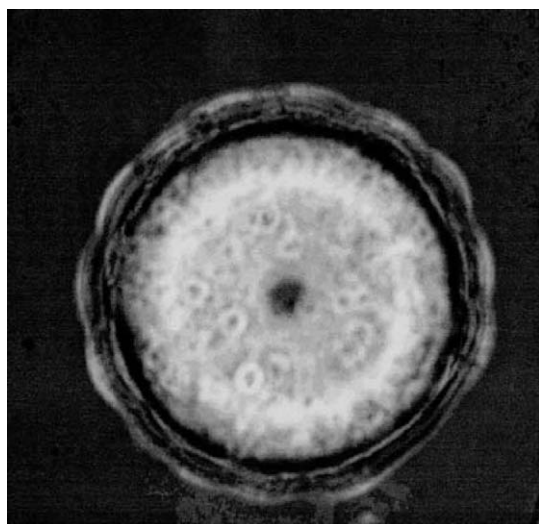


Fig. 12. Photomicrograph of bead coated with 50 kDa recombinant human gelatin and populated with Vero cells.

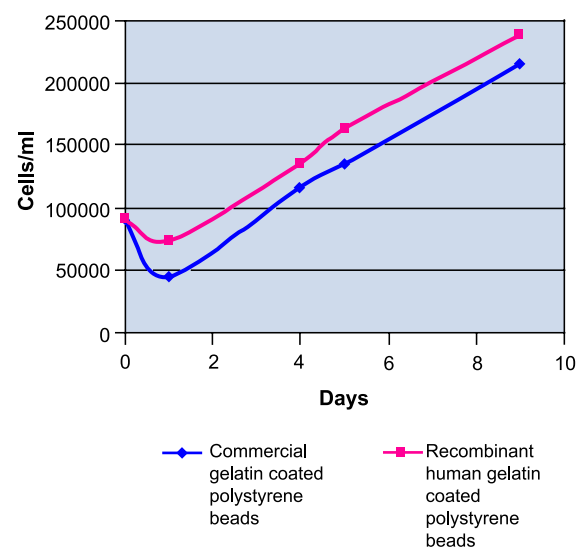


Fig. 13. Comparison of Vero cell growth on polystyrene beads (125–212 μ m diameter) coated with animal-derived gelatin and 50 kDa recombinant human gelatin fragment. Spinner flasks were inoculated with $\sim 1 \times 10^5$ Vero cells and growth was monitored by counting cells over the course of 9 days.

tunity to consider the use of transgenic systems for manufacturing, rather than use of conventional bioreactor-based microbial systems, as discussed above. Transgenic systems could be the preferred production technology for recombinant gelatins considering the high volume and low cost required for production of capsules and nutraceuticals. In addition to production cost savings, use of transgenic plants or animals should result in significant reductions in capital requirements for manufacturing. Additionally, these systems provide virtually unlimited protein capacity required for a high volume product [62].

5.3. Gelatins as substrates for cell attachment

Extracellular matrices are known to play a key role in cell attachment, proliferation, and differentiation. Collagens provide an ideal substratum for attachment of a variety of different cell types *in vitro* [101–103]. The ability of recombinant gelatin fragments, expressed in *Pichia*, to support *in vitro* attachment of Vero cells was studied. Vero cells were chosen for analysis because these cells are often grown on gelatin-coated microcarriers to prepare high-titer viral stocks during the manufacturing of vaccines [104]. Recombinant gelatin fragments (shown in Fig. 10) were able to support attachment of Vero cells at levels similar to those provided by commercial bovine type I collagen (Fig. 11).

5.4. Gelatins as coatings for microcarriers

Microcarrier beads used for propagation of Vero cells in bioreactors are coated with bovine gelatin to enhance cell attachment [104]. To determine if recombinant gelatin could replace the bovine material a series of experiments was performed using the 50 kDa gelatin fragment (Fig. 10, Panel A, fragment shown in dark blue with a *pI* of 8.08), which was shown to support cell attachment (Fig. 11). Polystyrene beads were coated with recombinant gelatin and mixed with Vero cells in a spinner flask and allowed to incubate for several days. Microscopic analysis revealed the beads were populated by cells (Fig. 12) and were capable of supporting proliferation (Fig. 13).

We further demonstrated the utility of beads coated with the 50 kDa gelatin fragment by demonstrating

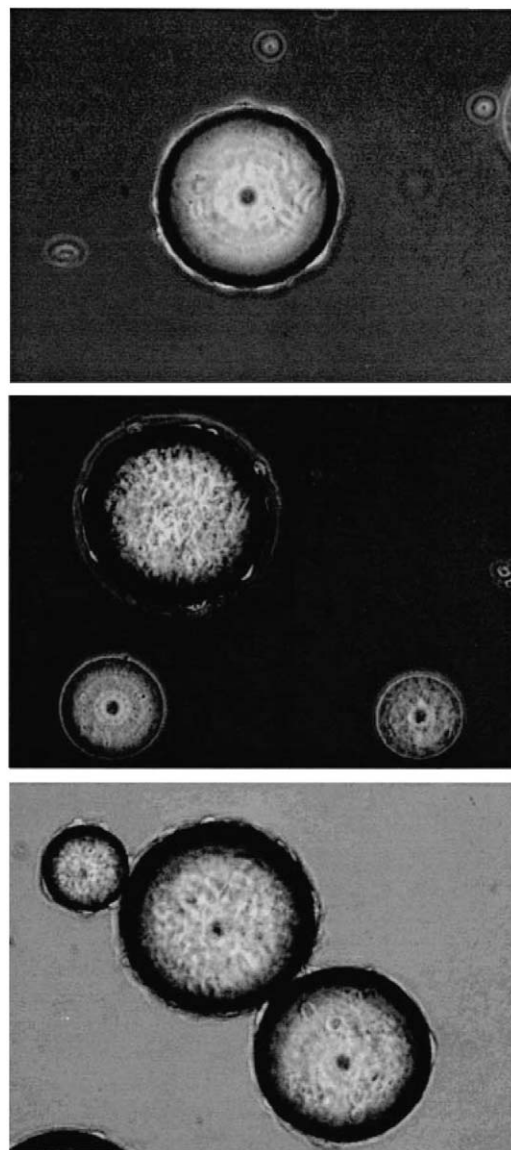


Fig. 14. Demonstration of “cell jumping” using beads coated with recombinant human gelatin. Vero cells were added to a spinner culture containing serum-free media and 50 kDa recombinant human gelatin-coated polystyrene beads with a diameter of 125–210 μm . The culture was grown to confluency over the course of several days (upper panel). The spinner culture was further supplemented with gelatin-coated beads with a diameter of 75–90 μm (middle panel). After several more days of growth, the smaller-diameter beads became populated with cells (lower panel), demonstrating the ability of cells to be passaged from one bead to another without the use of trypsin.

passage of Vero cells from one bead to another without the use of trypsin, an enzyme typically employed in passaging cells (Fig. 14). The culture containing attached Vero cells was mixed with 50 kDa gelatin-coated beads of smaller diameter (75–90 μm) and incubated for 48 h. Microscopic evaluation of the culture demonstrated cells on the surface of both the original beads (125–210 μm diameter beads) and the new beads of smaller diameter.

These studies demonstrate the effectiveness of recombinant human gelatin as a microcarrier coating, as well as a method of passaging cells in a bioreactor without using an animal-derived protease, such as trypsin.

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References

- [1] W. Friess, Collagen-biomaterial for drug delivery, *Eur. J. Pharmacol.* 54 (1998) 54113–54136.
- [2] L. Lachman, H.A. Liverman, J.L. Kaing (Eds.), *The Theory and Practice of Industrial Pharmacy*, Lea & Febiger, Philadelphia, 1986.
- [3] C.H. Lee, A. Singla, Y. Lee, Biomedical applications of collagen, *Int. J. Pharm.* 221 (2001) 1–22.
- [4] D.M. Asher, The transmissible spongiform encephalopathy agents: concerns and responses of the United States regulatory agencies in maintaining the safety of biologics, *Dev. Biol. Stand.* 100 (1999) 103–118.
- [5] J.W. Wilesmith, J.B.M. Ryan, M.J. Atkinson, Bovine spongiform encephalopathy: epidemiological studies on the origin, *Vet. Rec.* 128 (1991) 199–203.
- [6] D.J. Prockop, K.I. Kivirikko, Collagens: molecular biology, diseases, and potentials for therapy, *Annu. Rev. Biochem.* 64 (1995) 403–434.
- [7] L. Cooperman, D. Michaeli, The immunogenicity of injectable collagen: I. A 1-year prospective study, *J. Am. Acad. Dermatol.* 10 (1984) 638–646.
- [8] K.I. Kivirikko, Collagen biosynthesis: a mini-review cluster, *Matrix Biol.* 16 (1998) 355–356.
- [9] M. Sakaguchi, S. Inouye, Systemic allergic reactions to gelatin included in vaccines as a stabilizer, *Jpn. J. Infect. Dis.* 53 (2000) 189–195.
- [10] M.P. Bernard, M.L. Chu, J.C. Meyers, F. Ramirez, E.F. Eikenberry, D.J. Prockop, Nucleotide sequences of complementary deoxyribonucleic acids for the pro α 1 chain of human type I procollagen. Statistical evaluation of structures that are conserved during evolution, *Biochemistry* 22 (1983) 213–223.
- [11] T. Shirai, S. Hattori, M. Sakaguchi, S. Inouye, A. Kimura, T. Ebihara, S. Irie, Y. Nagai, H. Hori, The complete cDNA coding sequence for the bovine pro α 2(I) chain of type I procollagen, *Matrix Biol.* 17 (1998) 85–88.
- [12] W. de Wet, M. Bernard, V. Benson-Chanda, M.L. Chu, L. Dickson, D. Weil, F. Ramirez, Organization of the human pro- α 2(I) collagen gene, *J. Biol. Chem.* 262 (1987) 32–36.
- [13] Zyderm Collagen Implant, Package Insert, Collagen Aesthetics (International), Palo Alto, CA, 2002.
- [14] H. Lindsley, M. Mannik, P. Bornstein, The distribution of antigenic determinants in rat skin collagen, *J. Exp. Med.* 133 (1971) 1309–1324.
- [15] M. Sakaguchi, H. Hori, S. Hattori, S. Irie, A. Imai, M. Yanagida, H. Miyazawa, M. Toda, S. Inouye, IgE reactivity to α 1 and α 2 chains of bovine type I collagen in children with bovine gelatin allergy, *J. Allergy Clin. Immunol.* 104 (1999) 695–699.
- [16] J. Myllyharju, K.I. Kivirikko, Identification of a novel proline rich peptide-binding domain in prolyl 4-hydroxylase, *EMBO J.* 18 (1999) 306–312.
- [17] E.J. Miller, S. Gay, The collagens: an overview and update, *Methods Enzymol.* 82 (1982) 3–32.
- [18] R.A. Berg, D.J. Prockop, Thermal transition of a non-hydroxylated form of collagen. Evidence for a role for hydroxyproline in stabilizing the triple-helix of collagen, *Biochem. Biophys. Res. Commun.* 52 (1973) 115–120.
- [19] D.J. Prockop, L. Ala-Kokko, A. Fertala, A. Sieron, K.I. Kivirikko, A. Geddis, T. Pihlajaniemi, inventors; Thomas Jefferson University and FibroGen, assignees. Synthesis of human procollagens and collagens in recombinant DNA systems. US Patent 5 593 859. January 14, 1997.
- [20] A.E. Geddis, D.J. Prockop, Expression of human COL1A1 gene in stably transfected HT1080 cells, *Matrix* 13 (1993) 399–405.
- [21] A. Fertala, A.L. Sieron, A. Ganguly, S.W. Li, L. Ala-Kokko, K.R. Anumula, D.J. Prockop, Synthesis of recombinant human procollagen II in a stably transfected tumor cell line (HT1080), *Biochem. J.* 298 (1994) 31–37.
- [22] A. Fichard, E. Tillet, F. Delacoux, R. Garrone, F. Ruggiero, Human recombinant α 1(V) collagen chain-homotrimeric assembly and subsequent processing, *J. Biol. Chem.* 272 (1997) 30083–30087.
- [23] S. Frischholz, F. Beier, I. Girkontaite, K. Wagner, E. Poschl, J. Turnay, U. Mayer, K. von der Mark, Characterization of human type X procollagen and its NC-1 domain expressed as recombinant proteins in HEK293 cells, *J. Biol. Chem.* 273 (1998) 4547–4555.
- [24] M. Chen, F.K. Costa, C.R. Lindvay, Y.-P. Han, D.T. Woodley, The recombinant expression of full-length type VII collagen and characterization of molecular mechanisms underlying dystrophic epidermolysis bullosa, *J. Biol. Chem.* 277 (2002) 2118–2124.
- [25] E. Tillet, H. Wiedemann, R. Golbik, T.-C. Pan, R.-Z. Zhang,

- K. Mann, M.-L. Chu, R. Timpl, Recombinant expression and structural and binding properties of $\alpha 1(VI)$ and $\alpha 2(VI)$ chains of human collagen type VI, *Eur. J. Biochem.* 221 (1994) 177–185.
- [26] A. Lamberg, T. Helaakoski, J. Myllyharju, S. Peltonen, H. Notbohm, T. Pihlajaniemi, K.I. Kivirikko, Characterization of human type III collagen expressed in a baculovirus system. Production of a protein with a stable triple helix requires coexpression with the two types of recombinant prolyl 4-hydroxylase subunit, *J. Biol. Chem.* 271 (1996) 11988–11995.
- [27] J. Myllyharju, A. Lamberg, H. Notbohm, P.P. Fietzek, T. Pihlajaniemi, K.I. Kivirikko, Expression of wild-type and modified proalpha chains of human type I procollagen in insect cells leads to the formation of stable $[\alpha 1(I)]_2\alpha 2(I)$ collagen heterotrimers and $[\alpha 1(I)]_3$ homotrimers but not $[\alpha 2(I)]_3$ homotrimers, *J. Biol. Chem.* 272 (1997) 21824–21830.
- [28] M. Tomita, T. Kitajima, K. Yoshizato, Formation of recombinant human procollagen I heterotrimers in a baculovirus expression system, *J. Biochem. (Tokyo)* 121 (1997) 1061–1069.
- [29] M. Tomita, N. Ohkura, M. Ito, T. Kato, P.M. Royce, T. Kitajima, Biosynthesis of recombinant human pro-alpha 1(III) chains in a baculovirus expression system: production of disulphide-bonded and non-disulphide-bonded species containing full-length triple helices, *Biochem. J.* 312 (1995) 847–853.
- [30] M. Nokelainen, T. Helaakoski, J. Myllyharju, H. Notbohm, T. Pihlajaniemi, P.P. Fietzek, K.I. Kivirikko, Expression and characterization of recombinant human type II collagens with low and high contents of hydroxylysine and its glycosylated forms, *Matrix Biol.* 16 (1998) 329–338.
- [31] P.D. Toman, G. Chisholm, H. McMullin, L.M. Giere, D.R. Olsen, R.J. Kovach, S.D. Leigh, B.E. Fong, R. Chang, G.A. Daniels, R.A. Berg, R.A. Hitzeman, Production of recombinant human type I procollagen trimers using a four-gene expression system in the yeast *Saccharomyces cerevisiae*, *J. Biol. Chem.* 275 (2000) 23303–23309.
- [32] D.R. Olsen, S.D. Leigh, R. Chang, H. McMullin, W. Ong, E. Tai, G. Chisholm, D.E. Birk, R.A. Berg, R.A. Hitzeman, P.D. Toman, Production of human type I collagen in yeast reveals unexpected new insights into the molecular assembly of collagen trimers, *J. Biol. Chem.* 276 (2001) 24038–24043.
- [33] P.R. Vaughn, M. Galanis, K.M. Richards, T.A. Tebb, J.A. Ramshaw, J.A. Werkmeister, Production of recombinant hydroxylated human type III collagen fragment in *Saccharomyces cerevisiae*, *DNA Cell Biol.* 17 (1998) 511–518.
- [34] A. Vuorela, J. Myllyharju, R. Nissi, T. Pihlajaniemi, K.I. Kivirikko, Assembly of human prolyl 4-hydroxylase and type III collagen in the yeast *Pichia pastoris*: formation of a stable enzyme tetramer requires coexpression with collagen and assembly of a stable collagen requires coexpression with prolyl 4-hydroxylase, *EMBO J.* 16 (1997) 6702–6712.
- [35] M. Nokelainen, H. Tu, A. Vuorela, H. Notbohm, K.I. Kivirikko, J. Myllyharju, High-level production of human type I collagen in the yeast *Pichia pastoris*, *Yeast* 18 (2001) 797–806.
- [36] J. Myllyharju, M. Nokelainen, A. Vuorela, K.I. Kivirikko, Expression of recombinant human type I-III collagens in the yeast *Pichia pastoris*, *Biochem. Soc. Trans.* 28 (2000) 3532–3557.
- [37] D.D. Buechter, D.N. Paoletta, B.S. Lelie, M.S. Brown, K.A. Mehos, E.A. Gruskin, Co-translational incorporation of tran-4-hydroxyproline into recombinant proteins in bacteria, *J. Biol. Chem.* 278 (2003) 645–650.
- [38] C. Yang, M. Bodo, R. Chang, M. Perala-Heape, E. Hamalainen, D. Russell, J. Polarek, Development of recombinant gelatin by expression of hydroxylated collagen in yeast and plants, *AAPS Annual Meeting* (1999) Abstract Number: 3722.
- [39] F. Ruggiero, J.Y. Exposito, P. Boumat, V. Gruber, S. Perret, J. Comte, B. Olagnier, R. Garrone, M. Theisen, Triple helix assembly and processing of human collagen produced in transgenic tobacco plants, *FEBS Lett.* 469 (2000) 132–136.
- [40] S. Perret, C. Merle, S. Bernocco, P. Berland, R. Garrone, D.J.S. Hulmes, M. Theisen, F. Ruggiero, Unhydroxylated triple helical collagen I produced in transgenic plants provides new clues on the role of hydroxyproline in collagen folding and fibril formation, *J. Biol. Chem.* 276 (2001) 43693–43698.
- [41] C. Merle, S. Perret, T. Lacour, V. Jonval, S. Hudaverdian, R. Garrone, F. Ruggiero, M. Theisen, Hydroxylated human homotrimeric collagen I in *Agrobacterium tumefaciens*-mediated transient expression and in transgenic tobacco plants, *FEBS Lett.* 515 (2002) 114–118.
- [42] D.C. John, R. Watson, A.J. Kind, A.R. Scott, K.E. Kadler, N.J. Bulleid, Expression of an engineered form of recombinant procollagen in mouse milk, *Nat. Biotechnol.* 17 (1999) 385–389.
- [43] P.D. Toman, F. Pieper, N. Sakai, C. Karatzas, E. Platenburg, I. de Wit, C. Samuel, A. Dekker, G.A. Daniels, R.A. Berg, G.J. Platenburg, Production of recombinant human type I procollagen homotrimer in the mammary gland of transgenic mice, *Transgenic Res.* 8 (1999) 415–427.
- [44] M. Tomita, H. Munetsuna, T. Sato, T. Adachi, R. Hino, M. Hayashi, K. Shimizu, N. Nakamura, T. Tamura, K. Yoshizato, Transgenic silkworms produce recombinant human type III procollagen in cocoons, *Nat. Biotechnol.* 21 (2003) 52–56.
- [45] R.V. Datar, T. Cartwright, C.-G. Rosen, Process economics of animal cell and bacterial fermentations: a case study analysis of tissue plasminogen activator, *Bio/technology* 11 (1993) 349–357.
- [46] I. Keizer-Gunnink, A. Vuorela, J. Myllyharju, T. Pihlajaniemi, K.I. Kivirikko, M. Veenhuis, Accumulation of properly folded human type III procollagen molecules in specific intracellular membranous compartments in the yeast *Pichia pastoris*, *Matrix Biol.* 19 (2000) 29–36.
- [47] D.J. Prockop, A.L. Sieron, S.W. Li, Procollagen N-proteinase and procollagen C-proteinase. Two unusual metalloproteinases that are essential for collagen processing probably

- have two important roles in development and cell signaling, *Matrix Biol.* 16 (1998) 399–408.
- [48] E.J. Miller, R.K. Rhodes, Preparation and characterization of the different types of collagen, *Methods Enzymol.* 82 (1982) 33–64.
- [49] G.H. Wegner, Emerging applications of the methylotrophic yeasts, *FEMS Microbiol. Rev.* 7 (1990) 279–283.
- [50] E. Chung, E.J. Miller, Collagen polymorphism: characterization of molecules with the chain composition $[\alpha 1(\text{III})_3]$ in human tissues, *Science* 183 (1974) 1200–1201.
- [51] B.R. Williams, R.A. Gelman, D.C. Poppke, K.A. Piez, Collagen fibril formation. Optimal in vitro conditions and preliminary kinetic results, *J. Biol. Chem.* 253 (1978) 6578–6585.
- [52] R.A. Gelman, D.C. Poppke, K.A. Piez, Collagen fibril formation in vitro. The role of the nonhelical terminal regions, *J. Biol. Chem.* 254 (1979) 11741–11745.
- [53] H.S. Mason, H. Warzecha, T. Mor, C.J. Arntzen, Edible plant vaccines: applications for prophylactic and therapeutic molecular medicine, *Trends Mol. Med.* 8 (2002) 324–329.
- [54] Y. Yamamoto, N. Rajbhandari, X. Lin, B. Bergmann, Y. Nishimura, A. Stomp, Genetic transformation of duckweed, *In Vitro Cell Dev. Biol.* 37 (2001) 349–353.
- [55] C.L. Cramer, J.G. Boothe, K.K. Oishi, Transgenic plants for therapeutic proteins: linking upstream and downstream strategies, *Curr. Top. Microbiol. Immunol.* 240 (1999) 95–118.
- [56] M.B. Hein, Y. Tang, D.A. McLeod, K.D. Janda, A. Hiatt, Evaluation of immunoglobulins from plant cells, *Biotechnol. Prog.* 7 (1991) 455–461.
- [57] M. Young, Production of biopharmaceutical proteins in milk, *Biopharm* 10 (1998) 34–38.
- [58] L.M. Houdebine, Transgenic animal bioreactors, *Transgenic Res.* 9 (2000) 305–320.
- [59] J.K. Ma, A. Hiatt, M. Hein, N.D. Vine, F. Wang, P. Stabila, C. von Dolleweerd, K. Mostov, T. Lehner, Generation and assembly of secretory antibodies in plants, *Science* 268 (1995) 716–719.
- [60] L.J. Richter, Y. Thanavala, C.J. Arntzen, H.S. Mason, Production of hepatitis B surface antigen in transgenic plants for oral immunization, *Nat. Biotechnol.* 18 (2000) 1167–1171.
- [61] L. Miele, Plants as bioreactors for biopharmaceuticals: regulatory considerations, *Trends Biotechnol.* 15 (1997) 45–50.
- [62] D. Mison, J. Curling, The industrial production costs of recombinant therapeutic proteins expressed in transgenic corn, *Biopharm* 13 (2000) 48–54.
- [63] D.T.A. Lampert, The isolation and partial characterization of hydroxyproline-rich glycopeptides obtained by enzymic degradation of primary cell walls, *Biochemist* 8 (1969) 1155–1163.
- [64] M. Tanaka, K. Sato, T. Uchida, Plant prolyl hydroxylase recognizes poly(L-proline) II helix, *J. Biol. Chem.* 256 (1981) 11397–11400.
- [65] R. Hieta, J. Myllyharju, Cloning and characterization of a low molecular weight prolyl 4-hydroxylase from *Arabidopsis thaliana*. Effective hydroxylation of proline-rich, collagen-like, and hypoxia-inducible transcription factor α -like peptides, *J. Biol. Chem.* 277 (2002) 23965–23971.
- [66] S. Perret, J.A. Eble, P.R. Siljander, C. Merle, R.W. Farndate, M. Theisen, F. Ruggiero, Prolyl hydroxylation of collagen type I is required for efficient binding to integrin $\alpha 1 \beta 1$ and platelet GPVI but not to $\alpha 2 \beta 1$, *J. Biol. Chem.* 278 (2003) 29873–29879.
- [67] D.E. Birk, F.H. Silver, R.L. Trelstad, Matrix assembly, in: E.D. Hay (Ed.), *Cell Biology of the Extracellular Matrix*, Plenum, New York, 1991, pp. 221–253.
- [68] D.F. Holmes, C.J. Gilpin, C. Baldock, U. Ziese, A.J. Koster, K.E. Kadler, Corneal collagen fibril structure in three dimensions: structural insights into fibril assembly, mechanical properties, and tissue organization, *Proc. Natl. Acad. Sci. U. S. A.* 98 (2001) 7307–7312.
- [69] M.E. Nimmi, D. Cheung, B. Strates, M. Kodama, K. Skeikh, Chemically modified collagen: a biomaterial for tissue replacement, *J. Biomed. Mater. Res.* 21 (1987) 741–771.
- [70] L. Balleisen, S. Gay, R. Marx, K. Kuhn, Comparative investigation on the influence of human and bovine collagen types I, II, and III on aggregation of human platelets, *Klin. Wochenschr.* 53 (1975) 903–905.
- [71] M. Maeda, K. Kadota, M. Kajihara, A. Sano, K. Fujioka, Sustained release of human growth hormone (hGH) from collagen film and evaluation of effect on wound healing in db/db mice, *J. Control. Release* 77 (2001) 261–272.
- [72] W. Serruys, P. de Jaegere, F. Kiemeneji, C. Macaya, W. Rutsch, G. Heyndrickx, H. Emanuelsson, J. Marco, V. Legrand, P. Materne, A comparison of balloon expandable stent implantation with balloon angioplasty in patients with coronary artery disease. Benestent Study Group, *N. Engl. J. Med.* 331 (1994) 489–495.
- [73] L. Maillard, M. Hamon, K. Khalife, P.G. Steg, F. Beygui, J.L. Guernonprez, C.M. Spaulding, J.M. Boulenc, J. Lipiecki, A. Lafont, P. Brunel, G. Grollier, R. Koning, P. Coste, X. Favereau, B. Lancelin, E. Van Belle, P. Serruys, J.P. Monassier, P. Raynaud, A comparison of systemic stenting and conventional balloon angioplasty during primary percutaneous transluminal coronary angioplasty for acute myocardial infarction. STENTIM-2, *J. Am. Coll. Cardiol.* 34 (1999) 1498–1506.
- [74] Y. Nakayama, J.Y. Kim, N. Shogo, H. Ueno, M. Takehisa, Development of high-performance stent: gelatinous photo-gel-coated stent that permits drug delivery and gene transfer, *J. Biomed. Mater. Res.* 57 (2001) 559–566.
- [75] J. Yuan, R. Gao, R. Shi, L. Song, J. Tang, Y. Li, Intravascular local gene transfer mediated by protein-coated metallic stent, *Chin. Med. J. (Engl.)* 114 (2001) 1043–1045.
- [76] M.J. Wissink, R. Beermink, A.A. Poot, G.H. Engbers, T. Beugeling, W.G. van Aken, J. Feijen, Improved endothelialization of vascular grafts by local release of growth factor from heparinized collagen matrices, *J. Control. Release* 64 (2000) 103–114.
- [77] J. Soper, Method of encapsulating food or flavor particles using warm water fish gelatin and capsules produced therefrom, US Patent 5,603,952, 1997.
- [78] K.A. Piez, J. Gross, The amino acid composition of some fish collagens: the relationship between composition and structure, *J. Biol. Chem.* 235 (1960) 995–1000.

- [79] K. Asa, Studies of hypersensitivity to fish: a clinical study, *Int. Arch. Allergy Appl. Immunol.* 29 (1989) 990–994.
- [80] M. Sakaguchi, M. Toda, T. Ebihara, S. Irie, H. Hori, A. Imai, M. Yanagida, H. Miyazawa, H. Ohsuna, Z. Ikezawa, S. Inouye, IgE antibody to fish gelatin (type I collagen) in patients with fish allergy, *J. Allergy Clin. Immunol.* 106 (2000) 579–584.
- [81] J.-Y. Exposito, D. Le Guellec, Q. Lu, R. Garrone, Short chain collagens in sponges are encoded by a family of closely related genes, *J. Biol. Chem.* 266 (1991) 21923–21928.
- [82] J.-Y. Exposito, R. Garrone, Characterization of a fibrillar collagen gene in sponges reveals the evolutionary appearance of two collagen gene families, *Proc. Natl. Acad. Sci. U. S. A.* 87 (1990) 6669–6673.
- [83] R. Garrone (Ed.), *Phylogenesis of Connective Tissue, Morphological Aspects and Biosynthesis of Sponge Intercellular Matrix*, Karger, Basel, 1978.
- [84] S. Junqua, L. Robert, R. Garrone, M. Pavans de Ceccatty, J. Vacelet, Biochemical and morphological studies on collagen of horny sponges. *Ircinia* filaments compared to sponginess, *Connect. Tissue Res.* 2 (1974) 193–203.
- [85] D. Swatschek, W. Schatton, J. Kellermann, W.E.G. Mueller, J. Kreuter, Marine sponge collagen: isolation, characterization and effects on the skin parameters surface-pH, moisture and sebum, *Eur. J. Pharm. Biopharm.* 53 (2002) 107–113.
- [86] A.P. Sclafani, T. Romo III, A. Parker, S.A. McCormick, R. Cocker, A. Jacono, Homologous collagen dispersion (dermalogen) as a dermal filler: persistence and histology compared with bovine collagen, *Ann. Plast. Surg.* 49 (2002) 181–188.
- [87] G.K. Naughton, From lab bench to market: critical issues in tissue engineering, *Ann. N.Y. Acad. Sci.* 961 (2002) 372–385.
- [88] A. Asghar, R.L. Henrickson, Chemical, biochemical, functional, and nutritional characteristics of collagen in food systems, *Adv. Food Res.* 28 (1982) 231–372.
- [89] C.A. Farrugia, I.V. Farrugia, M.J. Groves, Comparison of the molecular weight distribution of gelatin fractions by size-exclusion chromatography and light scattering, *Pharm. Pharmacol. Commun.* 4 (1998) 559–562.
- [90] J.M. Saddler, P.J. Horsey, The new generation gelatins. A review of their history, manufacture and properties, *Anesthesiology* 42 (1987) 998–1004.
- [91] A. Courts, The N-terminal amino acid residues of gelatin: 2. Thermal degradation, *Biochem. J.* 58 (1954) 74–79.
- [92] Y. Sakai, R. Yamato, M. Onuma, T. Kikuta, M. Watanabe, T. Nakayama, Non-antigenic and low allergenic gelatin produced by specific digestion with an enzyme-coupled matrix, *Biol. Pharm. Bull.* 21 (1998) 330–334.
- [93] R.W. Moskowitz, Role of collagen hydrolysate in bone and joint disease, *Semin. Arthritis Rheum.* 30 (2000) 87–99.
- [94] M.W.T. Werten, T.J. van den Bosch, R.D. Wind, H. Mooibroek, F.A. de Wolf, High-yield secretion of recombinant gelatins by *Pichia pastoris*, *Yeast* 15 (1999) 1087–1096.
- [95] E.C. de Bruin, F.A. de Wolf, N.C. Lane, Expression and secretion of human alpha 1 (I) procollagen fragment by *Hansenula polymorpha* as compared to *Pichia pastoris*, *Enzyme Microb. Technol.* 26 (2000) 640–644.
- [96] E.C. de Bruin, M.W.T. Werten, C. Laane, F.A. de Wolf, Endogenous prolyl 4-hydroxylation in *Hansenula polymorpha* and its use for the production of hydroxylated recombinant gelatin, *FEMS Yeast Res.* 1 (2002) 291–298.
- [97] A.J. Brake, J.P. Merryweather, D.G. Coit, U.A. Heberlein, F.R. Masiarz, G.T. Mullenbach, M.S. Urdea, P. Valenzuela, P. Barr, Alpha-factor-directed synthesis and secretion of mature foreign proteins in *Saccharomyces cerevisiae*, *Proc. Natl. Acad. Sci. U. S. A.* 81 (1984) 4642–4646.
- [98] M.W.T. Werten, W.H. Wisselink, T.J. Jansen-van den Bosch, E.C. de Bruin, F.A. de Wolf, Secreted production of a custom-designed, highly hydrophilic gelatin in *Pichia pastoris*, *Protein Eng.* 14 (2001) 447–454.
- [99] I. Goldberg, A.J. Salerno, T. Patterson, J.I. Williams, Cloning and expression of a collagen-analog-encoding synthetic gene in *Escherichia coli*, *Gene* 80 (1989) 305–314.
- [100] H. Hori, S. Hattori, S. Inouye, A. Kimura, S. Irie, H. Miyazawa, M. Sakaguchi, Analysis of the major epitope of the $\alpha 2$ chain of bovine type I collagen in children with bovine gelatin allergy, *J. Allergy Clin. Immunol.* 110 (2002) 652–657.
- [101] T. Nakagawa, P. Pawelek, F. Grimmel, Extracellular matrix organization modulates fibroblast growth and growth factor responsiveness, *Exp. Cell Res.* 182 (1989) 572–582.
- [102] S.L. Schor, Cell proliferation and migration on collagen substrata in vitro, *J. Cell Sci.* 41 (1980) 159–175.
- [103] D. Carey, Control of growth and differentiation of vascular cells by extracellular matrix proteins, *Annu. Rev. Physiol.* 53 (1991) 161–177.
- [104] B. Montagnon, J.C. Vincent-Falquet, B. Fanget, Thousand litre scale microcarrier culture of Vero cells for killed polio virus vaccine: promising results, *Dev. Biol. Stand.* 55 (1984) 37–38.