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Collagen-based Biomaterials

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Introduction

The collagen family is one of the most abundant groups of proteins as collagens are the principal protein component of all connective tissues, including skin, tendon, ligament and cartilage. Because of its vital role in tissue, collagen has long been seen as having the potential to provide the basis of biomaterials, either as native, unmodified tissue grafts or as manufactured products for use in various medical areas. It is this second role, as prostheses and other products, which is discussed in this review.

Progress in the use of collagen as a biomaterial has depended to a great extent on an increased understanding of the chemistry and biology of collagen. Collagen sutures, catgut, have been used over centuries, while experiments with collagen based conduits (see Battista, 1949) and collagenous materials for wound repair (Davis, 1910; Sabella, 1913) were commenced around the turn of the century. However, it was the broad understanding of the methods for purification and characterisation of collagen from tissue (Nageotte, 1927; Leplat, 1933) that established core technologies which were extended to meet the war time demand for better wound and burns dressings. Thus, Schmitt and colleagues, from the 1940s, produced a variety of materials, principally in the form of films, tapes and threads, using techniques for the precipitation or coagulation of collagen solutions and dispersions (see Schmitt, 1985).

More recently, a broad range of potential medical products based on collagen have been suggested (for examples, see Chvapil, 1979; Pachence, Berg and Silver, 1987; Ramshaw, Werkmeister and Peters, 1990), and a range of different product types is now available commercially. These products come in standard forms with reproducible properties and performance standards. Such products have generally proved very successful and include, for example, injectable collagen for soft tissue augmentation, haemostatic powder or fleece and replacement components for the cardiovascular system, such as bioprosthetic heart valves. These collagen-based heart valves are a good example of a collagen-based biomaterial product which has provided enhanced quality of life to thousands of people.

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Most recently, an understanding of the complex biosynthetic pathway for collagens has emerged, initially through studies of various heritable disorders of connective tissues. Also, the discovery of the great diversity in collagen types has indicated that collagen plays much more than a structural role in tissue. The various collagens have been shown to have complex roles in tissue, and are involved in a variety of important molecular interactions (see Kadler, 1994). This greater understanding of the cellular and molecular biology of collagen provides a broad range of new opportunities for collagen-based biomaterials. Genetic engineering can be used to provide either novel collagen-based materials, or other biological factors which can be used with collagen to give composite materials.

This present review does not aim to be comprehensive. Rather, it intends to draw attention to some of the special properties of collagens, how these properties have been used in certain collagen-based biomaterials and how they provide opportunities for development through genetic engineering of better products for the future.

Collagen in tissues

THE ROLE OF COLLAGEN

Collagen is the most abundant protein component of the extracellular matrix (ECM), and provides the insoluble fibres and microfibrils in this complex composite material. A key role for this collagenous network is to provide a scaffold for cell attachment and migration, as well as providing specific mechanical properties. The composition of the ECM varies substantially between different tissues due to different patterns of gene expression and due to variation in particular proteins where differential splicing effects may be present. The composition of the matrix can provide specific information to cells, and thus provides opportunity for development of novel composite biomaterials based on a collagen scaffold. In addition, the topological arrangement of the matrix can provide information to cells and can lead to changes in cell morphology and phenotypic expression. An example of this effect is seen with cells from cartilage which grow with a round, chondrocyte-like morphology in a three-dimensional matrix, such as a collagen gel, but become flattened, like fibroblasts in a two dimensional monolayer culture. This importance of the collagen lattice is thus another feature which can be exploited in development of collagen-based biomaterials.

COLLAGEN DIVERSITY

The name 'collagen' is often used as a generic term to cover a wide range of protein molecules that all share the same characteristic triple helical configuration as a major structural motif (see below). Until 1971, the wide variety of genetically distinct collagens in different tissues had not been realised (Miller, 1971). Subsequently, numerous distinct collagen types have been described. Thus, to date, at least 18 genetically distinct collagen types, comprised of at least 32 individual genetically distinct polypeptide α-chains have now been identified (*Table 1*). The amino acid sequence feature which is common to, and defines, all these collagens is the presence of a $(Gly\text{-}X\text{-}Y)_n$ repeat, where X and Y could be any amino acid, although these are frequently proline and hydroxyproline.

Table 1. Genetically Distinct Collagen Types.

Type	Chain(s)	Molecular Forms	Distribution and Features
I	$\alpha1(I)$	$[\alpha1(I)]_2\alpha2(I)$	Very widespread, being the main constituent of major fibre bundles that give strength to connective tissues; most abundant collagen type, particularly in dermis, bone, ligament and tendon.
	$\alpha2(I)$		
		$[\alpha1(I)]_3$	Appears to be a minor form, found in dermis and dentin.
II	$\alpha1(II)$	$[\alpha1(II)]_3$	The main collagen of cartilage, where it forms the main, thin fibrils. Also found in intervertebral disk.
III	$\alpha1(III)$	$[\alpha1(III)]_3$	Frequently associated with type I, in extensible tissues such as blood vessels, dermis and intenstine. More abundant in foetal tissues.
IV	$\alpha1(IV)$	$[\alpha1(IV)]_2\alpha2(IV)$	Located in basement membranes, as a non-fibril forming collagen. Appears to form a two-dimensional network.
	$\alpha2(IV)$	$[\alpha3(IV)]_2\alpha4(IV)$(?)	
	$\alpha3(IV)$	$[\alpha5(IV)]_2\alpha6(IV)$(?)	
	$\alpha4(IV)$ (?)		
	$\alpha5(IV)$		
	$\alpha6(IV)$		
V	$\alpha1(V)$	$[\alpha1(V)]_3$	Widespread in low quantity; appears associated with type I collagen fibrils where it may form a core filament.
	$\alpha2(V)$	$[\alpha1(V)]_2\alpha2(V)$	
	$\alpha3(V)$	$\alpha1(V)\alpha2(V)\alpha3(V)$	
VI	$\alpha1(VI)$	$\alpha1(VI)\alpha2(VI)\alpha3(VI)$	Widespread, forming beaded filaments. Different alternatively spliced forms exist.
	$\alpha2(VI)$		
	$\alpha3(VI)$		
VII	$\alpha1(VII)$	$[\alpha1(VII)]_3$	Forms anchoring filament structure that links the epithelial basement membrane to underlying fibrillar tissue.
VIII	$\alpha1(VIII)$	(?)	A short chain length collagen, associated with endothelial cell layers, such as Descemet's membrane. In some tissues it may form a hexagonal lattice.
	$\alpha2(VIII)$		
IX	$\alpha1(IX)$	$\alpha1(IX)\alpha2(IX)\alpha3(IX)$	Has an interrupted triple helix collagen, and may contain a glycosaminoglycan chain. Found in cartilage and vitreous body, where it is associated with type II collagen.
	$\alpha2(IX)$		
	$\alpha3(IX)$		
X	$\alpha1(X)$	$[\alpha1(X)]_3$	A short-chain collagen, found in hypertrophic mineralizing cartilage, with a similar structure to type VIII collagen.
XI	$\alpha1(XI)$	$\alpha(XI)\alpha2(XI)\alpha3(XI)$	Forms fibrils which are associated with type II collagen in cartilage and intervertebral disc.
	$\alpha2(XI)$		
	$\alpha3(XI)$		
XII	$\alpha1(XII)$	$[\alpha1(XII)]_3$	A collagen with an interrupted triple helix, associated with type I collagen fibrils.
XIII	$\alpha1(XIII)$	(?)	Appears to be widespread in low quantity, showing different forms due to a complex pattern of alternative splicing.
XIV	$\alpha1(XIV)$	$[\alpha1(XIV)]_3$	A fibril-associated collagen with an interrupted triple helix found in skin.
XV	$\alpha1(XV)$	(?)	Is expressed in fibroblasts and smooth muscle cells; has a triple helix which is interrupted in several places.
XVI	$\alpha1(XVI)$	(?)	Is expressed in fibroblasts and keratinocytes; has a triple helix which is interrupted in several places.
XVII	$\alpha1(XVII)$	(?)	Bullous pemphigoid antigen. Is expressed at dermal-epidermal junction; has a triple helix which is interrupted in several places.
XVIII	$\alpha1(XVIII)$	(?)	Is expressed in highly vascularised tissues; has a triple helix which is interrupted in several places.

The size of the helical domain varies between different collagen types. For example, an uninterrupted triple-helical, $(Gly\text{-}X\text{-}Y)_n$ domain of about 1000 amino acids is present in the interstitial collagen group, whereas in the other collagen types, denoted as non-fibrillar collagens, the triple-helical domains are of varying lengths (van der Rest and Garrone, 1991). Also, in some of the non-fibrillar collagens, such as type IV collagen, several short interruptions to the $(Gly\text{-}X\text{-}Y)_n$ helical sequence occur, while in others of these collagens, such as type VIII, larger interruptions to the helical sequence produce a protein structure composed of a number of linked helical 'collagen cassettes' separated by non-collagenous sequence domains.

For some of the collagens, their distributions and major roles have been described, whereas for others, their structure, function and distributions are less clear. Of these various collagen types, only a few are abundant; these include type I collagen, the major collagen of skin, tendon and ligament, type II collagen, the collagen of cartilage, and type III collagen, important in blood vessels and other tissues which are particularly extensible. Along with collagen types V and XI, these collagens belong to the group of D-periodic, interstitial, fibril forming collagens. Most other collagens are only minor components overall, although in particular tissues or tissue locations they may be abundant. For example, type IV collagen, while in low abundance overall, is the predominant collagen component of all basement membranes.

Of the range of collagen types, type I collagen, which is the predominant component of most collagenous tissues, is the most studied, and therefore much of the following detail refers to this collagen type. Because of its abundance, accounting for up to 90% of the collagen found in the body, this collagen type is the one normally encountered in biomaterial applications. However, when it comes to evaluating the performance of biomaterials, and the potential replacement of the collagenous device by new functional tissue, then the distribution and roles of the other less abundant collagen types can be important.

COLLAGEN STRUCTURE

The triple-helical conformation is the defining structural element of all collagens. The basic conformation of the triple-helix has been deduced from high angle X-ray fibre diffraction studies on collagen in tendon (Rich and Crick, 1961; Fraser and MacRae, 1973). Each of the three polypeptide chains in the molecule forms an extended left handed polyproline II-type helix, which is stabilised by a high imino acid content. The three chains are staggered by one residue relative to each other, and are supercoiled about a common axis in a right handed manner to form the triple-helix. Every third residue of each chain is close to the central axis, and the close packing and hydrogen bonding between the three chains requires only glycine residues at this position. Thus, conformational requirements dictate that the amino acid sequence of a triple-helix can be represented as $(Gly\text{-}X\text{-}Y)_n$, where a large proportion of X residues are proline and a large proportion of Y residues are hydroxyproline. In collagen, sequences of Gly-Pro-Hyp are the most common tripeptides (about 12%), while sequences of the form Gly-Pro-Y and Gly-X-Hyp represent about 44% of tripeptides, and Gly-X-Y triplets with no imino acids constitute the remaining 44% (see Kadler, 1994).

BIOSYNTHESIS

The pathway of collagen synthesis from gene transcription to secretion and aggregation of collagen monomers into functional fibrils in tissues is complex (Kielty, Hopkinson and Grant, 1993; Bateman, Lamandé and Ramshaw, 1996). While the

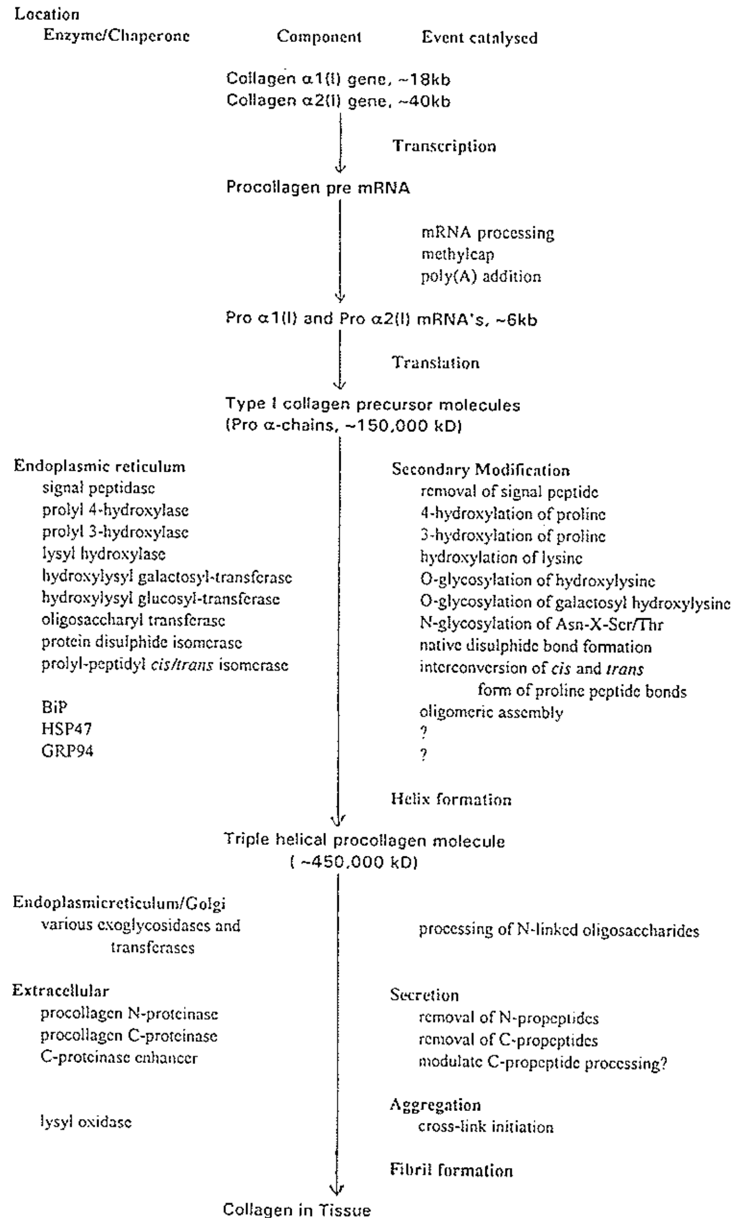

Figure 1. A summary of the pathway for type I collagen biosynthesis.

individual steps follow the general pattern of assembly and secretion of other multi-subunit proteins, the mRNA processing is particularly complex and the protein product is subject to extensive and diverse post-translational modifications. Thus collagen biosynthesis requires the co-ordinating of a very large number of biochemical events, both temporally and spatially.

The biosynthesis pathway has been most carefully studied in the fibrillar collagens, and the discussion here relates specifically to type I collagen (*Figure 1*). While many of the general pathways are probably common to all collagens, highly specialised collagens may require additional components or steps unique to their own structure.

The fibrillar collagen chains are encoded by large and complex genes comprising 51–53 exons. The initial steps of biosynthesis comprise the nuclear RNA processing events common to the vast majority of protein-encoding RNA molecules transcribed from eukaryotic genes. Transcripts are modified by capping at their 5' end, polyadenylation occurs at the 3' end of the transcript after the poly(A) additional signal, and the non-coding intronic sequences are spliced out. Like other secreted proteins, type I collagen translation products are initially directed into the endoplasmic reticulum (ER) by a hydrophobic N-terminal signal peptide, which is cleaved from the nascent polypeptide chain, probably as it emerges into the lumen of the ER, by a signal peptidase.

The polypeptide chain then undergoes a series of chemical modification reactions which are critical for the resulting properties of the collagen molecule. Particularly important is the hydroxylation of certain proline residues, as hydroxyproline is important for the stability of the final collagen helix. The denaturation temperature of the collagen is dependent on the extent of proline hydroxylation. If the extent of proline hydroxylation is not sufficient, the denaturation temperature is lower and the collagen is not stable at physiological temperatures (Berg and Prockop, 1973). In addition, certain lysine residues are hydroxylated, some also being subsequently glycosylated. Hydroxyproline, hydroxylysine and glycosylated hydroxylysine are found almost exclusively in collagens and other proteins with collagen-like domains, and the formation of these amino acids is catalysed by three specific hydroxylases and two specific glycosidases (*Figure 1*).

In the next step in biosynthesis, three precursor chains combine and fold from the C-terminus to form procollagen. This folding step terminates the hydroxylation reactions and the specific glycosylation of hydroxylysine. The possibility of simultaneous synthesis by a single cell of several fibrillar collagen types introduces a critical step in the assembly process, appropriate chain recognition and selection. Intracellular protein folding clearly is not a spontaneous, uncatalysed process; it requires interactions with enzymes and polypeptide chain binding proteins or molecular chaperones that catalyse slow folding steps and assist in oligomer assembly (see Kielty, Hopkinson and Grant, 1993; Bateman, Lamandé and Ramshaw, 1996).

The initial type I procollagen molecule after folding is still a precursor in that it has additional globular domains at each end of the molecule, the N- and C-propeptides (*Figure 2*). The secretion pathway of triple helical procollagen is similar to that of other extracellular proteins. The molecules pass through the Golgi and are then packaged into secretory vacuoles which move to the cell surface and release the procollagen by exocytosis. Procollagen aggregates have been observed within the secretory vacuoles suggesting that procollagen is probably secreted in an aggregated

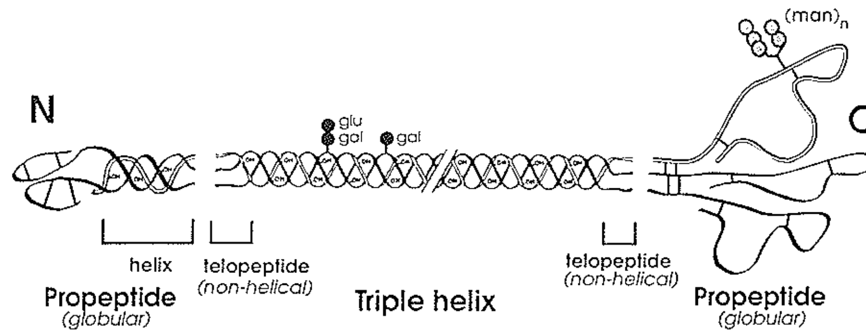

Figure 2. A diagram showing the structure of type I collagen and the relationship of the triple helix to the telopeptides and the propeptide domains. (Drawing courtesy of Dr John F. Bateman, University of Melbourne.)

form and not as monomeric molecules. Following (or possibly during) secretion the N- and C-propeptides are cleaved by specific endopeptidases, a procollagen N-proteinase (Tuderman and Prockop, 1982) and the procollagen C-proteinase (Hojima, van der Rest and Prockop, 1985). These cleavage reactions leave short, non-triple-helical domains, called telopeptides, at each end of the collagen molecule. The telopeptides play an essential role in the maturation of the collagen matrix by intermolecular cross-link formation (see below).

TISSUE ARCHITECTURE AND STABILISATION

In the extracellular matrix, collagen is incorporated into fibrils, which represent the functional unit of collagen. Individual fibrils, for example in tendon, can be examined by transmission electron microscopy and show a characteristic banding pattern along the fibre axis of about 67 nm, called the D period (see Fraser and MacRae, 1973). X-ray diffraction shows that for tendons, the D period is 67.0 nm, whereas the fibrils in skin and certain other tissues have a shorter D period of about 65.5 nm (see Brodsky and Ramshaw, 1994). This packing difference may reflect a more complex composition of collagen types and proteoglycan components in these tissues. As individual collagen molecules are about 300 nm long, the structure for the fibril is of a staggered overlap arrangement (Fraser and MacRae, 1987). Since each molecule is about 4.4 times the D period, this model leads to gaps between successive molecules and stain uptake into these gap regions leads to the characteristic banding pattern observed by transmission electron microscopy. In functional tissue, the fibrils further associate to form larger structures called fibre bundles.

Individual collagen molecules will spontaneously 'self-assemble' into ordered fibrillar structures (see Veis and Payne, 1988). This process can occur *in vitro* as well as *in vivo*. Thus the amino acid sequence of the collagen defines all the information to enable collagen to form into its native, fibrillar structure. Much of what is known about the factors which may regulate the size and shape of collagen fibrils has come from manipulation of *in vitro* fibrillogenesis model systems, with many of the findings now supported by ultrastructural and biochemical studies of tissues. *In vitro* collagen

fibrillogenesis is modulated by the extent of propeptide processing, the ratio of co-assembled collagen types and by the presence of other matrix molecules. Cleavage of the type I collagen N- and C-propeptides appears essential for regular fibril morphology. On the other hand, for type III collagen, the partially processed molecule retaining its N-propeptide is a normal component of tissues, where it appears to coat the type I collagen fibrils (Fleischmajer *et al.*, 1981). These observations have led to the idea that the type III collagen N-propeptide may limit fibril diameter by steric hindrance. It is likely that fibril formation *in vivo* occurs in close association with the cell surface and that the cell, along with other ECM molecules such as decorin, play a fundamental role in controlling the nature and extent of fibril formation (Birk *et al.*, 1990; Fleischmajer *et al.*, 1991).

The formation of intermolecular cross-links is an important step in the formation of stable collagenous tissues. The cross-links confer physical and mechanical properties to the fibrils and provide the tensile strength fundamental to the structural role of collagen fibrils in connective tissues (see Yamauchi and Mechanic, 1988).

The initial stages of cross-link formation are highly specific and result from the action of the copper containing enzyme, lysyl oxidase, on the single lysine or hydroxylysine residues found in the telopeptides. The aldehydes produced by this enzymatic step then undergo a series of further, non-enzymatic, condensation reactions which lead to the formation of a variety of intra- and intermolecular cross-links. The other amino acids involved in these reactions are also highly specific to particular collagen chains and sequence positions. The amounts and distributions of the various cross-links vary between different tissues, probably being related to the physiological requirements since the genetic types of collagen present are often the same in different tissues.

Three pathways for cross-link formation have been well established (Yamauchi and Mechanic, 1988), two involving lysine-derived aldehydes and one involving hydroxylysine-derived aldehyde. In one pathway, an aldol condensation product between two aldehydes is formed, whereas in the other pathways, condensations with hydroxylysine-derived amino groups occur. These initial bifunctional cross-links, which can be quantified after reduction by sodium borotritide, are found in greatest quantity in young and immature animals. During maturation and aging, other multifunctional cross-links are formed which link the collagen molecules into an extended cross-linked network. Only a limited number of these subsequent cross-links have so far been identified and it is probable that others, some possibly derived by further reactions of those already identified, will exist. In addition, during aging additional cross-links may form, independent of this process, as a result of non-enzymatic glycosylation of the collagens.

REGULATION OF COLLAGEN SYNTHESIS

Changes in collagen gene expression occur during development, between tissues and in a number of pathological conditions, and it is likely that precise temporal, spatial and quantitative regulation is critical for normal morphogenesis and differentiation. Collagen expression is known to be sensitive to modulation by growth factors, cytokines, glucocorticoids, and viral and chemical transformation (Bornstein and Sage, 1989; Raghow and Thompson, 1989; Slack, Liska and Bornstein, 1993).

Gene transcription appears to be modulated by multiple promoters, enhancers, and

silencers, and it is clear that the level of gene expression for a particular collagen type in a given tissue involves complex interactions between multiple, sometimes distant, DNA sequences that are mediated by the availability and binding of multiple transcription factors. For translation, a highly conserved nucleotide sequence surrounding the translation initiation site in the α1(I), α2(I), and α1(III) mRNAs has been proposed to modulate translational efficiency (Yamada, Mudryj and de Crombrugghe, 1983).

Both the N- and C-terminal propeptides have been proposed to play roles through feedback regulation of collagen synthesis (Schlumberger *et al.*, 1988; Wu, Walton and Wu, 1991). When added to the medium of cultured cells the free N-propeptides of type I and III collagens specifically inhibit collagen synthesis by fibroblasts but not chondrocytes (Paglia *et al.*, 1981). Other evidence to support the regulatory role of the N-propeptide has come from expression of this subunit in fibroblasts, leading to inhibition of the synthesis of type I procollagen. The type I collagen C-propeptides have also been implicated in feedback regulation of procollagen synthesis, possibly through a direct effect on transcription, although other effects may also occur (Wu, Walton and Wu, 1991).

Advantages of collagen as a biomaterial

In practice, there is no ideal biomaterial (Williams, 1987). A particular material which may be well suited to a specific application must be selected and then, with further research, optimised for clinical use. Thus, when looking for potential applications for collagen, it is important to examine its specific properties and advantages and choose those applications where these are not readily available through use of other materials. Inevitably, collagen-based products are in competition with those derived from synthetic polymers, so, apart from a primary need to be cost-effective, the materials must have advantages which can be clearly identified. In general, such advantages derive from the unique structural, biochemical and biological properties of collagen.

DIVERSITY OF PRODUCT FORMS

Although collagen-based biomaterials are normally discussed as a single group, the different products (see later, *Table 3*) form a wide range of materials with distinct formats and characteristics. The advantages of the collagen which are being exploited

Table 2. Advantages for different collagen formats for biomaterial applications.

Collagen format	Tissue-based	Purified (soluble or comminuted)
Advantages	Low immunogenicity	Low immunogenicity
	Durability and Persistence	Contolled turnover
	Cell-matrix interactions	Cell-matrix interactions
	Strength	Platelet interactions
		Fibril reformation
Examples of applications*	Heart valves	Injectable collagen
	Blood vessels	Haemostats
		Wound dressings
		Burns dressings

* See *Table 3* for an extended list

vary between these different products (*Table 2*). Most notably, there are two distinct groups of collagen-based biomaterials. These are the tissue-based biomaterials, where the intact tissue is adapted so that the product meets the medical needs, and purified collagen, either as a comminuted product or as a soluble product, which may then be reformed into a particular shape or form.

Collagen possesses several unique chemical and biological properties which can be exploited in the development of collagen-based biomaterials. In general, although some properties are common to both these formats, certain advantages are specific to each type (see *Table 2*).

LOW IMMUNOGENICITY

First and foremost, a collagen-based medical implant has to be immunologically inert, irrespective of whether tissue-based or purified collagen is used. Compared with proteins that are clearly immunogenic, collagens are particularly poor immunogens. When the immunogenicity of various collagen products was examined, it was found that the very small quantities of non-collagenous proteins that were present in some of the materials led to the dominant immunological response (DeLustro *et al.*, 1986a). Another potential source of immunogenicity is the presence of denatured collagen, since individual chains may be more immunogenic than intact helical molecules and previously hidden determinants could evoke an immune response.

Prior to the mid-1950s, collagen was thought to be totally non-immunogenic. Recently, however, collagens have been shown to be immunogenic. For example, there have been various studies to develop monoclonal antibodies (MAbs) to mammalian collagens (see Glattauer *et al.*, 1990). In addition to demonstrating that it is possible to develop MAbs, these studies have indicated that type I collagen, the most abundant collagen and the predominant collagen of biomaterials, is an extremely poor immunogen (Werkmeister, Ramshaw and Ellender, 1990), and is much less likely than other collagen types such as III, V and VI to lead to an antigenic response (Werkmeister and Ramshaw, 1991a,b; Werkmeister *et al.*, 1993).

Of the domains found in tissue collagens, the telopeptides and the helical domain, the telopeptides are the most immunogenic, perhaps due to their greater flexibility. Thus, for soluble collagens it is possible to minimise the immunogenicity of the product by removing these regions through enzyme treatment. For tissue-based biomaterials, these regions cannot be removed since they are the sites for the cross-links which give the tissue its structural integrity. Instead, the immunogenicity of these materials can be partly reduced by cross-linking the collagens, for example with glutaraldehyde.

CONTROL OF TURNOVER

An advantage of both forms of collagen-based devices is that the rate of collagen turnover can be controlled, and hence the long-term persistence and durability of the device determined. The rate at which a collagenous device may be absorbed after implant is clearly a very important property. *In vivo*, natural collagen turnover is tissue-dependent, and has been reported to occur in a few days in periodontal ligament (Sodek and Ferrier, 1988) and over several months or even years in skin (Nissen,

Cardinale and Udenfriend, 1978). For non-stabilised collagenous implants, the collagen is resorbed rapidly, over a period of weeks through months depending on the size and nature of the implant (Burke, Naughton and Cassai, 1985; Ellender *et al.*, 1992).

The intention of the control of turnover is generally quite different for tissue-based compared with purified collagen products. For tissue-based devices, long term durability is generally a highly desirable feature, for example in heart valves. Since native tissue would be resorbed in a fairly short time frame, in order to prolong the turnover of the collagen the material is extensively crosslinked, normally by glutaraldehyde. This leads to devices, such as heart valves, which remain durable over extended periods, exceeding 10 years (Ferrans *et al.*, 1988). For devices based on purified collagen, in most applications the material is intended to resorb. For example, for membranes for guided tissue regeneration in dentistry, or for adhesion barriers, collagen membranes will resorb in only a few weeks (Ellender *et al.*, 1992), and longer duration is not required. For other applications, for example certain wound or burns dressings longer duration may be required. In such cases, mild, controlled crosslinking can be used to extend the turnover time (Yannas *et al.*, 1977, 1980).

CELL-MATRIX INTERACTIONS

A clear advantage of both tissue and purified collagen devices is their ability to interact with the host and either act as a scaffold for new tissue formation prior to resorption or become assimilated into the host, being augmented by new host tissue. In these processes, collagen-cell interactions are of importance since the precise interactions and interplay between cells and the surrounding extracellular matrix are integral to the normal physiology and functioning of the cells and tissue. It has been known for a long time that collagen can enhance the growth of different cell types (Ehrmann and Gey, 1956) and a vast amount of evidence has emerged demonstrating the use of collagen substrates, particularly type I collagen, for culture of a variety of different cell types (Kleinman, Klebe and Martin, 1981). The mechanism of cell interaction with collagen depends on the cell type, and may occur directly through specific receptors or, more commonly, may be mediated by specific adhesion proteins such as fibronectin (Hahn and Yamada, 1979; Kleinman, Klebe and Martin, 1981). Another key feature which gives collagen materials a particular advantage is that collagen substrates, apart from augmenting cell adhesion, can also promote subsequent growth, differentiation and migration of cells (Doillon, Silver and Berg, 1987).

The ability of collagen, in conjugation with other extracellular matrix molecules, to support the growth and function of numerous different cell types, has accentuated the development and application of collagen-based biomaterials in several areas, but particularly for wound and burn dressings, where other desirable features, such as delay in wound contraction (Yannas and Burke, 1980), have emerged as additional advantages.

These specific biological advantages of collagens are not found in synthetic polymers. Consequently, composites including collagen or surface coatings of collagens have been developed for non-biological materials in order to enhance their performance characteristics (Civerchia-Perez *et al.*, 1980).

MECHANICAL PROPERTIES

A key advantage which is being used in devices based on tissue collagen is the inherent strength of the material. Collagen is the principal protein component to provide strength to connective tissues, and has to be able to accommodate a wide variety of mechanical demands. This is achieved by the collagen in each tissue having distinct architecture, with specific variations in composition, cross-linking patterns, fibril size and orientation (Hukins, 1984). For example in tendons, the collagen is almost entirely type I collagen which is aligned along the length of the tendon, in the direction of the force. On the other hand, in skin, which has to be able to change shape in all directions, the collagen fibres are arranged in all directions, and other collagen types, including type III collagen, are also found (Ramshaw, 1986; Werkmeister, Peters and Ramshaw, 1989).

The presence of natural cross-links is an important factor in the strength of collagen fibres, and these are maintained in tissue-based devices. The proteoglycan content of the tissue and the natural crimp of collagen fibres should also be preserved, if possible, during manufacture. These features help the tissue to dissipate energy before it leads to fracture and thus they further enhance the mechanical advantages of the material.

The mechanical advantages of tissue are lost when purified collagen is produced. Thus, reconstituted fibrils of soluble collagen lack the strength of native tissues as the native cross-links are no longer present. The native strength cannot be adequately replaced by introduced cross-linking since the high levels of introduced cross-linking that would be required lead to an unacceptable stiffness.

PLATELET INTERACTIONS

Platelet aggregation by collagens is an important event in haemostasis terminating in the formation of a haemostatic plug and arrest of bleeding. Following damage to the endothelial lining of the vascular wall, blood platelets adjacent to the injured site come into direct contact with the underlying subendothelial connective tissue components. Although the exact mechanism remains incompletely understood (see Ramshaw, Werkmeister and Peters, 1990), adhesion to collagen is a pre-requisite for subsequent platelet activation and release of its granular contents (Hugues, 1960; Zucker and Borelli, 1962).

This physiological property of collagen has been exploited in manufacture of haemostats. However, it is an obvious hazard in the use of collagen or collagen-composites in cardiovascular devices. In these instances, the interaction of the collagen with platelets needs to be inhibited or masked. One approach has been to cross-link the device with glutaraldehyde (Nimni *et al.*, 1987); it is not clear whether this approach works by reacting with a lysine residue(s) which is important for the platelet interaction (Wilner, Nossel and LeRoy, 1968), or by more generally covering the collagen surface with polymeric cross-links. Another approach has been to coat the collagen surface with compounds, such as heparin (Venkataramani *et al.*, 1986), which are inhibitory to platelet function.

FIBRIL REFORMATION

An important property of purified, soluble collagen molecules is that they are able to reform *in vitro* into the ordered fibrillar structures found for native fibrils, although the

mechanism of *in vitro* fibril formation may be significantly different to the *in vivo* process. This fibril forming process occurs even though the telopeptides have been removed by enzyme digestion, although the time taken increases (Hayashi and Nagai, 1974). Also, when telopeptides are absent, there is an increased tendency for the collagen to pack in a symmetric manner and in other non-native packing modes (McPherson *et al.*, 1985).

This process can be used as an advantage for collagen since the purified material, where the immunogenicity can be substantially reduced, can be reformed into fibrils which regain the native packing which is necessary for many of the molecular processes which are essential for the cell-matrix interactions. These materials can then be prepared either as pastes suitable for implanting by injection (Wallace *et al.*, 1988) or as dry sponges or similar formats (Miyata, Taira and Noishiki, 1992).

Preparation of collagen-based biomaterials

INSOLUBLE COLLAGEN

For tissue-derived devices and those made from comminuted collagen, the preparative steps for the collagen component are generally not complex. In both cases, washing steps to remove non-collagenous components may be included. However, immunological evidence indicates that unless additional treatments are included, these products could elicit a positive immunological response in recipients (DeLustro *et al.*, 1986b). Mild enzyme treatments, for example using ficin, have been used to digest away the non-collagenous proteins, while aiming to minimise the level of degradation of the collagen (Rosenberg *et al.*, 1956). However, while removal of protein components will be beneficial from an immunological perspective, removal of other components such as proteoglycan may be detrimental to the mechanical properties of devices through increasing the stiffness of the material. For tissue-based devices, it is important to minimise the time between tissue harvest and stabilisation to reduce the extent of autolysis and degradation.

SOLUBLE COLLAGEN

The general principles for the preparation of purified, soluble collagen have been described previously (see, Miller and Rhodes, 1982; Ramshaw, Werkmeister and Peters, 1990). However, each company and laboratory tends to develop variations on the broad themes, and these variations are often not fully documented for commercial in-confidence reasons.

Soluble collagen can be prepared easily and purified to provide medical grade material. An enzyme treatment of minced tissue such as skin, is frequently used in collagen preparation (Miller and Rhodes, 1982); normally the acid protease pepsin is used and this enzyme acts by cleaving the telopeptide cross-linking regions. The acid pH used for pepsin digestion leads to a swelling of the tissue which helps in dissolution. If the soluble collagen is taken to neutral pH, the pepsin is inactivated and may be removed during collagen purification procedures. The most frequently employed laboratory method for collagen purification is precipitation of collagen fractions by NaCl, at both acid and neutral pH (Trelstad, 1982). However, for the

production of commercial materials, a variety of additional proprietary approaches exist, some of which are described in the patent literature.

Other methods for making soluble collagen have been described, such as use of either acid or alkali treatments (see Chvapil, Kronenthal and van Winkle, 1973). Compared with the enzyme method, these have the disadvantage of causing cleavage in the collagen helix domain and not removing the telopeptides with great efficiency. The use of enzyme solubilisation provides benefits through reducing the immunogenicity, both by reducing the non-collagenous contaminants and by removing the telopeptides from the collagen.

A wide variety of methods are available for the characterisation of soluble collagen intended for medical application (see Ramshaw, Werkmeister and Peters, 1990). These include:

1. Polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulphate (SDS-PAGE) (Laemmli, 1970), with and without collagenase digestion of the sample.
2. Amino acid analysis, with specific attention to the tyrosine content as this is present only in the telopeptide regions of type I collagen (see Kadler, 1994).
3. Immunological assays, for example Enzyme Linked Immunoabsorbent Assay (ELISA), to detect specific impurities when appropriate antibodies are available. For example, residual levels of pepsin or of serum albumin can be quantified.
4. Spectroscopy, particularly circular dichroism spectroscopy where triple helical collagen gives a characteristic peak at 223 nm (Hayashi, Curran-Patel and Prockop, 1979).
5. Transmission electron microscopy, which may give an estimate of the proportions of cross-linked molecules, other aggregates or collagen fragments.

For insoluble, comminuted collagen, these methods are generally not applicable. Instead, refractive index has been suggested as a method for determining purity (Jayabalan, 1985)

INTRODUCED CROSS-LINKING

Whereas native collagen tissues possess significant strength, this strength is lost when collagen products are made from soluble collagen. These reconstituted products may therefore require chemical treatment with cross-linking agents so as to regain adequate strength for particular applications. Alternatively, when a biomaterial is based on collagen from the native, intact matrix, the immunogenicity of the product could be a major problem. This can also be overcome, in part, by treatment with chemical cross-linking agents which minimise these effects. In addition the cross-linking also serves the function of controlling the rate of turnover of the material. Thus, in both approaches to using collagen as a biomaterial, chemical cross-linking of the collagen is important in the manufacture of a successful product.

A range of different approaches have been described for cross-linking of collagens, including both chemical and physical approaches. From the point of view of biomaterials, the cross-linking ability of a particular method is not the only criterion and other criteria must be considered, including stability, toxicity, tendency to cause

calcification and resistance to enzymic degradation. Some examples of the chemical methods which have been described are:

1. Formaldehyde (for example Stenzel, Miyata and Rubin, 1974).
2. Di aldehyde starch (Bowes and Cater, 1968; Rosenberg *et al.*, 1966).
3. Glutaraldehyde (see Nimni *et al.*, 1988).
4. Other bis aldehydes (Cater, 1965; Bowes and Cater, 1968).
5. Di-isocyanates, particularly hexamethylene di-isocyanate (Olde Damink *et al.*, 1995).
6. Water soluble carbodiimides (van Wachem *et al.*, 1994)
7. Aliphatic epoxides (Tu *et al.*, 1993; Lee, Pereira and Kan, 1994).
8. Cyanuric chloride (Cater, 1965).
9. Acyl azides (Petite *et al.*, 1990).
10. Non-enzymatic glycosylation (Brodsky *et al.*, 1990).
11. Dye-mediated photo-oxidation (Moore *et al.*, 1994; Ramshaw, Stephens and Tulloch, 1994).

In general, these chemical methods have the advantages of specificity and reproducibility. To date, by far the most important of these is glutaraldehyde cross-linking, and this is used in the majority of the current commercial products. Of the other methods, some are still being developed and may well increase in importance; these include the use of carbodiimides, aliphatic epoxides, hexamethylene diisocyanate and dye-mediated photo-oxidation. On the other hand, some of the other methods have already proved unsatisfactory; thus formaldehyde shows toxicity and stability problems, while dialdehyde starch gave products with poor stability and durability.

Although glutaraldehyde is the preferred reagent, it still has performance limitations (see below). The mechanism of stabilisation by glutaraldehyde, although probably based initially on aldimine formation, is considerably more complex as a result of the numerous components which make up the reagent (Nimni *et al.*, 1988). Glutaraldehyde exists in solution as a mixture of chemical species of which only a small proportion is the dialdehyde itself (Hardy, Nicholls and Rydon, 1969: Holloway and Dean, 1975). At higher pH, increasing amounts of addition products are formed, and polymers based on the cyclic hemiacetal (Hardy, Nicholls and Rydon, 1969). The formation of polymers is a problem for stabilisation of biomaterials as the polymer may become trapped in the structure or may bind through single attachment points to the collagen, hindering penetration into the full thickness of the material (Nimni *et al.*, 1987, 1988). Although the amount of polymer may be reduced by washing procedures, and the level of free aldehyde eliminated by a glycine wash, residual polymer in the implanted material degrades over an extended period of time, releasing monomers or small polymers which are cytotoxic. This may have short-term benefits as an anti-bacterial agent, but in the longer term the lack of cellular infiltration appears detrimental to the product performance. Another problem with glutaraldehyde, discussed further below, is its tendency to induce calcification of the implant.

As well as chemical approaches for cross-linking, physical methods have also been proposed. These include,

1. Irradiation, particularly by UV light (Miyata *et al.*, 1971).
2. Dry heat (Yannas and Tobolsky, 1967).

These methods are less versatile than the chemical approaches, and are generally limited to products reconstituted from purified collagen. When a product with the desired properties is obtained, these methods have the advantage of not introducing any potentially toxic reagents. Whichever method is chosen, chemical or physical, it is the *in vivo* performance of the product which defines the suitability of the cross-linking process which has been used.

Examination of collagen biomaterial performance

What is common with any type of biomaterial used as a medical device is the mandatory assessment of performance criteria to establish safety and efficacy. In recent years, there has been renewed awareness of such a need continually to assess and monitor the performance of implanted devices. Establishment of quality assurance systems is emerging as a means to keep check on devices. Sadly in such instances like the failure of some Bjork-Shiley heart valves, the silicone gel filled breast implants and the recent fracture of the retention wire in atrial 'J' pacing leads, the situation has been accentuated by the lack of appropriate, if any, tracking and data records of patients and devices (see Werkmeister and Ramshaw, 1995). Implants made from collagenous materials are subject to the same regulatory restrictions and guidelines for testing, outlined by appropriate ISO or FDA standards.

The term which incorporates the assessment of safety and efficacy of materials or devices is 'biocompatibility'. At a second consensus meeting on definitions held in Chester (Williams, Black and Doherty, 1991), it was re-defined as the 'ability of a material to perform with an appropriate host response in a specific application'. While it is commonly defined in negative terms as the property of the material which should not be able to initiate an intolerable clinical response, biocompatibility encompasses a far broader view since most materials like collagens are interactive and are specifically designed to evoke a specific desirable response in a specific environment.

IN VITRO TESTING

As with any other biomaterial intended to interact with biological systems, collagen-based materials must undergo mandatory screening for toxicity. The simplest and most economic means of assessment are the battery of *in vitro* cytotoxicity tests which are available (von Recum, 1986; Silver and Doillon, 1989). These initial biocompatibility screening assays are performed in cell culture using a variety of standard continuous cell lines. Cell culture techniques monitor the degree of cell lysis, the inhibition of cell growth and other morphological effects caused by the collagen implants and/or extracts from the material. Generally, there are three types of testing which can be performed and the rules for which test to use are not well defined. These are the direct contact test, the indirect test (agar overlay assay), and the extract test. Most of these tests involve simple microscopic evaluation or the use of a dye to assess the degree of cell clearance, although quantitation can be monitored using radioactive markers.

As previously indicated, one of the major advantages with the use of collagen as a biomaterial is that it is a natural and essential component of living systems. As such in its unmodified form it is not toxic, although there have been reports that collagen-

based biomaterials may induce cytotoxic effects (Speer *et al.*, 1980; Speer and Chvapil, 1981; van Luyn *et al.*, 1991). Without exception, the primary reason for the induced toxicity has been due to the cross-linking agent used for stabilisation. As discussed previously, the main agent used is glutaraldehyde and for some applications of collagen-based biomaterials like heart valves, this is a primary concern of toxicity and implant failure (Ferrans *et al.*, 1980; Schoen and Levy, 1984).

The cell culture assays are also a convenient means of assessing performance of the collagen-based biomaterial. The property of the collagen which allows effective and beneficial interactions with cells is of critical importance in the development and use of collagen as a biomaterial. These interactions which occur in biological systems and particularly within the surrounding extracellular matrix are integral to the normal physiology and functioning of the cell.

Collagens, particularly type I collagen used for medical implants, can support the growth of different cell types (Kleinman, Klebe and Martin, 1981). The mechanism of adhesion of fibroblasts and other cell types to collagen is partly dependent on the form of the protein, namely whether it is present in a native or denatured state (Schor and Court, 1979). Collagen substrates, apart from augmenting cell adhesion, can promote subsequent growth, differentiation and migration of cells (Kleinman, Klebe and Martin, 1981; Doillon, Silver and Berg, 1987). The major cell receptor for collagen interactions is the $\alpha_2\beta_1$ integrin which is present in fibroblasts, platelets and leucocytes (see Hemler, 1990; Tuckwell, Weston and Humphries, 1993). This receptor belongs to a supergene family of cell adhesion receptors comprising at least 13 α subunits and 8 β subunits. The integrins mediate cell-cell and cell-extracellular interactions in a divalent cation-dependent manner (Hemler, 1990; Haas and Plow, 1994). The $\alpha_2\beta_1$ integrin is responsible in part for the three dimensional type I collagen gel contraction and the associated re-organisation of the collagen matrix by the cells (Klein *et al.*, 1991).

These properties of the collagen molecule are critical features for the application of this protein to a biomaterial implant. They have been utilised to promote the adhesive properties of other biomaterial implants, for example, hydrogels of poly(hydroxyethyl methacrylate) (polyHEMA) and sepiolate (magnesium silicate) complexes, which do not themselves readily support attachment and growth of mammalian cells (Civerchia-Perez *et al.*, 1980). It is these same properties which makes collagen an ideal material for use as an haemostat but which must be controlled in the application of collagens for use as a vascular prosthesis (see later section).

IN VIVO TESTING

Examination of the performance of collagen biomaterials also extends to animal implantation and explant analysis. While *in vitro* testing has the capacity to screen for a large number of samples, the *in vivo* testing is confined to selected materials which have proved satisfactory in the initial screening procedures. These tests can be designed to evaluate the general toxicity and performance of the collagen material. In general, a variety of animals including rats, guinea pigs, or rabbits are used and the primary assessment is for short and long-term acute or chronic inflammation, as well as immunological testing, both humoral and cell-mediated responses. The alternative choice is to evaluate the particular prototype biomaterial or device in a specific

functional model. This test has the advantage that both the 'negative' aspects of biocompatibility are examined as well as the proposed 'positive' functional criteria in the same animal model. These tests establish the safety of the collagen-based biomaterial and the proper performance in a specific end-use application. With respect to most collagen-based biomaterials, a significant proportion of the data on evaluation of performance has emerged from clinical appraisal. In particular, the largest studies on the immunological responses to collagen has come from the dermal Zyderm and Zyplast implants from patients undergoing predominantly aesthetic cosmetic surgery for correction of scars or wrinkles (DeLustro *et al.*, 1986b; Keefe *et al.*, 1992).

Conventional histological examination

An examination of the nature and type of extracellular matrix components and the host response to collagen-based biomaterials is an appropriate means of assessing the biocompatibility of the implant. While the host cellular response can be easily evaluated by conventional histology, it is somewhat harder to assess the matrix responses which occur and contribute to the final functional outcomes of the implant (see immunohistological examination).

The *in vivo* biological response to biomaterial implants is important for examination of the short-term effects and the long-term patency of implants. For example, in many biomaterial applications, fibrous tissue capsule formation is not uncommon. This type of host response, which involves a persisting granulomatous host tissue response and the formation of foreign body giant cells, can be detrimental to the tissue and the implant. The success of an implant will often depend on approaches to minimise the host response, particularly the chronic type of inflammatory response. In the extreme case of capsule formation, where the implant remains encapsulated and non-interactive with the surrounding tissue, methods to alter the capsule properties are essential. For collagenous implants it is important to be able to control the rate of resorption and to monitor the extent of replacement (or not) by host-derived tissue. The extent and rate of host invasion can indicate the level of cytotoxicity/immunogenicity. In addition, the degree of host response could compromise the physical properties of the device.

Perhaps the most widely histochemically characterised example of a purified and re-constituted collagen explant material is the injectable bovine collagen dermal implants, Zyderm and Zyplast, used for soft tissue contour augmentation (DeLustro *et al.*, 1986b; Keefe *et al.*, 1992). Most of these investigations have shown a typical wound healing response irrespective of the site of implantation and the type of collagen. There is an initial mild perivascular lymphohistiocytic response which resolves over a period of 3 months or so (Burke, Naughton and Cassai, 1985; Kligman and Armstrong, 1986). Fibroblasts are common but usually limited to the implant periphery during the initial healing. As the inflammatory phase subsides, fibroblasts are seen to infiltrate the collagen implant and presumably are associated with the deposition and re-organisation of new collagen. Explant analyses of collagen biomaterials based on the native structure of the collagenous tissue have also been investigated and include examples of heart valves and vascular prostheses. The tissue response to most of these biomaterials is related to the glutaraldehyde used for stabilisation. In the case of biological heart valves, this response is associated with

calcification (Ferrans *et al.*, 1980; Schoen and Levy, 1984), in the case of vascular prostheses this appears less severe (Casagranda *et al.*, 1994). Moderate infiltration was found in all glutaraldehyde-treated collagenous prostheses; a more severe cellular infiltration was observed in low-pH stabilised samples compared with those treated with glutaraldehyde at neutral pH (Casagranda *et al.*, 1994). Like the example of the reconstituted collagen implant, the tissue response subsided with time of implantation.

A variety of staining procedures are available for the histochemical detection of collagens and proteoglycans in tissues (Montes and Junqueira, 1988). Of these, the trichrome stains and the picrosirius-polarisation method are the most useful. The most commonly used of the trichrome stains are the Masson and van Gieson methods. Although these will generally stain collagen fibres well, they can give variable results. The main problem is that these stains are not sensitive enough to detect basement membranes and other small fibrillar collagenous structures, and they do not distinguish between the genetically distinct collagen types (Montes and Junqueira, 1988). The picro-sirius staining method is a specific and common histochemical method for collagen examination in tissue sections. The Sirius Red component is an elongated, strongly acidic dye which binds to the basic collagen molecule, leading to a significant enhancement of the natural birefringence (Junqueira, Bignolas and Brentani, 1979). Unlike the trichrome stains, it has been reported that this stain can differentiate between some of the different types of collagens which produce different interference colours and intensities of birefringence in a tissue section (Junqueira *et al.*, 1983).

There are significant limitations in the histochemical approach for performance evaluation of implants. The thickness of the section, for example, will have an important bearing on the birefringence status observed. For collagen-based implants, the use of these simple staining procedures is further restricted since it is doubtful how these stains can differentiate the original collagenous material from the host collagenous response. In the example given above of the bovine collagen dermal implants, there are indeed conflicting reports on the rate of appearance of newly deposited collagen around the resorbing Zyderm collagen (Burke, Naughton and Cassai, 1985; Kligman and Armstrong, 1986).

Immunohistological examination

The methodology described above which has been available for the assessment of host-biomaterial interactions has been limited to identification of inflammatory responses and general histopathological staining procedures associated with these processes. While these methods are admirable for the assessment of the cellular reactions to the collagenous implants, they are limited and confusing when trying to analyse the host extracellular matrix response which is critical to the outcome of the implant.

The collagen that accumulates during tissue repair differs from that which originally existed at the site. Dermal scars are known to have a higher ratio of collagen type III to type I compared with normal tissue (Barnes *et al.*, 1976). Tissue repair is analogous to the early developmental processes where the ratio of collagen type III to type I is also high (Epstein, 1974). The tissue response to biomaterials is also likely to vary from normal to pathological wound healing. With collagenous implants there is

a problem in that there needs to be a way to distinguish unequivocally the original implant collagen from the type of newly deposited collagen.

The rationale for the development of monoclonal antibodies: The use of antibodies against many of the proteins of the extracellular matrix, particularly the various collagen types, provides an effective means for examining the host response to an implanted biomaterial. The high degree of specificity which can be achieved using monoclonal antibodies (MAbs), where different collagen types from individual species can be distinguished, enables collagen-based biomaterials to be examined after explant (Werkmeister, Peters and Ramshaw, 1989; Werkmeister *et al.*, 1991; Werkmeister, White and Ramshaw, 1994). New host-derived collagen, which may have surrounded or infiltrated into the matrix of a collagenous prosthesis, can be specifically identified from the original collagen of the prosthesis (Werkmeister *et al.*, 1991, 1993; Werkmeister, White and Ramshaw, 1994).

The use of polyclonal antibodies against different collagen types may be subject to some degree of error. While they may be useful in some cases for detecting selected types of collagen response in the surrounding tissue, polyclonal antibodies are not sufficiently specific to allow distinction between the original collagenous components of the implant and new host collagens. In addition, there will always be a problem with batch to batch variations in the supply of the antibodies which may lead to substantial variation in evaluation of the performance of the implant. The high degree of specificity which can be achieved by MAbs, coupled with batch to batch reproducibility, makes them the preferred approach.

We have had extensive experience in the successful development and use of a library of anti-collagen MAbs in retrieval and analyses of collagen-based biomaterials. The various types of collagens have largely proved to be very poor immunogens in the generation of MAbs (Werkmeister, Ramshaw and Ellender, 1990; Werkmeister and Ramshaw, 1991a,b; Werkmeister *et al.*, 1993). A major problem has been the high degree of structural homology between collagens. Examination of the observed amino acid sequence variation between different mammalian collagens (see Kadler, 1994) suggested that type specificity would be possible, but for any given collagen type, species specificity would be difficult and dependent on very limited differences, sometimes only a single amino acid substitution. Our results in mapping the epitopes of some of these MAbs have verified the minor changes between species which have resulted in our ability to produce novel MAbs to collagen type III and to type VI which can distinguish between selected species.

Performance evaluation of a collagen tissue-based biomaterial: An example of the use of our MAbs in explant evaluation has been with the assessment of the performance of the 6mm Omniflow Vascular Prosthesis. The manufacture and design principles in this medical device will be described in detail later. This is a bio-synthetic composite material comprising sheep collagens and synthetic polyester fibres that is 'manufactured' in sheep using a silicone mandrel covered with polyester (see below). The sheep produces a normal wound healing response, which results in a fully integrated sheep collagen-polyester composite prosthesis (Ketharanathan and Christie, 1980; Ramshaw *et al.*, 1989).

During the development of the Omniflow prostheses, several modifications have

occurred, but all are stabilised in glutaraldehyde, using a proprietary procedure developed by the manufacturer (BioNova International, North Melbourne).

The biological component of the device has been well characterised and shown to comprise predominantly type I and type III collagens (Ramshaw *et al.*, 1989; White *et al.*, 1993). Of the minor collagens, type V was not readily detected by immunohistology, while type VI was localised around cellular infiltrates near the interface and around the polyester fibres. Evaluation of the performance of these prostheses in xenogenic species including dogs, rats and humans has been possible with MAb probes which are reactive with either the donor (always ovine) or recipient components but not both. The important criteria in our novel antibody technology is that the MAbs have been developed to detect new collagen deposition amongst the existing collagen framework of the prosthesis, as well as persistence of the original components of the device. The probes have proved invaluable in evaluating the importance of collagen types III, V and VI in tissue stability around and within the implanted device.

In animal and human retrieval studies, the prosthesis has demonstrated long-term patency rates comparable and often better than alternate surgical replacements (Ketharanathan and Christie, 1981; see Werkmeister, White and Ramshaw, 1994), with minimal aneurysmal or thrombotic problems which are the reported major problems associated with biological grafts (Rosenberg *et al.*, 1970; Dardik *et al.*, 1988). Much of the performance characteristics can be assessed immunohistological with MAbs and the data provide good evidence for the excellent *in vivo* performance. Two performance components which are essential for good biocompatibility of a blood vessel are structural durability and haemocompatibility. The explant analyses from both animal and human studies using immunohistochemical analyses indicate that the Omniflow prostheses is a safe and effective blood vessel replacement with scope to adapt to the next level of stringency, namely the smaller diameter coronary vessels.

Correlation of structural durability with immunohistological data: A structural weakening of the main vessel wall can lead to development of dilatations and aneurysms, resulting in a non-functional graft with major clinical sequelae. In this case of a collagenous tissue-based biomaterial, the structural integrity of the prosthesis are dependent on the persistence of the original biological components of the prosthesis, and the augmentation of these components with new host tissue.

Animal explants retrieved up to 4 years demonstrated synthesis of new dog collagen type III, prominent throughout the entire wall of the device (Werkmeister *et al.*, 1991). Persisting collagenous components from the original implant were also detected. Collagen type VI, a relatively minor component of the original device, can also be shown to persist and is augmented by newly deposited layers in pockets throughout the vessel wall after long-term implantation (Werkmeister *et al.*, 1993, Werkmeister, White and Ramshaw, 1994). Host tissue deposition of collagens, particularly type VI and later type III, in the vessel wall could be detected as early as 1 to 3 months after implantation (Werkmeister *et al.*, 1995). These early changes serve as powerful performance indicators for the further development of such collagen-based blood vessels.

The other major collagen component, collagen type I, is also present in all explants examined from 1 month to 4 years after implantation.

Investigations of human explants retrieved from periods of 2.5 to 5 years have shown similar results to the animal studies, namely tissue augmentation by new collagen type III and VI and some degree of persisting collagen component (Werkmeister *et al.*, 1993; Werkmeister, White and Ramshaw, 1994). These explants were obtained as a result of revision surgery or upon autopsy where patient death was unrelated to the implant. Like the animal studies, aneurysms and dilations were not found and the Omniflow prosthesis performs as a robust replacement blood vessel due to excellent tissue augmentation and persistence of the original components.

Correlation of haemocompatibility with immunohistological data: The Omniflow prosthesis becomes lined by a cellular neo-intimal lining which is in direct contact with the blood. The thickness of the intima varies depending on anatomical location and length of implantation time of the device. In general, the neo-intima is slightly thicker at the two anastomoses, although this does not lead to functional occlusion of the prosthesis.

The major collagen types I and III, along with collagen type VI and V, laminin, fibronectin and microfibril-associated elastic proteins are all present in the neo-intimal region of explants retrieved from dogs and humans (Werkmeister *et al.*, 1993; Werkmeister, White and Ramshaw, 1994).

Collagen type V could be detected very early during the development of the neo-intima and was associated with areas of new tissue formation particularly localised at the blood interface (Werkmeister and Ramshaw, 1991b). This was interpreted as a good sign of biocompatibility since compared with other interstitial collagen types, platelets adhere less efficiently to collagen type V (Morton, Peachey and Barnes, 1989) and subsequent platelet aggregation and thrombosis are not observed. Collagen type V is not readily detected in explants examined at longer periods of implantation and appears to be readily detected only with the early tissue growth. The distribution of collagen type VI in animal and human explants is different, being readily detected throughout the entire developing neo-intima and clearly associated with cell (fibroblast) infiltration (Werkmeister *et al.*, 1993).

The deposition of this stable connective tissue matrix in the developing neo-intima allows for endothelialisation of the blood vessel which is critical for a non-thrombogenic, haemocompatible interface. In addition, at the anastomotic junction, pannus ingrowth does occur but the extent of smooth muscle cell proliferation is not unusually prolific and is limited to these junctions (Werkmeister, White and Ramshaw, 1994). In the dog model, endothelial cells can be found continuously along the blood-contacting surface of the neo-intima, distant from the anastomoses, while in the human explants examined the blood contact zone was more complicated and consisted of an accumulation of smooth muscle cells, some endothelial cells and a cell-fibrin layer (Werkmeister, White and Ramshaw, 1994).

Calcification

A major problem which occurs in certain collagen-based biomaterials is calcification. This is particularly pertinent to the two biological xenograft heart valve devices, the glutaraldehyde-treated porcine aortic valves and the bovine parietal pericardial valves (Ferrans *et al.*, 1980; Schoen and Levy, 1984). The development of these bioprosthetic

collagen-based biomaterials has proceeded for over 30 years and current efforts are targeted towards methods which will minimise the extent of calcification. Allograft aortic and pulmonary valves also of biological origin and containing collagen do not generally suffer from calcification dysfunction (O'Brien *et al.*, 1987). Similarly, the autologous tissue valves including those fabricated from fascia lata and dura mater which are now not in clinical use were not associated with the problems of calcification (Silver, Hudson and Trimble, 1975).

Normal mineralisation occurs by two independent mechanisms, one associated with a cell, membrane or matrix-vesicle, the other involving nucleation of hydroxyapatite crystals by collagen fibrils (Strates, Lian and Nimni, 1988). Pathological calcification can be either dystrophic or metastatic, the former relating to tissue changes *per se*, the latter associated with oversaturation of calcium phosphate in tissue fluids. In the case of bioprosthetic heart valves, calcification can be either intrinsic or extrinsic. Intrinsic calcification is primarily concerned with the valve components like devitalised cells, collagen and elastin fibres, extrinsic calcification involves other components like thrombus. Strates and colleagues initially speculated on the formation of hydroxyapatite crystals around collagen and believed that the nucleation site is associated with the 65–67 nm periodicity of the native collagen fibril (Strates, Lian and Nimni, 1988). Alternatively, it has been proposed that the initiation of calcification is dependent on devitalised cells or matrix-vesicle components and collagen is involved in subsequent deposition and growth (Schoen *et al.*, 1985).

While the pathogenesis of calcification remains unclear, there are certain features which appear to be fundamental. Calcification in these bioprosthetic valves increases with time and, in agreement with animal studies, is more severe in children and young adults (Schoen and Levy, 1984). Extrinsic calcification, which is rapid and occurs on surface microthrombi appears to be less of a problem clinically (Schoen and Hobson, 1985) than the slower accumulation of calcific deposits associated with intrinsic calcification. Probably the single most important determinant associated with calcification in any bioprosthesis is the tissue preparation, in particular collagen cross-linking by glutaraldehyde (Levy *et al.*, 1983; Golomb *et al.*, 1987). This was not unique to heart valves *per se* since similar extents of calcification could be obtained with subcutaneously implanted aldehyde-treated type I collagen sponges (Levy *et al.*, 1986).

There are a variety of anti-calcification treatments which have been tried. These include treatment with surfactants, including sodium dodecyl sulphate and polysorbate 80, use of diphosphonates, various chemicals including magnesium, ferric and aluminium chlorides, and others (Levy *et al.*, 1985; Golomb *et al.*, 1987). None of these treatments have an established record in clinical trials although it would appear that the surfactants at least do work in appropriate models like the juvenile sheep model for biological assessment of calcification.

The occurrence of calcification may well be associated with the treatment process, which involves glutaraldehyde, as well as with other factors. However, the exact mechanism of how calcification occurs in these collagen-based heart valves remains unclear. It certainly appears limited to only certain applications and sources of tissue. Extensive animal and human studies with another tissue-based biomaterial, the Omniflow Vascular Prostheses, have shown no indication of calcification being a major problem (Werkmeister *et al.*, 1991; Werkmeister, White and Ramshaw, 1994). Even in

the standard non-functional, subcutaneous rat model, which is designed to accentuate the potential problems of calcification, the Omniflow prosthesis does not have a tendency to calcify (Casagranda *et al.*, 1994). Even more puzzling is the significantly lower extent of calcification in glutaraldehyde-treated autologous pericardial valves compared with the equivalent treated xenograft replacements (Jones *et al.*, 1989).

Immunogenicity

The therapeutic use of collagenous implants is historically based on the reported low immunogenicity of the bovine collagen (Chvapil, Kronenthal and van Winkle, 1973). By and large this is correct and it has been difficult deliberately to induce an immune response as is the case in the generation of monoclonal antibodies to purified collagens as discussed in the previous section.

Immune response to collagen implants can be mediated by humoral and cellular reactions. The most immunogenic domains of the collagen molecule are the procollagen and non-helical telopeptide regions, at least with respect to induction of an humoral response (see Timpl, 1984). For the reconstituted collagen biomaterials, these domains are removed by enzyme treatment. Collagen can also induce a cellular response which does not seem necessarily to coincide with the same immunogenic regions of the molecule like the telopeptide domains (Adelman, 1972). In most instances, the implant is further stabilised with glutaraldehyde which masks immunogenicity as assessed by a reduced cellular (usually in the form of hypersensitivity, particularly a delayed type IV class) and humoral (usually by the titre of antibodies in the sera) response (Oliver *et al.*, 1980; DeLustro *et al.*, 1986b; DeLustro, Mackinnon and Swanson, 1988). Natural collagenous tissues used as implants are also treated largely with glutaraldehyde to stabilise the tissue. This process also acts as a sterilant and lowers the immunogenic potential of the implant. Regardless of these dampening processes, there have been reports of adverse immune responses in a small percentage of patients receiving collagen-based biomaterials.

The majority of information has arisen directly from clinical analyses of patients receiving injectable collagen implants (DeLustro *et al.*, 1986b; Keefe *et al.*, 1992). While there are also studies from animal implantation of collagenous materials, it is the vast studies on humans which have assessed both clinical reactions and antibody responses that are more pertinent (Cooperman and Michaeli, 1984; McCoy *et al.*, 1985; Keefe *et al.*, 1992). It is also important to state clearly that while there may be some evidence of immune responses, these may not be a predictive correlation with clinical manifestations which may sometimes be due to a normal physiological response to tissue damage. The most common response reported is one of hypersensitivity and around 3% of the population will have an underlying reaction (Cooperman *et al.*, 1985; DeLustro, Mackinnon and Swanson, 1988). It is for this reason that a pre-treatment skin test is routinely performed to assess the sensitivity and suitability of prospective patients. However these tests do not screen out some patients who will develop an immune response after subsequent injections. Around a further 1% to 2% of patients will develop clinical symptoms of delayed type hypersensitivity (Cooperman *et al.*, 1985; Keefe *et al.*, 1992). Typical symptoms include a local oedematous and erythematous reaction with induration and sometimes pruritis which usually lasts from 4 to 6 months but can persist up to periods in excess of one year.

There have been several reports demonstrating the presence of serum anti-collagen antibodies in these patients with clinical hypersensitivity symptoms (Cooperman and Michaeli, 1984; McCoy *et al.*, 1985; Hartmann, Charriere and Ville, 1992). The class of antibody is usually IgG, although in a small proportion of sera IgA is also detected; IgM is rare and IgE has not been detected (McCoy *et al.*, 1985; DeLustro, Mackinnon and Swanson, 1988). In patients where there were no visible clinical signs of reactivity, there have been reports of high percentage of sera with anti-collagen antibodies (McCoy *et al.*, 1985; Sellem *et al.*, 1987). The reason for this phenomenon is unclear but may well be related to a response to beef in the normal diet similar to what is seen with the detection of anti-milk protein antibodies (André *et al.*, 1975). While there is no reason to associate the presence of these anti-collagen antibodies to any subsequent clinical sequelae, screening for these may be beneficial to further select patients more likely to have an adverse reaction (Hartmann, Charriere and Ville, 1992). In this large retrospective study of 705 patients, antibodies to collagens correlated well with the presence of cellular type reactions as previously reported. More interestingly, in a prospective study of 420 patients, 6% presented with some degree of hypersensitive skin test and a further 8.3% with a negative skin reaction had antibodies to collagen in their sera. Collagen therapy proceeded only with patients who were negative in both cellular and humoral immunological testing. Under these stringent guidelines, no major clinical adverse reactions were detected in the study, although a small percentage (0.5%) did develop indications of some form of minor reactions.

There has been limited investigations on the nature of the epitopes being recognised in sera. Multiple bands after electrophoresis were detected with a variety of sera (McCoy *et al.*, 1985). Analyses of cyanogen bromide (CB) fragments of isolated collagen chains with reactive sera have highlighted certain hot spots particularly in α1(I)-CB6 and α2(I)-CB4 fragments (Ellingsworth *et al.*, 1986). Detailed molecular studies on genetic linkage and anti-collagen responses are unclear, but it appears that associations of certain HLA-DR types, DR2/DR4 (Cooperman, Garovoy and Sondel, 1986) may be important.

While most of the large patient studies have been performed on the bovine collagen implants (Zyderm or Zyplast, Collagen Corporation, Palo Alto), a range of other commercially available collagenous biomaterials also exist and have been examined for comparative immunogenicity (DeLustro *et al.*, 1986a). Zyderm was found to be the least immunogenic, closely followed by the Atelocollagen (Koken Company, Tokyo), gelatin haemostat foams (Gelfoam Upjohn Co., Michigan) and collagen haemostat sponges (Collastat, Kendall Co., Boston). Avitene microfibrillar collagen haemostats (Avitene, Avicon Inc., Humacao, Puerto Rico) produced the highest titres of antibody which were also reactive against bovine serum albumin. There is also good clinical serological data on two bone-grafting substitutes comprising collagen, Alveoform (Collagen Corporation, Palo Alto) and Collagraft (Collagen Corporation, Palo Alto; Zimmer, Warsaw, IN) (DeLustro *et al.*, 1990). For both these collagen-containing implants there was an induced humoral response in 6.5% and 7.5 % of patients receiving either Alveoform or Collagraft, respectively. In both studies, the elevated immune response was not associated with the onset of any adverse effect, particularly the ability to interfere with fracture healing.

The immune response to bioprosthetic tissue-based implants, mostly glutaralde-

hyde-treated, has also been investigated. Collagen constitutes the tissue component in vascular grafts and heart valves and it is important that any response to this biological component is monitored. Some studies have shown that glutaraldehyde-treated xenografts are capable of eliciting both humoral and cell-mediated responses (Slanczka *et al.*, 1979; Bajpai and Stull, 1980), while others have shown little response (Christie, Ketharanathan and Perloff, 1980; Werkmeister *et al.*, 1991). Occasionally there has been the scattered report of a possible autoimmune response (Sheikh, Tascon and Nimni, 1980).

The most important question regarding immunogenicity of collagen-based biomaterials is indeed whether there is cross-reactivity in the immune response to allow the development of auto-immune diseases. Although concern has been expressed as to the possible binding of these antibodies with human collagens and the development of an autoimmune disease, no cross-reactivity has been demonstrated in studies with the injectable bovine collagen implants (Cooperman and Michaeli, 1984; Ellingsworth *et al.*, 1986). A study on the response to human placental type I collagen in rats has demonstrated both humoral and lymphoblastic cellular responses (Quteish and Dolby, 1991). In this particular report, cross-reactivity was found against collagens from other species including the host which may be a sign of an auto-immune response. Two conflicting studies have also been documented on the immunological response to collagen-impregnated vascular grafts (Canadian Multicenter Hemashield Study Group, 1990; Norgren *et al.*, 1990). Unlike the Canadian study, Norgren *et al.* (1990) found that 4 of the 11 patients who developed antibodies to bovine collagen also reacted with human collagen type I. The significance of this remains unclear as no clinical symptoms of auto-immune disease were documented. There have been reported incidences, which have appeared in the lay press, of putative associations of the development of auto-immune connective tissue disorders in a small proportion of patients receiving injectable collagen implants. In particular, the incidence of polymyositis/dermatomyositis, a very rare disease which occurs in around six out of every one million people annually, has been alleged to be elevated in the population of collagen implant recipients. However, no scientific correlations have been published to confirm such extreme linkages.

Overall, the problems with the possible immunogenicity of collagen implants seems to attract a minor but persistent audience bent on undermining the integrity of the manufacturers and the usefulness of the biomaterial under certain applications and guidelines. Apart from a small percentage of the population who will respond with mild to moderate adverse reactions, collagen as a biomaterial is largely regarded as non-immunogenic. Furthermore, with today's technology it is possible to engineer and express less immunogenic types of collagens or at least to use human collagens which would implicitly avoid the question of xeno-immunisation (see later section).

Examples of collagen-based biomaterials

In the scientific and technical (patent) literature, a diverse range of devices have been described which use collagen as the key (or only) component. A selection of these, given in *Table 3*, illustrates the broad range of medical applications where collagen-based products have potential application. To date, many of these examples have been pilot studies, in many cases limited to animal model systems, and relatively few of

Table 3. Examples of applications of collagen-based biomaterials in a range of medical areas.

Medical Area	Application	Form*	Examples
Cardiovascular surgery	Vessel replacement	T	Dardik *et al.* (1988); Ramshaw, Edwards and Werkmeister (1995)
	Heart valves	T	Ferrans *et al.* (1980); Schoen and Levy (1984)
Dentistry	Periodontal attachment	P	Yaffe, Ehrlich and Shoshan (1984); Ellender *et al.* (1992); Quteish and Dolby (1992)
	Alveolar ridge augmentation	P	Mehlisch *et al.* (1988)
Dermatology	Tissue augmentation		Knapp, Kaplan and Daniels (1978); Wallace *et al.* (1988)
Dressings		P	Yannas *et al.* (1977); Yannas and Burke (1980); Doillon *et al.* (1986); Doillon (1992)
Esophageal surgery	Augmentation	P	Remacle, Hamoir and Marbaix (1990); Natsume *et al.* (1993)
General surgery	Hernia repair	P	Adler *et al.* (1962)
		T	James *et al.* (1991)
	Adhesion barriers		Young *et al.* (1991)
	Hemostasis	P	Peacock, Siegler and Biggers (1965); Hait, (1970); Hait *et al.* (1973); Rudakova and Zaikov (1987)
	Tissue adhesives		Tardy *et al.* (1995)
Neurosurgery	Nerve conduits	P	Yannas (1988); Li *et al.* (1992)
	Nerve repair	P	Rizvi, Pins and Silver (1994)
Ophthalmology	Corneal graft	P	Tanner *et al.* (1968)
		T	Ruffini, Aquavella and LoCascio (1989)
	Vitreous replacement	P	Stenzel *et al.* (1969a); DeVore *et al.* (1994)
	Retinal retachment	P	L'Esperance (1965)
Orthopaedics	Bone repair	P	Benfer and Struck (1973); Moskow, Gold and Gottsegen (1976); Deporter *et al.* (1988); Stastny, Hora and Stol (1993)
	Articular cartilage reconstruction	P	Speer *et al.* (1979); Stone *et al.* (1990)
	Cruciate ligament reconstruction	P	Chvapil *et al.* (1993); Suganuma *et al.* (1994)
		T	Roe *et al.* (1992)
Otology	Tympanic membrane replacement	P	Salen and Simbach (1965); Abbenhaus and Hemenway (1967)
Urology	Ureter replacement	P	Tachibana, Nagamatsu and Addonizio (1985)
	Renal repair		Tanner, Marcucci and Bradley (1968)
	Dialysis membrane		Stenzel *et al.* (1969b)
	Urinary incontinence	P	Herschorn, Radomski and Steele (1992)
Other	Drug delivery	P	El-Samaligy and Rohdewald (1983); Gilbert and Kim, (1990); McPherson (1992)

* Form of device: T: tissue-based, P: purified collagen-based

these applications have been fully developed to the stage where they can provide commercially available materials. In some cases, the likelihood of products becoming commercially available is probably low since either the demand is too low to justify the development and registration costs, or alternative materials at lower cost are available. However, the versatility of collagen, particularly in the way it can be made into composites with other biological factors, provides opportunities which may not be as readily obtained by synthetic polymers. Of the commercially available products, some illustrative examples, based on the different formats which are found, are described briefly below.

TISSUE-BASED DEVICES

The main medical area where tissue-based collagen devices have been used is cardiovascular applications. The examples show three different approaches to the manufacture of tissue-based devices. These are:

1. Manufacture of a device which uses the natural form and shape of tissue.
2. Use of tissue in sheet form to manufacture a device with a more complex format.
3. Growth of tissue, using a mandrel, to provide tissue in the desired shape and size.

In each of these strategies, glutaraldehyde has been used as a stabilising agent, to give long-term durability to the devices.

Tissue-based devices have clearly been very beneficial; for instance, cardiac valve replacement has significantly improved the average life expectancy of patients suffering from valvular heart disease, giving enhanced cardiovascular function and improved quality of life. With the continued increase in the incidence of cardiovascular disease, it may be expected that research to improve these devices will continue to be important.

Heart valves

The first aortic and mitral valve replacements were performed in 1960/1961 using mechanical valves (Starr and Edwards, 1961), and shortly after, the use of aortic allograft (heterograft) valves was introduced (Carpentier *et al.*, 1969). Subsequently, a variety of xenograft, tissue-based devices have been developed as commercial products, which provide effective alternatives to mechanical devices for particular patient groups.

The most widely used natural tissue heart valve substitutes have been the porcine aortic valves (particularly the Hancock and Carpentier-Edwards) and bovine pericardium (particularly the Ionescu-Shiley). The porcine valve devices consist of the porcine aortic valve and the adjacent region of the aortic root (Ferrans *et al.*, 1988), thus taking advantage of the natural tissue architecture for this complex device. As part of manufacture, the porcine device undergoes changes, some post-mortem and some due to the subsequent processing. These changes include complete loss of the endothelial layer which covered the tissue, partial loss of proteoglycans particularly from the spongiosa of the cusp and a loosening of the connective tissue matrix. Pericardial devices are mostly made from bovine pericardial tissue, which is cut and arranged to give a valve (predominantly trileaflet) with an architecture which approxi-

mates that of a natural valve. Compared with the aortic valve, the thickness of the cusps is greater and the junctions show defined edges. The collagenous structure of these devices is well defined, and similar changes to those which occur for porcine valves are also found during processing.

All tissue-based valves are stabilised with a cross-linking agent, with glutaraldehyde being the predominant choice of reagent. The specific conditions used in stabilisation will effect the product. For example, while low concentrations of glutaraldehyde are used to prevent an excessively stiff product, even then, the products are less compliant than native tissue.

In both these devices, a major advantage of using collagen in the device is its strength and the durability, while for the porcine valves the natural architecture of the tissue is also being exploited. While these valves frequently show deterioration over time, this is slow and is unlikely to result in a sudden, catastrophic failure such as has been observed with certain mechanical devices.

One of the main advantages seen for the biological devices compared to the mechanical devices is that thromboembolic complications are less frequent and hence long-term anticoagulant therapy is not normally required. However, since about one third of patients do require such treatment for other reasons, this advantage is not always present. In some cases, the choice of whether to use a tissue-based or mechanical device is determined by specific patient needs. For example, women of child-bearing age would preferably avoid anti-coagulation therapy which would cross the placental barrier.

In general, tissue devices have finite durability, so the advantage with low thromboembolism advantage is balanced by the need for future additional operations to correct for degenerative failures. Tissue devices undergo degenerative processes which limit their long-term durability. Short-term mortality, within 1 month of surgery, is normally due to the poor condition of the heart prior to surgery, whereas in late mortality, about 50% is due to valve failure (Ferrans *et al.*, 1988). Detailed statistics are now available defining the problems, and show that calcification is a problem in 86% of cases, with both calcification and cuspal tears being present in 75% of cases. Calcification increases with time after implant; it is evident in most patients after 3 years, although in some patients it is absent even after 10 years. It is most evident in children and young adults (Schoen and Levy, 1984). Calcification frequently occurs at the site of cuspal attachment, the location of the greatest mechanical stress (Ferrans *et al.*, 1980), leading to stiffening of the cusps. This in turn, reduces their mobility, leads to an increase in regurgitation and eventually leads to tears and perforations, although tearing may also occur in the absence of calcification. Thus tissue-based valves are possibly more favourable for the first 10 years, but not necessarily beyond.

Other potential performance problems for tissue-based valves include fibrous sheathing, where a sheath of host origin spreads over both surfaces of the cusps. At low levels, it may provide a benefit in reinforcing the cusp structure, but when too thick, it can be deleterious leading to stiffness and associated problems. All devices, tissue or mechanical, have a non-biological sewing ring which surrounds the device at the orifice at the base and which is used for suturing the device into the surgically prepared location. While endocarditis associated with this ring is infrequent, it is a major problem when it does occur, leading to abscesses and serious sequelae (Ferrans *et al.*, 1988).

Vascular prostheses

The general increase in cardiovascular disease has led to a demand for a range of vascular replacement devices. A wide range of diameters are required for varying surgical needs. These include micro-arterial replacements, < 1.5 mm, where manipulation under a microscope would be required, small-arterial replacements, 1.5 mm–4 mm, where normal surgical techniques may be used, and larger diameter devices (Loisance, 1984). Whereas satisfactory larger diameter devices made from synthetic polymer materials, such as polyester, are now available for thoracic, abdominal and, to some extent for peripheral surgery, the main demands on biomaterial technology are still for adequate small diameter devices. In particular, while the use of autologous saphenous vein or mammary artery has proved an extremely valuable approach for coronary by-pass surgery, in many potential patients, perhaps up to 30%, suitable autologous material is unavailable.

The main potential for tissue-based vascular devices is for small (<5 mm) diameter applications, where the versatility of the biological material presents opportunities for improvement which may not be available for synthetic polymers. Two categories of tissue-based conduits have been used for vascular replacement; these are modified and stabilised, naturally occurring, biological tubes, such as human umbilical vein, and fabricated collagen tubes. In practice, the various devices based on natural vessels, for example bovine artery prostheses, have generally lacked durability and failed due to aneurysm or dilatation problems (Rosenberg *et al.*, 1970; Dardik *et al.*, 1988).

Despite the problems with devices based on natural vessels, a successful tissue-based device has been developed in which the tissue is grown into a tube through use of a mandrel. This device, the Omniflow Vascular Prosthesis, is a fibre-reinforced, mandrel-grown, collagen conduit (Ketharanathan and Christie, 1980,1981). This device is produced by allowing *in vivo* collagen deposition to occur around a polyester mesh on a silicone mandrel which has been implanted in a sheep. After explant, the conduit is stabilised by glutaraldehyde and the silicone tube removed. This process fully embeds the polyester mesh within the collagen matrix. A detailed review of the development of this device has been presented elsewhere (Ramshaw, Edwards and Werkmeister, 1995).

Collagen-based devices may have certain advantages compared with the synthetic biomaterial devices, including a lower risk for infection, no need for a highly porous structure since host tissue invasion can readily occur (Werkmeister, Peters and Ramshaw, 1989), and a better match of physical properties to those of natural vessels. It has been suggested that a mismatch of mechanical properties can be detrimental to prosthesis performance, particularly at the anastomoses as this leads to increased turbulent flow and an increased risk of thrombosis (Kidson, 1983). Improved compliance matching, which can be achieved by collagen-based devices may be advantageous for long-term patency (Werkmeister *et al.*, 1991; see Werkmeister, White and Ramshaw, 1994).

PURIFIED COLLAGEN-BASED DEVICES

Injectable collagen

The important properties of collagen that are being utilised in these products are that

they are non-immunogenic and can reform into fibrils analogous to native collagen fibrils, retaining normal interactions with cells and other connective tissue components. This fibril formation can take place either *in situ*, when a collagen solution in an appropriate buffer warms to body temperature, or prior to placement into tissue. In the latter form, however, the material still retains appropriate rheological properties which allow it to be injected in a fluid form.

The demonstration that moulded, fibrous gels of collagen could be reconstituted by warming purified soluble collagen (Gross and Kirk, 1958) provided a method whereby the response to heterologous, purified collagen could be evaluated in animal models (Grillo and Gross, 1962). These materials were evaluated by subcutaneous implantation which indicated that there was a moderate fibrotic response, with apparent gradual replacement of the material by host connective tissue.

Later, based on the same principle of thermal gelation of collagen solutions, it was proposed that the formation of the insoluble fibre mass could occur *in situ* after injection of the collagen solution into a desired site (Knapp, Kaplan and Daniels, 1997). It was suggested that this approach would be useful for soft tissue augmentation or restoration, and in particular for the correction of various skin contour defects. Subsequently, instead of using a collagen solution/gel for injection, it was suggested that a dispersion of preformed, fine collagen fibrils could be used as the injectable form of the collagen since under appropriate conditions this material would be fluid for injection (Wallace *et al.*, 1988).

Commercial products based on these technologies have been used extensively in dermal applications, and a substantial body of information on their biological performance is now available (see above). Also, further possible applications have been suggested, including use in correction of problems of the oesophageal sphincter, of the vocal cord and in various dental conditions including periodontal disease.

Wound and burns dressings

The development and application of collagen, specifically type I collagen, as a wound or burns dressing is largely due to the inherent biological benefits of the molecule which have been discussed in detail in the previous section. Like any biomaterial intended for use as a dressing, collagen dressings have to allow an optimal healing environment, be able to control heat and water loss, act as an effective microbial barrier, handle excess exudate and come in various forms. They have to be easily sterilised and stable and, most of all, they must be non-toxic, non-allergenic and non-immunogenic.

The process of wound healing and tissue repair is a complex pathological process, encompassing inflammation, granulation tissue formation, tissue remodelling and extracellular matrix modification. The process is dependent on the interplay between many different cell types, growth factors and the extracellular matrix. Optimal repair requires a rapid replacement of both epidermal and dermal constituents (where required) with controlled or delayed formation of granulation tissue, which will lead to minimal hypertrophic scar tissue and wound contraction. To be successful as a wound/burns dressing, the collagen biomaterial must be biocompatible and able to maintain an effective tissue repair. In the case of partial thickness wounds, spontaneous healing and tissue repair follows, but in more

severe cases which necessitate full thickness dressings, a further skin replacement is usually required.

Collagen dressings can be prepared in a variety of different forms including membrane sheets, sponges and powder (Miyata, Taira and Noishiki, 1992; Ellender *et al.*, 1992), are resorbable and have the essential biological properties which make them prime candidates for such an application (Chvapil, 1982; Ramshaw, Werkmeister and Peters, 1990). In particular these materials use the advantages of collagen to interact with the extracellular matrix to promote cell adhesion, migration, growth and deposition of new connective tissue (Chvapil, 1977), to induce differentiation (Kleinman, Klebe and Martin, 1981), to induce chemotaxis of fibroblasts (Postelthwaite, Seyer and Kang, 1978), to delay wound contraction (Yannas and Burke, 1980), to accelerate wound repair (Doillon *et al.*, 1984) and under certain fabrications, the ability to absorb wound fluids (Chvapil, 1982).

The idea of using collagen as a dressing dates back to the early part of the century where natural tissue in the form of porcine skin and amnion was used for the treatment of burns (Davis, 1910; Sabella, 1913). What is more useful as a wound dressing today is the reconstituted forms of collagen which can be purified and fabricated into a number of specific designs (Miyata, Taira and Noishiki, 1992; Ellender *et al.*, 1992; Doillon, 1992; Yannas, 1992).

Multilayered 'synthetic or artificial' skin incorporating solubilised collagen and glycosaminoglycans (GAGs) has been extensively developed by Yannas and colleagues and has recently been reviewed (Yannas, 1992). The dressing comprises an inner highly porous biodegradable layer of glutaraldehyde cross-linked bovine collagen type I reconstituted with chondroitin-6-sulphate from shark cartilage and an outer layer of polydimethylsiloxane (Yannas *et al.*, 1977). The collagen dressing was designed to enhance wound closure and minimise infection and fluid loss (Yannas and Burke, 1980; Yannas *et al.*, 1980). This type of dressing referred to as a stage 1 membrane (Yannas, 1992) is unique because of its ability to induce normal dermis formation. A variety of modifications to this principle have also been documented, for example the development of a less immunogenic material (Suzuki *et al.*, 1990), the use of alternative GAGs to produce differing collagen biodegradation rates (Yannas *et al.*, 1975), and methods to control the thickness and pore size (Doillon *et al.*, 1986; Yannas *et al.*, 1989; Berthod *et al.*, 1994) which is vital for optimal tissue ingrowth. In the latter case, chitosan was included to enhance the mechanical and wound healing properties of the material which comprised collagen type I and III, and GAGs without any chemical (glutaraldehyde) cross-linking agent (Berthod *et al.*, 1994). In all these cases of stage 1 collagen 'artificial' skin substitutes, the dressing acts as template for new matrix formation by allowing infiltration of fibroblasts and also serving as a potential template for re-epithelialisation. The silicone is normally removed after around 20 days and autologous epidermal cells are grafted to the newly formed dermis.

A second approach described by Bell and colleagues introduced cell seeding into skin replacements of hydrated rat tail collagen lattices (Bell *et al.*, 1981, 1983) Autologous fibroblasts were incubated with collagen and after gel contraction, this dermal layer was seeded with autologous epidermal cells which had been first propagated *in vitro*. The main problem with this approach is that two skin biopsies are required and the whole process involves cell culture and valuable time, usually around

1 month or more to generate the finished product. Allogeneic fibroblasts with autologous epidermal seeding appears to be a satisfactory compromise which reduces the time, does not result in immune rejection (Bell *et al.*, 1983) and performs well in clinical trials (Hull, Finley and Miller, 1989).

Another advance has combined the Yannas stage 1 membranes with the concept of cell seeding with autologous basal (epidermal) cells to generate stage 2 skin replacements (Yannas *et al.*, 1982). In this development, dermal tissue is again generated in the patient but is accompanied by a mature, keratinised epidermis within 2 weeks as well (Yannas *et al.*, 1982; Yannas, 1992). Modifications to this approach have included using the collagen matrix as a biological and mechanical support for the growth of epidermal cells and then transferring these cells onto the wound (Boyce and Hansbrough, 1988; Heck, Bergstresser and Baxter, 1985). In this procedure, the epidermal cells become well differentiated (Yoshizato, Nishikawa and Taira, 1988) on a developing basement membrane (Doillon, 1992).

Other more complex connective tissue matrices have also been developed, some comprising mixtures of collagen type I and III with elastin or elastin-solubilised peptides (Lefebvre *et al.*, 1992). In addition, various supplements to the collagen-based dressings have been formulated. These include anti-microbial additives (Rao and Joseph, 1988) and matrix enhancing agents like hyaluronic acid and fibronectin (Doillon and Silver, 1986) or heparin (McPherson *et al.*, 1988) which enhance the wound healing response. More recent developments have involved the supplementation with growth factors like basic fibroblast growth factor (bFGF), platelet-derived growth factor (PDGF), and epidermal growth factor (EGF) (Boyce, Stompro and Hansbrough, 1992; Roy, DeBlois and Doillon, 1993; DeBlois, Côté and Doillon, 1994) also to promote optimal enhanced wound healing.

In general, collagen-based wound/burns dressing biomaterials are not major commercial products yet, even though the market for such products is large and the experimental research has been extremely promising. Clinical success stories date back to the early part of the 1980s, for instance in the case of Mark Walsh who received severe burns to 80% of his body and survived through use of this pioneering technology (see Maugh, 1985), to more recent clinical trials with modified dermal substitutes like those made from collagen-GAG-chitosan (Damour *et al.*, 1994). In the absence of availability of an appropriate autograft, collagen-based dressings of various types offer a true and beneficial outcome to the patient. Further refinements of the basic material to include additional matrix components or growth factors must take into account the cost-effectiveness of the final commercial product. Apart from the application for specific burns areas, there are a number of other specific applications which have been documented. For example, porcine dermal collagen (Zenoderm, Ethicon) has been used as a successful dural substitute (Pietrucha, 1991) and collagen conduits have been used for successful nerve repair (Li *et al.*, 1992).

Collagen as a haemostat

The important property of collagen that is being utilised in these products is that rapid coagulation of blood may be achieved through its interaction with platelets, with the subsequent associated aggregation and release phenomena. The mechanical effect of providing a temporary framework while the host cells regenerate their own fibrous

stroma can also be important in ensuring the success of the products, as is their ability to be resorbed within a few weeks after application.

The products available come in various physical forms, including powders, fleece and sponge. Commercially, these products have to compete with a range of other materials, including cellulose, gelatin and fibrin-derived products, many of which are less costly to produce. In addition, numerous collagen-based products are competing for the same market niche. These include Avitene, Tachotop, Pangen, Helistat, CollaCote and several others (Ramshaw, Werkmeister and Peters, 1990).

Initial studies on a collagen-based haemostat (Goldberg and Burgeson, 1982) indicated that it performed effectively, and was superior to previously available materials. The material used comprised insoluble collagen from bovine tendon which was pretreated with ficin to digest non-collagenous proteins. It was then dispersed and homogenised in an acid medium to give a swollen fibrillar mass which was then lyophilised to give a sponge. To provide extra resiliency, the sponge was stabilised by 0.15% formaldehyde.

Subsequently, a 'microcrystalline' collagen product was described which was shown to be a powerful haemostat and substantially better at inducing coagulation than reconstituted collagen (Hait, 1970). This early commercial product, Avitene, was prepared from bovine corium by controlled and combined conditions of pH and attrition. Other products have now appeared based on collagen from other species, for example horse, and using non-dermal tissue, such as tendon (Adelmann-Grill and Otto, 1987).

Most products are made from native, cross-linked collagen which retains its telopeptides; this type of material is more cost effective to produce compared with soluble collagen products. Despite the retention of the telopeptides, the potential immunogenicity of the materials has not proved detrimental to their use. On the other hand, in the preparation of comminuted collagen products it is the contaminating non-collagenous proteins which can sometimes prove to be the major immunogenic impurities (DeLustro *et al.*, 1986a).

The use of collagen-based haemostats has been proposed for reducing blood loss in generalised bleeding in a wide number of tissues, and the use of the collagen haemostats is particularly useful for management of wounds to cellular organs, such as the liver or the spleen. These tissues tend to lack connective tissue support, and thus provide examples of a haemorrhaging, exuding, cellular surface for which a fibrous protein framework is needed while the process of repair occurs (Schiele, Kuntz and Riegler, 1992).

A variety of studies have shown the effectiveness of collagen haemostats and have compared them with alternative materials such as oxidised cellulose. In general, these studies demonstrate that the collagen-based haemostats perform well and are useful products. The comparative studies, however, while enthusiastic about the advantages of the collagen haemostats, do not show a clear cut superiority of performance, and the other products may well be equally acceptable in many procedures.

A potential disadvantage of the collagenous materials is that they could lead to an increased risk from infection compared with oxidised cellulose products which are less supportive of bacterial growth due to a more acidic pH. However, the risk of infection may be accentuated when an excess of haemostat is used, since excess of any foreign material favours the promotion of infection (Ramshaw, Werkmeister and Peters, 1990).

Future directions

To date the only fabricated collagens approved for commercial applications are derived from animal sources, primarily bovine. While there is ample evidence of successful uses of collagen biomaterials, it is nonetheless a foreign protein which raises some concern regarding safety, including immunogenicity and transmission of infective diseases like bovine spongiform encephalopathy (BSE) or Creutzfeldt-Jacob disease. The general consensus has been that human collagens are likely to be more biocompatible and perform better than the animal counterparts, which already have distinct advantages as outlined in the previous sections. The problem until recently has been mainly one of availability. Traditionally, the only reliable source has been from human placenta (Tayot and Tardy, 1987), although the idea of extracting collagen from dead human tissue is subject to ethical and health issues. Fabrication and use of collagen from human tissue will need mandatory stringent testing for viruses and other transmissible infective agents.

A viable alternative in the light of today's advancement in molecular biology techniques is the expression and purification of human collagens by recombinant DNA or engineered technologies. The full exploitation of collagen as a biomaterial would be greatly accelerated by the development of effective methods to achieve large scale production of recombinant collagen at a competitive price. There are many patents relating to new developments of collagen for various applications. However, until recently, all these patents have been based on developments and improvements of the animal tissue derived collagens.

Perhaps it is time to take the next leap forward to the next level of development using more sophisticated technologies like recombinant DNA processing. This goal, if one looks at the current activities and patents appearing, may not be very far away.

Expression of many exogenous genes is now commonplace and has been achieved in a variety of recombinant host-vector systems, including bacterial, yeast, baculovirus, mammalian and others. The problem with collagen molecules is that expression and production of functional protein is complicated by the many post-translational processing events which are involved; these have been discussed in previous sections (*Figure 1*). Of the modification enzymes, prolyl-4-hydroxylase, a tetramer comprising two α and two β subunits, is a critical enzyme which hydroxylates prolyl residues in the Y-position of the repeating -G-X-Y- sequences to 4-hydroxyproline. This enzyme which is essential for collagen stability has been recently expressed in baculovirus (Vuori *et al.*, 1992). For commercial applications of recombinant human collagens for medical applications, it would appear that the genes encoding this enzyme need to be co-expressed as well.

In all, it is essential to develop vectors and expression systems which will allow high level expression of a functional protein. Two patents have appeared in the public domain which have taken into account these requirements and claimed expression of human recombinant procollagen/collagen. The first approach (Prockop *et al.*, 1993) involves various gene constructs comprising COL1A1, COL2A1 and hybrids of these with appropriate promoters and signal sequences. The invention includes transfection/transformation and expression of various collagen gene constructs into selected competent cells of yeast, insect or mammalian origin, and where necessary, incorporation of the genes encoding the prolyl-4-hydroxylase.

An alternate approach (Berg, 1994) would be to produce human recombinant collagen in the milk of transgenic animals. The invention is directed to developing expression systems compatible with the mammary glands of a non-human mammal comprising the genes for the human (pro)collagen and the prolyl-4-hydroxylase. Several suitable promoters for milk-specific proteins are available for ligation to the collagen and enzyme constructs, and these can be generated from human genomic or cDNA libraries. The idea in this approach is to micro inject the appropriately designed expression systems into fertilised oocytes or embryonic stem cells *in vitro* and re-implant the cells into surrogate mothers.

In both these systems, the genetic materials for use in either approach are well documented. While the currently available collagen-based biomaterials are based on type I collagen, the availability of the genes for all the other collagen types may well provide opportunities for materials targeted to the specific anatomical applications where they are normally present (*Table 1*).

Once obtained, the recombinant approach has the flexibility and potential to produce a line of second generation products, engineered to be intrinsically more biocompatible for specific end-use applications. For instance, the vast knowledge now available on the structure, function and regulation of collagen, particularly in relation to its interactions with biological systems, would allow one to create specifically a molecule and biomaterial with increased or decreased stability, immunogenicity, haemostatic capacity and selective binding ability. Two other possibilities also emanate from this line of thought. One is to design and express a totally synthetic analogue of the natural protein, the other is to develop entirely novel biologically enhanced biomaterials.

To date, no protein designed *de novo* with a folding pattern to mimic the natural properties of complex proteins has been expressed. Certainly the tools of modern molecular biology and fermentation technologies should permit this development. Already there is emerging evidence that it is feasible to design and produce protein polymers. One example of this approach is with silk which is composed of long repeats of the dyad alanine-glycine. Using recombinant protein polymers, it is possible to control the length of the dyad repeat and also to introduce hydrophilic amino acids such as glutamic acid to engineer a structural turn between β-sheets (see Hubbell, 1995). There is also evidence of this in relation to designing synthetic collagen or gelatine molecules (Cappello, 1993), although in this case there is no evidence for formation of a triple helical structure stable at body temperature from the expressed synthetic collagen chains.

The development of biofunctional or biomimetic materials appears to be a logical step in the progression of this developing technology cascade. The wealth of growing information on the importance of integrins and cell attachment ligands makes them good candidates for grafting onto the collagen materials to create biomaterials with enhanced or selectively directed biological properties with novel functions. This concept is not without precedent. Ligands like the ubiquitous RGD and YIGSR sequences from fibronectin and laminin for instance have been engineered onto exposed surface of proteins like silk (Cappello, 1992; see Hubbell, 1995). The repeating pentapeptide of elastin (GVGVP) has also been chemically oligomerised and expressed as a recombinant protein with engineered cell attachment sequences (Nicol *et al.*, 1992).

Future design and generation of collagen-based products could also incorporate cytokines or growth factors which are critical for wound healing. The process of tissue repair is complex and involves continual interactions between cells, matrix and growth factors. Indeed there are emerging reports discussed in previous sections on the incorporation of growth factors like transforming growth factor beta (TGF-β), platelet-derived growth factor (PDGF), fibroblast growth factor (FGF) and epidermal growth factor (EGF), all of which are well characterised and known to accelerate wound repair. As we learn more about the mechanisms and control of these growth factors, it should be possible to design technologies to incorporate the protein or preferably the genetic code directly into the engineered biomaterial to generate a bioactive/biomimetic material. For example, it was recently shown that a pentapeptide from type I procollagen has a positive effect on switching on collagen synthesis in a variety of mesenchymal cells (Katayama *et al.*, 1993). It is important that both the matrix (Berthod *et al.*, 1993) and growth factor (Garner *et al.*, 1993) be part of the equation in designing advanced biomimetic materials since the healing cellular responses from different matrix environments will respond differently to the same cytokine. Human fibroblasts taken from a matrix environment of hypertrophic scarring will respond differently to EGF and TGF-β when compared with normal dermal fibroblasts (Garner *et al.*, 1993).

Overall, it is clear that many factors need to be taken into account in order to optimise the biocompatibility of emerging future collagen-based biomaterials. Engineering the wound healing response as well as the material *per se* would seem to be an appropriate goal. Recombinant DNA technology and our knowledge of collagen and tissue repair can be combined to generate biologically-enhanced collagen-based biomaterials with specifically designed characteristics to allow interaction and manipulation of the intending tissue environment.

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