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REVIEW

Structure and function of ECM-inspired composite collagen type I scaffolds

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Collagen I is one of the most abundant molecules in vertebrates constituting major parts of the fibrillar extracellular matrix (ECM), thus providing structural integrity and mechanical resilience. It has therefore become an almost ubiquitous biomolecule to use in contemporary biomimetic cell culture scaffolds and in tissue engineering scenarios where new functions for biomedical applications are sought. As collagen I easily self-assembles into fibrillar structures, a number of approaches aim to integrate new functionalities by varying the compositional complexity of the developed scaffolds. Such composite matrices make use of the abundant knowledge about the fibrillar collagen I structure and its binding sites for other ECM molecules. This review gives an overview of the reconstitution of collagen I scaffolds by the implementation of other organic biomolecules. We focus on the self-assembly and structure of the collagen I fibrils affected by the interaction with cofactors and comment on mechanics and biomedical use of such composite scaffolds.

1 Introduction

Supramolecular collagen assemblies provide the most fundamental platform in vertebrate organisms for the attachment of cells and extracellular matrix (ECM) molecules, thus being crucial for the mechanical stability of tissues.[1] Collagens form a large protein family containing more than 40 genes encoding various alpha chains which can form at least 29 members. They are characterised by three alpha chains with repeating Gly-X-Y sequences, where X and Y are often proline and 4-hydroxyproline, respectively.[2] In tissues, 'monomeric' triple-helical collagens assemble into higher-ordered structures such as fibrils or fibres, and networks or sheets. One of the most remarkable features of collagens is their ability to endow resilience to hierarchical structures. Therefore depending on their function the arrangement of the fibres can vary significantly. This is often mediated by interaction with other ECM molecules, such as proteoglycans

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(PG), glycosaminoglycans (GAG), and elastic fibrils, which cooperatively modulate the structure and mechanics of such supramolecular complexes.[3,4] The existence of such heterotypic collagen structures is necessary to tune structural properties, including fibril size, banding periodicity, and interfibrillar connectivity.[4–6]

One of the most challenging tasks of tissue engineering is to develop constructs that are functionally equivalent to damaged or lost tissues.[7] To ensure the structurally related functions of such systems it is pivotal to comply with their biomechanical and bioadhesive requirements.[8] In that sense, collagen type I fibrils are the most abundant and major tensile element of the ECM in a number of animal connective tissues, including tendon and bone. They provide the necessary strength to accommodate the uniaxial and multiaxial mechanical loading to which these tissues are commonly subjected.[9] Due to its superior mechanical properties, ability to self-assemble *in vitro*, and hierarchical structure, collagen I has become an ubiquitous molecule for the development of biomechanical scaffolds and a biomaterial for controlled cell attachment and tissue engineering.[10,11] The development of artificial and reconstituted scaffolds based on natural biopolymers, such as collagen I, is an established technique which is often preferred to the recently emerging and promising approach to use decellularised xenogeneic native ECMs. The latter is advantageous, as it provides an acellular and preserved three-dimensional structure and composition of the particular tissue of interest, but is still under debate due to the potential immunogenicity of particular ECM components and epitopes.[12] Furthermore, the development of novel bioinspired ECM scaffolds has the potential to selectively modulate their structural complexity, number of components, and correspondingly functions. The utilisation of collagen I in tissue engineering applications is further spurred by its importance to tissue integrity, as well as ability to interact with nearly 50 known binding partners.[9]

The development of such bioinspired matrices for tissue engineering has to comply with the complexity and structural variety of native ECMs. As mentioned already, this is mainly attributed to the structural regulation of collagen I by other molecules, such as other collagens, non-collagenous proteins, GAGs and PGs. The considerable amount of research studies carried out on pure collagen systems have made a major contribution to our current understanding of the kinetics of collagen I fibril formation, and its influence by a number of factors, as well as the initial cell adhesive behaviour in such cell culture systems. Nevertheless, it is pivotal to address the influence of other ECM constituents, as such interactions represent more closely the *in vivo* situation, and provide additional opportunities to control structural organisation.

This review focuses on the development and application of composite collagen type I-based matrices which allow additional and new functionalities to be used in contemporary tissue engineering strategies by implementation of other organic ECM molecules. We do not discuss the usage of inorganic materials in composite collagen I scaffolds, as such approaches like the bone tissue engineering scenario do not directly relate to the fibril assembly process and fibrillar structure of collagen I in most currently reconstituted scaffolds. To this end we start with an overview of the classical collagen type I self-assembly and its structural model, focusing on some of the factors involved in its fibril growth and size. We further review the mechanical properties of composite scaffolds, by emphasising the relationships among their biochemical composition, structural organisation, and nanomechanical properties. Finally, we highlight the use of collagen-based matrices in biomedical applications and give examples for their potential to direct cell behaviour by matrix characteristics.

2 Classical collagen I self-assembly and structural hierarchies

The versatility of collagen as a building material is mainly due to its complex hierarchical structure originating from its molecular sequence. The collagen hallmark is a 300 nm long and 1.5 nm thick molecule called tropocollagen, composed of three polypeptide alpha chains, each with a regularly repeating amino acid motif (Gly-X-Y), where X and Y can be any amino acid but mostly proline and hydroxyproline, respectively.[13] Such a redundant motif allows the formation of the right-handed triple helical structure accepted today, where all glycine residues are buried within the core of the protein and residues X and Y are exposed to the surface. This arrangement allows for a single interstrand helical bond per triplet and a tenfold helical symmetry with a 2.86 nm axial repeat (10/3 helical pitch).[14–16] Depending on the type of alpha chains which comprise the tropocollagen molecule and are characteristic of each collagen type, the triple helices can be either homo- or heterotrimeric. Typically collagen type I is mainly found in nature as a heterotrimer composed of two α1(I) and one α2(I) chains, but the α1(I) chain is also able to form homotrimers. Although homotrimeric *vs.* heterotrimeric molecular composition seems to have minor effects on the stability of the triple helices, certain hypotheses exist that it might be related to fibrillar diameter and mechanical properties of collagen type I.[17] The three α chains are held together by a number of interchain hydrogen bonds, while highly ordered hydration networks surround the tropocollagens.[18,19]

The central part of tropocollagen type I is comprised of a central triple-helical region consisting of the abovementioned Gly-X-Y repeats ($n = 337$–343, depending on the source) further flanked by short non-helical regions called telopeptides, about 20–30 residues in length, at both N- and C-termini.[13] *In vivo*, all fibrillar collagens are normally expressed as soluble precursors, namely procollagens, with additional large N- and C-terminal propeptide domains. The propeptides serve a role in the initial assembly of the tropocollagen monomers, and ensure the solubility of the procollagen molecules.[18] The initiated self-assembly of collagen fibrils is ensured at later stages by the enzymatic removal of the C- and N-propeptides, by specific metalloproteinases, leaving the short C- and N-telopeptides. The N-terminal processing of collagen I is typically complete, leaving the short N-telopeptides playing a role in fibrillogenesis at a later stage.[10,13] Furthermore, it has become almost ubiquitous that the telopeptides in collagen I are removed during isolation protocols as they carry an immunogenic potential upon the introduction of newly developed scaffolds into xenogeneic hosts (Fig. 1A).

Despite numerous efforts, it remains unclear how collagen type I triple helices precisely self-assemble into D-periodic cross-striated micron length fibrils with a regular axial pattern of 67 nm.[20] The general notion is that fibrillogenesis is an entropy-driven

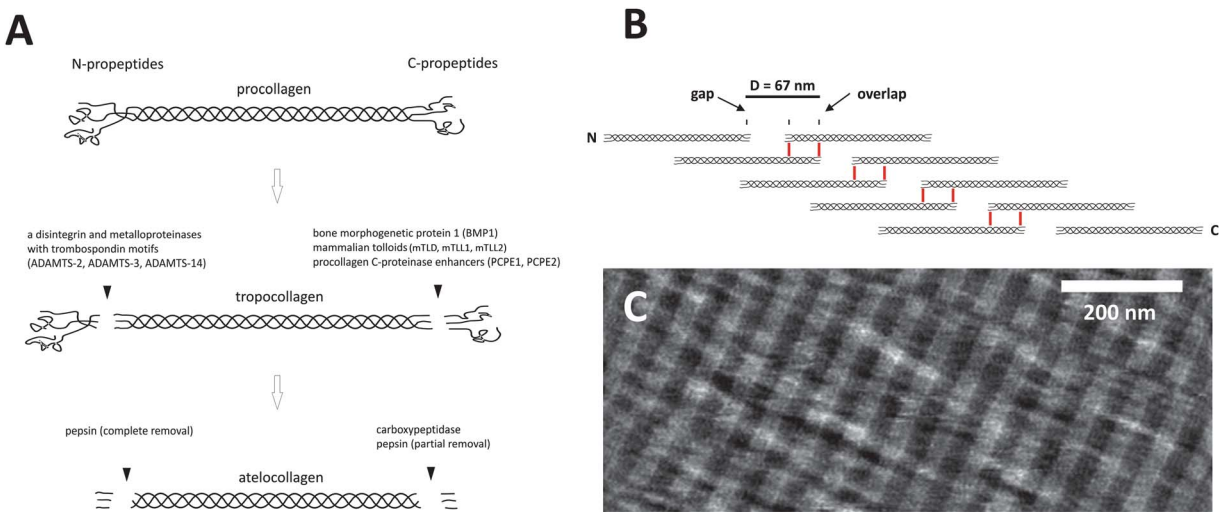

Fig. 1 Enzymatic processing and axial structure of collagen type I. N- and C-terminal *in vivo* processing of soluble procollagen type I to tropocollagen monomers (A) which are ready to self-assemble into fibrils. For tissue engineering (*in vitro*) applications, tropocollagen is often further processed by a non-specific (pepsin) or specific (carboxypeptidase) hydrolytic removal of the telopeptides, leading to formation of atelocollagen. The typically unidirectional lateral staggering of the 300 nm-long tropocollagen monomers results in the formation of high and low electron-dense regions, often referred to as 'overlap' and 'gap' (B). Some characteristic cross-links, involving telopeptide lysine/hydroxylysine and near-end triple-helical hydroxylysine residues are shown here in red. (C) A high-resolution scan of collagen I reconstituted from atelocollagen, showing that the regular axial D-pattern of 67 nm can be reconstituted even in the absence (almost complete removal) of telopeptides.

process, as also seen in other protein self-assembly systems.[10,21] It is driven by the loss of solvent molecules from the surface of protein molecules and results in assemblies of long fibrillar structures with tapered ends and circular cross-section, which minimize the surface area to volume ratio of the final assembly. The least critically discussed aspect of collagen molecular packing is the axial structure within a fibril. The frequently observed 67 nm density step function repeat of fibrils is explained by the molecular stagger between molecules, or an integer multiple of this. Since the tropocollagen molecular length of 300 nm corresponds to 4.4 D, the molecular stagger leads in projection to regions of high and low electron densities, typically referred to as overlap and gap regions (Fig. 1B).[22,23] The interactions that drive the association between tropocollagens are mostly electrostatic and hydrophobic with the 234-amino acid pseudoperiodicity (D-pattern) being crucial to optimal electrostatic pairings between adjacent triple helices, as well as responsible for maximising the contact between hydrophobic regions.[24,25] The stabilisation of such interactions is ensured by the existence of molecular cross-links, occurring between sites in the short non-helical N- and C-terminal telopeptides of the collagen molecules and the main chain of the helix.[26]

Although the correct 67 nm D-pattern as seen in microscopy studies appears to be the least discussed feature of collagen I fibrils, the literature still abounds with reports about their property to exhibit varying structural and hierarchical characteristics which are different from the classical collagen I, also known as collagen polymorphism, as observed both *in vitro*[27,28] and *in vivo*.[29,30] Amongst the major factors shown to affect it are the presence of GAGs/PGs[31–33] and other forms of collagen,[34,35] intactness of telopeptides,[36,37] purification protocols,[38] concentration,[39,40] temperature,[41] pH, and salinity of the solutions.[42,43] The numerous reports also account for the identification of novel banding patterns, growth mechanisms, and morphological properties of these systems deviating to a different extent from the classical collagen type I. There is no uniform classification, due to the heterogeneous nature of the collagen type I polymorphic forms, although there is a good overall agreement about the molecular structure of their axially projected tropocollagen arrangements. The most studied classical forms, apart from the native asymmetric collagen type I D-banding of 67 nm, feature various types of fibrous long spacing (FLS) collagens (utilising symmetric or antiparallel arrangement of tropocollagen),[44,45] as well as segment long spacing (SLS) fibrils (with an asymmetric pattern, or parallel staggering of collagen monomers),[46,47] both being non-D periodic. Examples of D-periodic fibrils are the D-periodic symmetric (DPS)[27,30] or oblique-striated asymmetric tactoids.[27,48] An updated overview of the last 5 decades of microscopical studies of the polymorphic forms of collagen *via* transmission electron microscopy (TEM) and scanning force microscopy (SFM) is modified following Doyle *et al.*[49] and can be summarised as follows:

(1) *Native collagen fibrils (D-pattern)*. The standard polymorphic form with an asymmetric banding of 67 nm (D) as a result of the parallel alignment of monomeric collagen units.[50–52]

(2) *DPS I–IV (D-periodic symmetric) fibrils*. The same as the native form but with a D-periodic centrosymmetric banding pattern.[27,30,50,53,54]

(3) *D/3 periodic fibrils*. A rare polymorphic form with periodic banding equal to 22 nm (D/3).[49]

(4) *D/6 periodic fibrils*. Tactoids with the smallest reported banding pattern to date of 11 nm (D/6).[49]

(5) *Oblique striated fibrils*. Tactoids of D-periodic polar subfibrils (67 nm). The composing subfibrils are staggered with respect to the nearest neighbours by a displacement of about 9 nm (D/7).[27,48,53,55,56]

(6) *SLS (Segment long-spacing) asymmetric polymorphic form*. Segments equal to the molecular length of collagen monomers (280–300 nm) with an asymmetric banding pattern, corresponding to parallel staggering.[57–60]

(7) *SLS symmetric polymorphic form*. Equivalent to SLS asymmetric segments, however with antiparallel arrangement of collagen monomers, resulting in symmetric banding periodicity.[31,61]

(8) *SLS (4D) fibrils*. Fibrils with a periodic asymmetric pattern, corresponding to about 268–270 nm.[32,62,63]

(9) *FLS (Fibrous long-spacing) I–IV*. A group of antiparallel non-D-periodic fibrillar patterns, ranging from 90 to 260 nm.[28,29,31,45]

Interestingly, experimental conditions are not the only ones that can lead to collagen polymorphism. Further variables influencing the process can be the amino acid sequence of collagen monomers, and the predominant type of interactions during the fibrillogenesis. For example, it was previously demonstrated that the native D-periodic (67 nm) asymmetric stagger of collagen I monomers originates in the pseudo-periodic distribution of hydrophobic and charged side chains along the main triple helical part of the molecules.[24] At the same time, D-periodic symmetric collagen is mainly produced either at low pH (4.3–5.5) or after enzymatic treatment. Due to the low degree of dissociation of glutamic and aspartic acid residues at low pH, the possible electrostatic interaction between collagen molecules is hindered, thus rendering the hydrophobic interactions as the only possible driving force for assembly of symmetric DPS structures.[49] Formation of FLS collagen appears to be related to the distribution of unpaired positively charged side chains, while the adjacent molecules are typically held together by a bridging interaction between these charges and a variety of polyanions.[49] The existence of polar SLS structures is attributed either to a direct hydrophobic interaction between large, uncharged side chains, or interaction between charged side chains *via* some non-collagenous molecules. Earlier studies have shown that small highly negatively charged molecules such as ATP[60] or synthetic polysulfonates[47] successfully participate in the formation of SLS fibrils by bridging adjacent net positive charges from the side amino acid residues.[49]

In this context, we previously reported a mechanism for the appearance of both symmetric and asymmetric polymorphic forms in a system comprised of pepsin-solubilised collagen type I monomers lacking telopeptides (atelocollagen) and the highly negatively charged GAG heparin. The transition and coexistence of 4 different polymorphic forms have a structural evolution which is summarised below (Fig. 2).

3 Factors influencing the formation of composite fibrillar matrices *in vitro*

Former studies on the kinetics of collagen I fibril formation (fibrillogenesis) have shown that it is a multistep process, normally separated into two phases – a lag (nucleation) phase with almost no or minuscule increase in turbidity of the solution, and a growth (exponential) phase which is distinguished by a sigmoidal increase of the solution optical density.[64,65] The nucleation phase, where soluble collagen monomers accrete to preform nuclei (activation centres), normally predominates over growth and was shown to have the major influence on fibrillar size, which is determined in that phase.[66] A characteristic of that stage is the appearance of linear dimers and trimers which contain 4D-staggered neighbouring tropocollagens. By further lateral accretion such molecules form narrow collagen fibrils, which subsequently grow into bigger fibrils (fibres) with a D-periodic pattern of 67 nm. This is also signified by an increase in solution turbidity.[64]

Collagen fibrillogenesis has been extensively studied in terms of parameters, such as temperature,[42,65,67–69] pH value,[42,43,54,64,65,70] concentration,[10,62,65,71,72] presence/absence of telopeptides,[37,67,73–78] *etc.* The majority of them affect the lengths of the lag and exponential phases, and as mentioned above also inadvertently influence the size, shape, and even diversity of polymorphic forms of the fibrils. Nevertheless, the focus of the current review is on the formation of composite fibrillar matrices; therefore we will emphasise the importance of other factors, which are heavily implemented in various scaffold engineering strategies. Among these, serious attention nowadays is received by molecules such as GAGs and PGs, as well as other types of collagens and proteins that have been shown to not only bind the collagen I fibrils, but also regulate their structure *in vitro*. Fig. 3 summarizes these cues and their outcome in the self-assembly process of ECM-inspired composite collagen I fibrils.

(A) Glycosaminoglycans and proteoglycans

The complex nature of collagen and GAG interaction normally involves both electrostatic and short range non-electrostatic (predominantly hydrophobic) forces. The electrostatic interactions are attributed to the high negative charge of the sulphate groups in GAGs, such as heparin, heparan sulphate, dermatan sulphate, and chondroitin sulphate, with positively charged residues in the collagen chains. The non-electrostatic interaction is associated with the protein core attached to some GAGs in nature (PGs) which are attracted by the non-cationic regions of the collagen helix.[79,80] It was previously shown that incubating monomeric collagen and GAGs/PGs can either accelerate or inhibit the kinetics of fibril formation,[81–83] with both the size and degree of sulphation influencing the size of the fibrils. In contrast, studies on collagen and PGs from bovine tendon and bovine nasal cartilage have shown no effect on the lag phase of fibril formation, where they predominantly control the lateral assembly of collagen I fibrils.[84]

A number of *in vivo* studies have shown that binding between collagen I and GAGs/PGs is exclusively interfibrillar. Due to the spatial hindrance of the closely packed monomeric tropocollagens, neither the protein core nor the GAG chains are able to localise inside the collagen fibrils. Pure GAGs and PGs normally bind collagen at an interfibrillar level, where supposedly by either electrostatic repulsion or sterical hindrance they increase the spacing between the fibrils. Interestingly, some recent works have shown that when working with atelocollagen (in which telopeptides are enzymatically removed) in the presence of the highly sulphated heparin it is possible to form cofibrils with a rather stoichiometric ratio between monomeric atelocollagen and heparin from 1 : 1.1 to 1 : 1.7, pointing towards a specific interaction and intercalation of heparin inside the fibrils.[83] Such an interaction is plausible due to the absence of

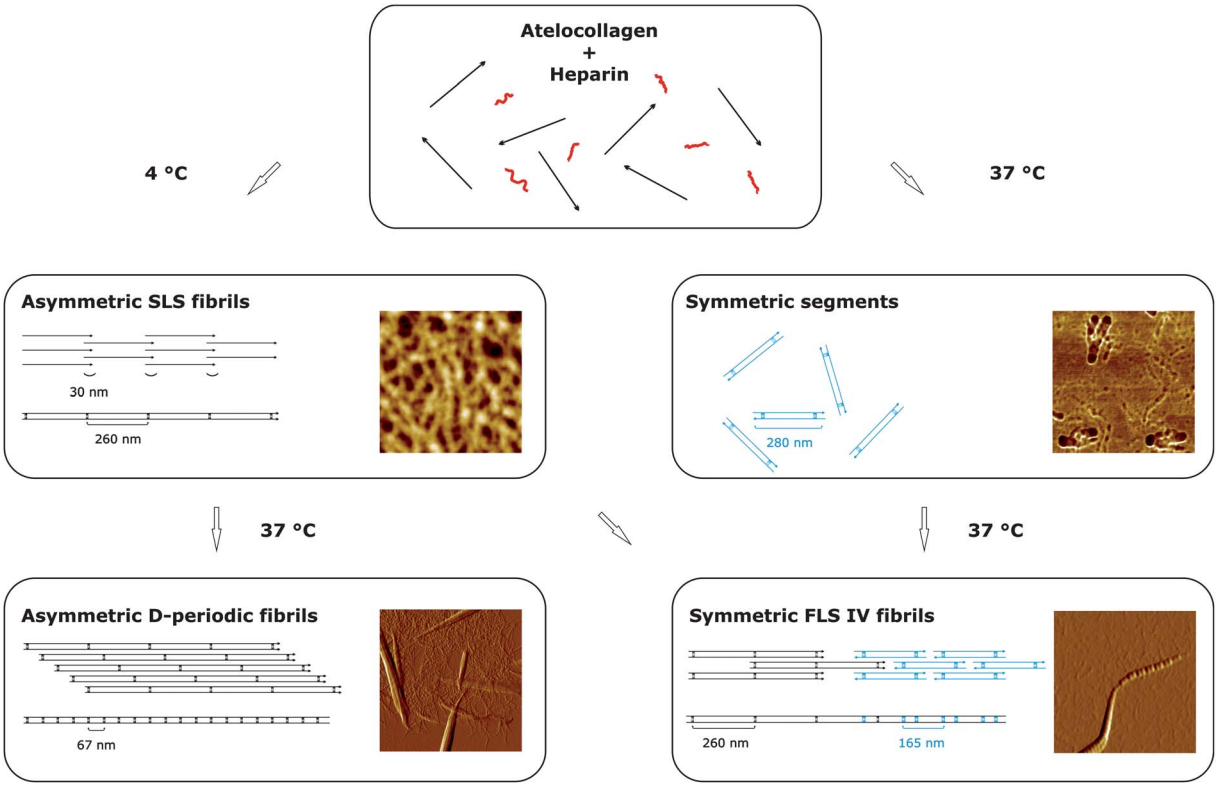

Fig. 2 Proposed mechanism for the hierarchical self-assembly of asymmetric D-periodic cofibrils and symmetric FLS IV nanofibrils from pepsin-treated bovine collagen type I and highly sulphated heparin. In the first phase (nucleation) the atelocollagens interact specifically with the highly negatively charged heparin thus leading eventually to the formation of asymmetric SLS fibrils with a banding of 250–260 nm. The growth to asymmetric D-periodic cofibrils is initiated after a temperature switch to 37 °C and involves a quarter staggering of the asymmetric SLS fibrils, a process expectedly driven by both electrostatic and hydrophobic interactions. On the other side the transition from asymmetric long-spacing fibrils to symmetric FLS IV nanofibrils is provided by the stepwise addition of symmetric segments of 280–300 nm, which are formed only after the nucleation phase. They use the tips of the already existing asymmetric SLS fibrils as a scaffold and continue to grow in a stepwise manner producing FLS IV banding (modified with permission from ref. 33).

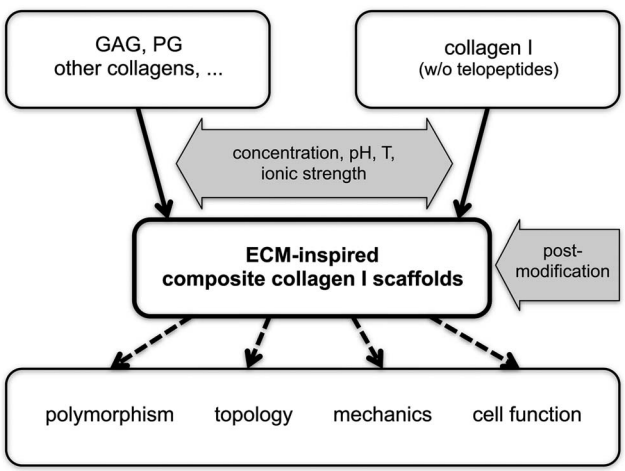

Fig. 3 Summarising scheme of influencing parameters on assembly of ECM-inspired composite collagen I scaffolds and the outcome in terms of structure and function.

intrafibrillar cross-links, leaving two major basic positive clusters at the places of the missing telopeptides which bind the heparin with a high specificity and do not rearrange the normal asymmetric collagen staggering.[33] The localisation of heparin inside the fibrils is only possible due to the absence of telopeptide-related cross-links which normally hinder the intrafibrillar binding of GAGs *in vivo*.[78] Although such interactions are mostly valid for some pathological cases *in vivo*, their importance in composite scaffolds is enhanced by the increasing use of less immunogenic pepsin-solubilised collagen type I. The presence of highly negatively charged GAGs was also shown to influence fibril morphology. Various GAGs were reported to lead to the formation of fibrils with spindle-like morphology using intact tropocollagen I[85] as well as atelocollagen.[47,86,87]

Another example for the functional diversity of collagen I is its ability to interact with a range of PGs. In this regard, the small leucine-rich repeat proteoglycans (SLRPs) have spurred a lot of attention during the last two decades as potential regulators of collagen fibril assembly.[88–90] SLRPs are two-component constructs consisting of an N-terminal variable domain containing either sulphated tyrosines[91] or stretches of amino acids, and a conserved domain of leucine-rich repeats (LRRs).[92] The protein core is further covalently attached to a small number of GAG chains, typically chondroitin/dermatan sulphate or keratan sulphate.[13] A number of SLRPs, such as decorin,[93,94] fibromodulin,[95] and lumican,[96,97] have been shown to regulate collagen fibrillogenesis *in vitro*, supposedly by sterically hindering the coalescence of collagen intermediates into axially

growing fibrils. *In vitro* studies have shown that decorin, fibromodulin and lumican inhibit fibrillogenesis, resulting in smaller fibril size, whereas biglycan did not have a significant impact on fibril formation.[13] Although most reports emphasise the importance of the protein core, as the major regulator of fibril formation, a few recent studies suggest different contributions of the protein core and the GAG chains to collagen fibril structure and mechanics.[98–100] The importance of such PGs for collagen fibrillogenesis is also highlighted *in vivo* where knock-outs of individual SLRPs have resulted in aberrant fibrillar structures.[101,102] In particular, mice lacking decorin exhibit severe congenital corneal stromal dystrophy[101] and renal fibrosis,[93,103] while lumican/fibromodulin deficiency is typically associated with high myopia (a common cause of blindness) as a result of corneal detachment and choroidal neovascularisation.[104] Because of their effect on collagen architecture, and suggested influence on fibril mechanics,[105–107] SLRPs currently get a lot of attention as potential tools for tuning the biophysical properties of *in vitro* assembled collagen-based scaffolds. Interestingly, studies with recombinant lumican were found to accelerate fibril formation *in vitro*,[96] similar to some heparan sulphate-containing PG (perlecan).[13,108] It is likely that such interactions stabilise the initial nuclei that form during the lag phase prior to subsequent fibril growth.[13]

(B) Interaction with other collagen types

Fibrillar size and interactions of collagen type I vary widely across tissues as a result of the heterotypic composition of these microenvironments. Previous studies have shown that a large number of molecules, such as heterotypic collagens, fibril-associated collagens, as well as cofibrillar macromolecules, can alter the accretion properties of available procollagen molecules, and thus affect the size and shape of the collagen I fibrils.[22] Heterotypic fibrils are characteristic of most connective tissues, *e.g.* collagens I, III and V in skin, as well as types I and V in cornea.[13] For example, partially N-terminally enzymatically processed forms of procollagen III or collagen V were shown to sterically control the assembly and fibril size of collagen I *in vitro*[35] and in cornea,[109] respectively. At the same time a complete lack of procollagen V leads to almost no formation of collagen type I fibrils, emphasising the importance of collagen V as a nucleator in collagen I fibrillogenesis.[110] This shows that although the presence of other collagen types, such as type III and V, might be required for the nucleation of collagen type I fibrils, their increasing concentrations lead to an overall decrease in collagen type I diameters.[13]

Fibrillar collagens, including collagen type I, II and XI, also associate with additional collagenous proteins, such as fibril associated collagens with interrupted triple helices (FACITs). Recent work on FACITs has emphasised novel structural roles they may play in the ECM of different tissues.[111] Collagen IX is an important FACIT component of cartilage collagen fibrils along with fibrillar collagens type II and XI. On its own, however, collagen IX does not self-assemble into fibrils.[112] The collagen IX molecule is a heterotrimer, sometimes carrying a chondroitin/dermatan sulphate glycosaminoglycan residue in its α2(IX) chain, thereby partly giving it properties of a PG.[13] The functional complexity of the molecule extends to its large N-terminal NC4 domain located in the α1(IX) chain which interacts with collagen II and XI and is supposedly available for interaction with other ECM proteins.[13,111] Two additional FACITs (collagen type XII and XIV) have also been shown to associate with the surface of collagen I and II fibrils, respectively.[113,114] Nevertheless, the detailed molecular mechanisms of the interaction between fibrillar and FACIT collagens remain to be elucidated.[111] A few reports account for the absence of covalent cross-links in these interactions thereby suggesting the presence of speculative mediators, such as the matrix SLRPs decorin and fibromodulin.[115,116] The current understanding is that most likely these molecules have an organisational/stabilising role in the fibrillar collagen network,[111] where they resist mechanical stress as seen from other studies.[117–120]

4 Mechanical properties of collagen composites

Collagen type I molecules in the forms of fibrils and bundles provide mechanical and structural stability particularly in connective tissues such as bone, tendon, as well as the ECM.[121] Although the origin of the structural stability of the fibres lies in the amino acid sequence of their molecular building blocks (tropocollagens), a number of studies emphasise their internal structure as the determining factor for their mechanical behaviour.[3,121] The structural hierarchy evolving from the single tropocollagen molecules (300 nm long and 1.5 nm in diameter), through the suggested 4.5 nm microfibrillar units, the few hundred nanometre fibrils, to the micrometre-sized bundles of fibrils (fibres) is a prerequisite for their unique mechanical properties.[122] Furthermore a line of evidence suggests that the internal structure of the collagen molecules morphologically resembles the one of a classical rope, which is expected to further impact its mechanical properties.[123] Native mature collagen structures are further enzymatically stabilised by intra- and intermolecular cross-links involving the lysine/hydroxylysine from their telopeptides and near-end triple-helical hydroxylysines.[26] Another hierarchical level is ensured by the stabilisation with GAGs/PGs, with the latter being localised typically between the fibrils.[124,125]

Recent decades have seen an increase in mechanical studies of collagen-rich tissues which corroborate quite clearly their viscoelastic properties.[126–130] Such experiments normally involve creep-relaxation (tensile) tests producing characteristic stress–strain curves as depicted in Fig. 4. These normally include three typical stages.[3,131] The initial stage, commonly referred to as the 'toe', is associated with the stretching of the macromolecular collagen I crimps, at the micrometre level and above.[132] Higher strains lead to a concave change in the curves which is supposedly associated with straightening of the molecular kinks arising from the gap regions between the thermally wiggling collagen molecules, the so-called 'heel' region.[133,134] The last linear elastic region is characterised by stretching of triple-helical tropocollagens and the cross-links between them, therefore resulting in the so-called intrafibrillar sliding, suggested to have major implications for the overall structure of the fibrils.[135,136] The typical elastic modulus of a tendon is in the range of 1–2 GPa, and the maximum applied strain rarely exceeds 10–20%. This suggests that the physiological range in collagen-rich tendon tissues is normally in the 'heel' or beginning of the linear elastic region.[137] A recent rheological study offered a novel insight into the early

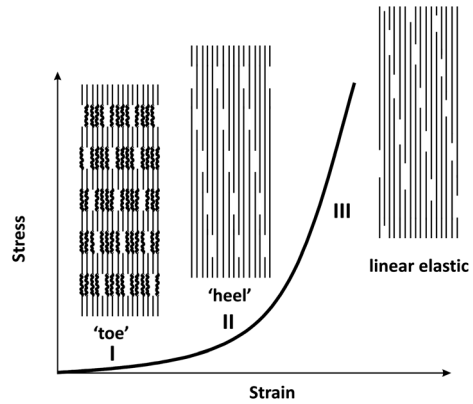

Fig. 4 Schematic representation of a typical stress–strain curve resulting from a creep-relaxation (tensile) test of a collagen I-rich tendon fascicle (following Fratzl *et al.*, ref. 136). The three different regimes represent distinct changes taking place at inter- (micro) and intrafibrillar (nano) levels.

complex deformation nonlinearity of collagen gels, revealing both strain softening as well as strain stiffening.[138] The results suggest that softening can be partially ascribed to cross-linking dynamics in the formed collagen networks, whereas stiffening at small tensile strains is largely due to network rearrangement and stretching of individual fibrils.

Separate studies on individual hydrated collagen type I fibrils carried out predominantly either by tensile stretching *via* atomic force microscopy (AFM)[139–142] or microelectromechanical systems (MEMS)[143–145] analysis have yielded values in the range of 0.1–3.0 GPa.[146] Interestingly, although representing quite high variations these values are similar to the ones of bending moduli obtained from micromechanical bending AFM experiments on hydrated collagen fibrils (0.07–0.38 GPa),[78,147] as well as dried and electrospun filaments (1.0–7.5 GPa).[147–149] Such significant differences are likely a result of the fact that micromechanical bending tests focus solely on the elastic properties of individual fibrils, leaving the viscous element coming from energy dissipation due to intrafibrillar monomeric sliding unaddressed.[146]

Single-molecule analysis of hydrated tropocollagen molecules has provided little evidence about the elastic behaviour of such structures, mostly derived either from light scattering experiments,[150,151] or atomistic modelling studies,[146,152–154] resulting in values in the range of 3–9 GPa. Recently an atomistic creep test provided evidence for the non-linear viscoelastic properties of collagen I at the single molecule level with values for the elastic modulus of 6–16 GPa for strains up to 20%.[146]

The impact of PGs and GAGs has been investigated in more detail due to their importance for structural regulation in developing collagen type I-rich tissues.[155] Both decorin or biglycan *in vivo* knockouts have been shown to result in differently impaired mechanical properties in developing tendons,[107,156] skin,[93] as well as lung tissues.[157] Straightforward conclusions on the individual contribution of each molecule however are difficult to make as their expression levels appear to be increased in single-molecule knockout *in vivo* studies, as also shown for another pair of PGs such as lumican and fibromodulin.[102] Most models refer to PGs binding to collagen type I with their protein core, while the GAG chains interact with one another to form inter-fibrillar cross-links.[80,106] Nevertheless, a direct specific or non-specific binding of the polysaccharide chains to collagens is also possible as shown previously for studies with pure GAGs. Similar to PGs, GAGs have also been suggested to have an elastic and viscoelastic contribution to tendon mechanics,[158] although experimental enzymatic degradation (50–95%) studies so far have mostly shown small physiological differences between treated and untreated samples.[99,159,160]

While the structural hierarchy of the collagen polymorphs is the main reason for its mechanical properties, furthermore particular cross-links play an essential role in their stabilisation, and therefore are important for tissue function and matrix remodelling.[105] Normally after fibrillogenesis, fibrils and fibres undergo a tissue-specific intermolecular cross-linking which involves lysine/hydroxylysine residues from the non-helical telopeptides, and near-end triple-helical hydroxylysine, and heavily depends on the enzyme lysyl oxidase (LOX).[26,78] A secondary non-enzymatic cross-linking mechanism is related to the reaction of collagens with sugars which leads to the formation of advanced glycation end-products (AGEs) which although responsible for the stiffening of the tissues is deemed mechanically disadvantageous and associated with impaired matrix remodelling and decreased matrix toughness.[105,161]

As discussed above, a number of contemporary tissue engineering studies utilise C- or N-terminally enzymatically processed collagen type I, in order to remove the telopeptide regions,[94,162,163] which have been associated with undesirable immunogenic responses.[164–166] Correspondingly this was shown to have an adverse effect on fibrillar stiffness *in vitro*, thus emphasising the importance of telopeptides for stabilisation of the collagen molecules.[78] Furthermore a number of artificial cross-linking techniques have been historically utilised to enhance the mechanical properties of collagen fibrils. Physical cross-linking approaches include dehydrothermal (DHT) or ultraviolet light (UV) treatments which have been shown to significantly improve the mechanical strength of *in vitro* reconstituted collagen I fibrils.[167–169] Unfortunately they also showed a substantial decrease in the rate of cell migration.[170] The same study showed a reversed effect of a commonly used chemical cross-linker, carbodiimide, resulting in suboptimal mechanical strength but increased cell migration.[170] Interestingly when collagen–GAG containing scaffolds were further treated with carbodiimide they showed both increase in stiffness of the developed scaffolds as well as increased cell spreading and proliferation.[171] Other common chemical cross-linkers used for collagen type I except carbodiimide[172–174] are glutaraldehyde,[147,149,175] acyl azides,[176] and glycidyl ethers,[177] which share a certain cytotoxicity. Furthermore, glycation schemes can also be applied to post-process collagen I scaffolds by crosslinking to increase mechanical stability.[178] An excellent overview of the effects of different physical and chemical cross-linkers on mechanics and size of extruded hydrated and dried collagen fibres can be found elsewhere.[179,180] An interesting alternative to most approaches is the use of a particular family of calcium-dependent transglutaminases, which catalyse the formation of covalent cross-links between lysine amino groups and glutamine carboxy groups, and were shown to both strengthen the mechanical properties[181] as well as stabilise the three-dimensional structure of collagen gels.[182]

Despite the versatility of most artificial cross-linking strategies, they should be applied with caution due to their potential adverse effect on receptor/ligand interaction bonds, scaffold degradation or impaired fibrillar functionality.

5 ECM-inspired collagen composite matrices in biomedical research

Recent decades have seen an increase in studies on xenogeneic scaffolds, which have become a ubiquitous tool in tissue engineering and biomedical applications.[7] Collagen type I is usually preferred in such systems due to its importance in maintaining tissue integrity, as well as its excellent adhesive properties – able to interact with nearly 50 other molecules.[9] Combined with their superior mechanical properties, collagen fibrils are suggested to modulate the overall ECM structure and mechanics.[4,22] The importance of matrix mechanics for cell survival, proliferation and differentiation[183,184] is known *in vivo*, but is also investigated *in vitro* for designing novel biohybrid ECM analogues.[185] Current strategies are used to implement and modify the properties of collagen type I into novel biomaterials and systems which are expected to have therapeutic application and promote tissue and organ regeneration.[8]

Decellularisation and harvesting of ECM from tissues rich in collagen I were recently shown to be quite a promising approach for generating relevant matrix structures.[186–188] The major advantage of such an approach is the preserved acellular three-dimensional matrix architecture which can be repopulated with the cells of interest.[12] Nevertheless, major concerns remain for the potential complication from the use of such xenogeneic ECMs, regarding the 'host–recipient' immune response to particular epitopes and constituents.[189–191] In the case of collagen type I, the immunogenicity of the non-helical telopeptides[164–166] has prompted an increase in the studies with enzymatically treated collagen I,[4,94,192] which however has been shown to have adverse effects on collagen mechanics, structure, and cell response.[78]

The ability of collagen-based matrices to direct cell fate and tissue formation has been demonstrated for a number of cell types, which include cardiomyocytes,[193,194] endothelial cells,[195] fibroblasts, nerve cells,[196] interstitial cells,[197] and tumour cells.[39] To ensure the tuning of scaffold properties, such as porosity, fibrillar size, interfibrillar connectivity, and availability of binding sites for growth factors and receptors, a number of production techniques have been previously applied depending on the particular tissue requirements.[198] Commonly, the forms of native collagen I in which it is used in cell culture systems are either swollen hydrogels or in more or less simplified two-dimensional lattice-like structures.[199] In the absence of other molecules, which supposedly control the three-dimensional structure of the matrix, and especially in the absence of telopeptides (atelocollagen), different approaches have been implemented to control the porosity and stability of such scaffolds. Separate studies, as also described in the previous section, have used various forms of physical or chemical cross-linking to improve the stability of such systems. Some of the most common collagen-based nanofibrous fabrication techniques feature *in vitro* self-assembly of collagen type I, phase separation to form foam-like structures or sponges,[200–202] as well as the relatively new electrospinning approach,[203–205] the advantages and levels of processability of which have been reviewed elsewhere.[198,199]

We recently demonstrated the effect that differences in mechanical properties (in terms of flexural rigidity and bending moduli) of two collagen preparations, such as reconstituted pepsin-solubilised collagen I (PSC) fibrils, as well as PSC–heparin composite fibrils can have on adhesive cell behaviour, which is highly relevant for cell proliferation and differentiation on such scaffolds.[78] The two different cell types (*i.e.*, mouse L929 fibroblasts and human endothelial cells) were shown to probe the microchannel suspended two-dimensional matrices of the pure PSC (Fig. 5B and D) and the composite PSC–heparin fibrils (Fig. 5A and C) in very different ways. In summary, cells grown on composite PSC–heparin scaffolds spread to a higher extent or slided directly over the freestanding suspended PSC–heparin cofibrils. The inability of cells to spread over the suspended pure PSC fibrils was correlated with their up to 10-fold lower flexural rigidity. In a control experiment where cells were cultured on planar substrates with both PSC and PSC–heparin fibrils (data not shown), no substantial differences in morphology of the adherent cells were seen, thus emphasising the mechanics of the freestanding fibrils as the major contributor to the above-mentioned differences in cell behaviour in comparison to other possible influences like altered receptor binding due to the presence of heparin.

Viscoelastic behaviour and mechanical and adhesive properties of collagen I-based scaffolds are considered insufficient for explaining the *in vivo* remodelling events taking place following implantation of such materials.[8] The incorporation and availability of GAGs and PGs in such systems, discussed above, ensure the modulation of tissue dynamics through their activity as coreceptors for soluble growth factors and cytokines.[206,207] The potential of various collagen–GAG/PG containing scaffolds to dock growth factors for tissue engineering applications has been demonstrated for transforming growth factor β1 (TGF-β1),[208,209] vascular endothelial growth factor (VEGF),[210] basic fibroblast growth factor (bFGF),[211,212] *etc.* Binding scenarios are nowadays also extended to the use of artificial GAG-analogues, such as sulphated hyaluronic acid, which was shown to dock effectively TGF-β1, and further promote collagen synthesis in human mesenchymal stromal cells (hMSCs).[213] Such recent developments can offer interesting alternatives, as hyaluronic acid is the only non-sulphated native GAG, which plays an important signalling role in the ECM, mostly mediated by binding to other molecules. An interesting alternative for introducing insoluble factors in collagen scaffolds is the so-called collagen mimetic peptides (CMPs) which can be prepared with high anionic charges and therefore attract signalling molecules.[214] Such structures were successfully shown to induce tubulogenesis of endothelial cells, by trapping VEGF in three-dimensional collagen gels.[215] Polyanionic CMPs were shown to not only mediate binding between collagen gels and the growth factors,[216] but also consider the three-dimensionality of collagen gels, which is expected to complement the efficient trigger of cell morphogenesis in such scaffolds. Further considerations should include not only collagen type I, but also a number of non-collagenous proteins which are able to bind secreted growth factors and cytokines, and further maintain them as repository.[217] One of the most prominent and best

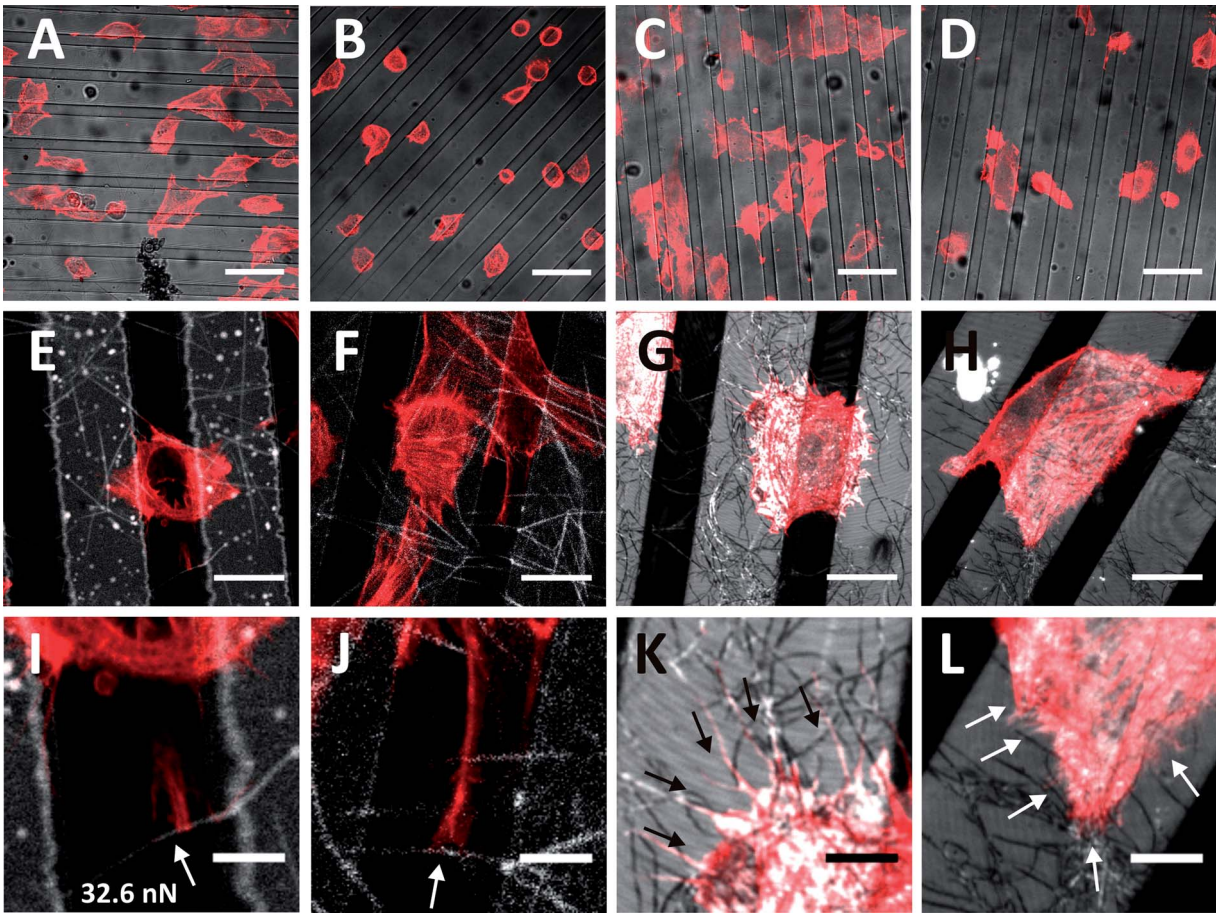

Fig. 5 Impact of the flexural rigidity of collagen and collagen–heparin fibrils on cell adhesion and morphology. Adhesion of mouse fibroblasts L929 (A and B) and human endothelial cells (C and D) after 4 h of cell culture on PSC (0.5 mg ml^{-1}) (B and D) and PSC–heparin fibrils (at concentrations of 1.2 and 0.1 mg ml^{-1} respectively) (A and C) suspended on PDMS gratings and investigated by confocal laser scanning microscopy. The actin cytoskeletons of the cells were stained with phalloidin-TRITC and are shown in red. In contrast to the results observed using fibrils made from pure collagen, cells that were grown on collagen–GAG assemblies were often spread over the channels and followed the linear topographic cues provided by the straight cofibrils. Both HUVECs (E and I) and L929 (F and J) were often found to pull on the significantly stiffer heparin-FITC-containing cofibrils (shown using a greyscale). Such behaviour was rarely observed for the PSC fibrils, where the HUVECs (G and K) and L929 cells (H and L) predominantly spread over aggregates of fibrils and adhered to the substrates. The directions of the traction forces exerted by the cells are shown using arrows. (G), (H), (K) and (L) were recorded in the RICM mode, providing signals from the PDMS gratings, the suspended and adherent PSC fibrils, and the adherent cells. The scale bars in (A–D), (E–H) and (I–L) represent 50, 15 and 5 μm, respectively (reproduced with permission from ref. 78).

understood examples is the involvement of the latent TGF-β binding proteins (LTBPs) for mediating the binding to TGF-β.[218] The requirement of a fibronectin matrix to initially incorporate LTBPs, which further carry binding sites for fibulins, fibrillins, and vitronectin,[219] once more brings about the important issue of considering the vast range of ECM components and interactions when designing scaffolds for biomedical applications.

Over the past few years, considerable efforts have also been spent to produce collagen I scaffolds with controlled fibril/fibre alignment. Such interest has been spurred by the ability of fibre orientation to guide cellular responses, which is inspired by the alignment of collagen fibrils in connective tissues, such as tendon, dermis, fascia, *etc.*[198,220] Most common approaches to drive fibril orientation include electrospinning,[17,221] microfluidic shear flow,[222,223] substrate contact guidance,[224–226] *etc.* The spatial guidance provided by such aligned fibrillar substrates was demonstrated by the ability of a range of cell types to follow the arrangement of the collagen molecules.[226–228]

As mentioned above, the development of three-dimensional collagen-containing hydrogel-based matrices, which provide more physiologically relevant conditions for tissue engineering and cell morphogenesis, has become a prerequisite in current scenarios. A few recent studies have further emphasised the importance of predefined microstructured orientation of three-dimensional collagen-containing hydrogels,[229–232] as compared to three-dimensional bulk-isotropic cell-seeded matrices. In addition, the cell orientation/positioning in such structurally predefined hydrogels is also of particular importance for cell–matrix and cell–cell contact interactions.[231] This once again comes to show that the more efficient development of collagen-based matrices should not only address the structure, but also the spatio-temporal parameters between the newly developed matrix and the seeded cells.[196,233]

6 Concluding remarks

Despite the wealth of collagen research and knowledge, gathered over the last five decades, a number of challenges still remain. Studies have already started to unravel the multiple binding sites along the collagen surface and their involvement in the time-dependent formation of collagen-containing ECMs. A number of binding sites are still buried within the fibrillar collagen structure and become available only after proteolysis, therefore, suggesting that collagen structures are highly dynamic.[234–236] Acquiring such knowledge will prompt the minimised use of atypical and cytotoxic cross-linkers while making way for the more efficient reconstitution of the real native three-dimensional organisation of such matrices.

The development of novel collagen-based composite scaffolds should make use of the knowledge about the collagen structure and constantly emerging specific binding sites for other molecules. The development of heterotypic scaffolds, which are more physiologically relevant than the pure collagen I, is fast becoming a standard in tissue engineering scenarios. Implementation of other structural glycoproteins, different collagen types, GAGs, PGs, *etc.*, is an indispensable approach in modulating the mechanical, topological, as well as the adhesive cues which such matrices offer to cells.

The source of collagen I used for preparation of such materials remains a point of concern, in particular its immunogenicity when xenogeneic sources are being used. However, the use of recombinant collagen type I is starting to make its way into more and more research studies at present.[237–241] Scaffolds using such collagen would definitely make use of the non-modified and conserved collagen type I sequence, which is expected to more fully represent the native collagen–cell interaction, and improve the biocompatibility of such materials.

The next generation of collagen I-based materials need to address more aptly the compositions of the particular tissue architectures, which they are supposed to substitute/mimic. It is pivotal to consider the multiple interactions emerging from studies related to such microenvironments, and their particular contribution to the integrity, structure, and mechanics of the newly developed systems. Current mechanotransduction studies on collagen-based scaffolds provide us with information about cell behaviour, migration, and adhesion, but more intense parallels to the situation *in vivo* are required, if we are to bridge the gap between the lab development of such scaffolds and future clinical trials.

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