

DSC XXII - Collagen Studies

Barbani N., Giusti P., Lazzeri L., Polacco G., and Pizzirani G. (1995) Bioartificial materials based on collagen: 1. Collagen cross-linking with gaseous glutaraldehyde. *J Biomater Sci Polym Ed* **7**, 461-469.

Abstract: The effect of exposure time of thin films of soluble collagen to glutaraldehyde (GTA) vapour was studied at 37 degrees C, and was evaluated by examining the thermal and biological stability and the swelling ratio. It was found that the collagen films treated with GTA vapour for 18 h showed the highest denaturation temperature, the lowest swelling ratio, and an enhanced proteolytic stability. This study shows that soluble collagen can be effectively cross-linked with GTA vapour and that the degree of cross-linking can be controlled by varying the exposure time.

Barbani N., Cascone M. G., Giusti P., Lazzeri L., Polacco G., and Pizzirani G. (1995) Bioartificial materials based on collagen: 2. Mixtures of soluble collagen and poly(vinylalcohol) cross-linked with gaseous glutaraldehyde. *J Biomater Sci Polym Ed* **7**, 471-484.

Abstract: Thin films of both pure soluble collagen (CLG) and poly(vinylalcohol) (PVA) and mixtures of the two, ranging from 20-80% PVA composition were studied to test the effects of PVA content and of glutaraldehyde vapour cross-linking. Both the thermal and mechanical behavior and, in addition, proteolytic stability were clearly influenced by the ratio of CLG/PVA. The experimental results indicate that no thermodynamic compatibility occurs between the two homopolymers. However, there is evidence that strong interactions, probably due to hydrogen bond formation, occur between the biological and synthetic polymers. The interactions appear stronger in those films with a lower PVA content and which were not cross-linked. Both the thermal and biological stability are increased and there is an improvement of the mechanical properties. The mutual intermolecular influence appears to allow the attainment of a good mechanical compatibility between CLG and PVA.

Barth D., Musiol H. J., Schutt M., Fiori S., Milbradt A. G., Renner C., and Moroder L. (2003) The role of cystine knots in collagen folding and stability, part I. Conformational properties of (Pro-Hyp-Gly)₅ and (Pro-(4S)-FPro-Gly)₅ model trimers with an artificial cystine knot. *Chemistry* **9**, 3692-3702.

Abstract: In analogy to the cystine knots present in natural collagens, a simplified disulfide cross-link was used to analyse the conformational effects of a C-terminal artificial cystine knot on the folding of collagenous peptides consisting of solely (Pro-Hyp-Gly) repeating units. Assembly of the alpha chains into a heterotrimer by previously applied regioselective disulfide-bridging strategies failed because of the high tendency of (Pro-Hyp-Gly)₅ peptides to self-associate and form homotrimers. Only when side-chain-protected peptides were used, for example in the Hyp(tBu) form, and a new protection scheme was adopted, selective interchain-disulfide cross-linking into the heterotrimer in organic solvents was successful. This unexpected strong effect of the conformational properties on the efficiency of well-established reactions was further supported by replacing the Hyp residues with (4S)-fluoroproline, which is known to destabilise triple-helical structures. With the related [Pro-(4S)-FPro-Gly]₅ peptides, assembly of the heterotrimer in aqueous solution proceeded in a satisfactory manner. Both the intermediates and the final fluorinated heterotrimer are fully unfolded in aqueous solution even at 4 degrees C. Conversely, the disulfide-crossbridged (Pro-Hyp-Gly)₅ heterotrimer forms a very stable triple helix. The observation that thermal unfolding leads to scrambling of the disulfide bridges was unexpected. Although NMR experiments support an extension of the triple helix into the cystine knot, thermolysis is not associated with the unfolding process. In fact, the unstructured fluorinated trimer undergoes an equally facile thermodegradation associated with the intrinsic tendency of unsymmetrical disulfides to disproportionate into symmetrical disulfides under favourable conditions. The experimental results obtained with the model peptides fully support the role of triple-helix nucleation and stabilisation by the artificial cystine knot as previously suggested for the natural cystine knots in collagens.

Belopol'skaia T. V. and Tsereteli G. I. (1996) [Melting of ordered structures in denatured collagen depending on biological age by differential scanning calorimetry]. *Biofizika* **41**, 665-671.

Abstract: Using the differential scanning calorimetry we were able for the first time to establish the correlation between the biological age of the collagen tissue (rat tail tendon, age from 20 days to 24 months) and the melting parameters of gels, which are formed after the collagen denaturation. We have found that the half-width of the gel melting curves $\Delta T_{1/2}$, as well as the ratio of the gel melting heat to the

denaturation heat Q_m/Q_d are sensitive to the biological age of the tissue. The gel melting temperature T_m , in distinction to the denaturation temperature, was found to be not sensitive to the age but entirely dependent on the gel formation temperature. As it was experimentally demonstrated the above mentioned correlations can be observed only at well characterized thermal prehistory of samples and strictly fixed regime of gel formation.

Bodian D. L., Madhan B., Brodsky B. and Klein T. E. (2008) Predicting the clinical lethality of osteogenesis imperfecta from collagen glycine mutations. *Biochemistry* **47**, 5424-5432.

Abstract: Osteogenesis imperfecta (OI), or brittle bone disease, often results from missense mutation of one of the conserved glycine residues present in the repeating Gly-X-Y sequence characterizing the triple-helical region of type I collagen. A composite model was developed for predicting the clinical lethality resulting from glycine mutations in the $\alpha 1$ chain of type I collagen. The lethality of mutations in which bulky amino acids are substituted for glycine is predicted by their position relative to the N-terminal end of the triple helix. The effect of a Gly \rightarrow Ser mutation is modeled by the relative thermostability of the Gly-X-Y triplet on the carboxy side of the triplet containing the substitution. This model also predicts the lethality of Gly \rightarrow Ser and Gly \rightarrow Cys mutations in the $\alpha 2$ chain of type I collagen. The model was validated with an independent test set of six novel Gly \rightarrow Ser mutations. The hypothesis derived from the model of an asymmetric interaction between a Gly \rightarrow Ser mutation and its neighboring residues was tested experimentally using collagen-like peptides. Consistent with the prediction, a significant decrease in stability, calorimetric enthalpy, and folding time was observed for a peptide with a low-stability triplet C-terminal to the mutation compared to a similar peptide with the low-stability triplet on the N-terminal side. The computational and experimental results together relate the position-specific effects of Gly \rightarrow Ser mutations to the local structural stability of collagen and lend insight into the etiology of OI

Burdzhanadze T. V. and Tiktopulo E. I. (2001) [Collagen denaturation enthalpy--nonlinear function of 4-hydroxyproline]. *Biofizika* **46**, 607-611.

Abstract: The results of calorimetric measurements of denaturation of collagens with different imino acid content are reported. In contrast to the existing point of view that denaturation enthalpy is a linear function of 4-hydroxyproline content, a nonlinear dependence was revealed. It is suggested that the reason for the observed nonlinearity is triplets of the (Gly-Pro-Hyp) type. An increase of their content can cause a decrease in the denaturation enthalpy in accord with the water-bridge structure and due to the minimum enthalpy effect of stabilization of the triplets as compared to triplets of other type.

Cabral W. A., Merts M. V., Makareeva E., Colige A., Tekin M., Pandya A., Leikin S., and Marini J. C. (2003) Type I collagen triplet duplication mutation in lethal osteogenesis imperfecta shifts register of α chains throughout the helix and disrupts incorporation of mutant helices into fibrils and extracellular matrix. *J Biol Chem* **278**, 10006-10012.

Abstract: The majority of collagen mutations causing osteogenesis imperfecta (OI) are glycine substitutions that disrupt formation of the triple helix. A rare type of collagen mutation consists of a duplication or deletion of one or two Gly-X-Y triplets. These mutations shift the register of collagen chains with respect to each other in the helix but do not interrupt the triplet sequence, yet they have severe clinical consequences. We investigated the effect of shifting the register of the collagen helix by a single Gly-X-Y triplet on collagen assembly, stability, and incorporation into fibrils and matrix. These studies utilized a triplet duplication in COL1A1 exon 44 that occurred in the cDNA and gDNA of two siblings with lethal OI. The normal allele encodes three identical Gly-Ala-Hyp triplets at aa 868-876, whereas the mutant allele encodes four. The register shift delays helix formation, causing overmodification. Differential scanning calorimetry yielded a decrease in $T(m)$ of 2 degrees C for helices with one mutant chain and a 6 degrees C decrease in helices with two mutant chains. An in vitro binary co-processing assay of N-proteinase cleavage demonstrated that procollagen with the triplet duplication has slower N-propeptide cleavage than in normal controls or procollagen with pro $\alpha 1(I)$ G832S, G898S, or G997S substitutions, showing that the register shift persists through the entire helix. The register shift disrupts incorporation of mutant collagen into fibrils and matrix. Proband fibrils formed inefficiently in vitro and contained only normal helices and helices with a single mutant chain. Helices with two mutant chains and a significant portion of helices with one mutant chain did not form fibrils. In matrix deposited by proband fibroblasts, mutant chains were abundant in the immaturely cross-linked fraction but constituted a minor fraction of maturely

cross-linked chains. The profound effects of shifting the collagen triplet register on chain interactions in the helix and on fibril formation correlate with the severe clinical consequences.

Cabral W. A., Makareeva E., Colige A., Letocha A. D., Ty J. M., Yeowell H. N., Pals G., Leikin S., and Marini J. C. (2005) Mutations near amino end of alpha1(I) collagen cause combined osteogenesis imperfecta/Ehlers-Danlos syndrome by interference with N-propeptide processing. *J Biol Chem* **280**, 19259-19269.

Abstract: Patients with OI/EDS form a distinct subset of osteogenesis imperfecta (OI) patients. In addition to skeletal fragility, they have characteristics of Ehlers-Danlos syndrome (EDS). We identified 7 children with types III or IV OI, plus severe large and small joint laxity and early progressive scoliosis. In each child with OI/EDS, we identified a mutation in the first 90 residues of the helical region of alpha1(I) collagen. These mutations prevent or delay removal of the procollagen N-propeptide by purified N-proteinase (ADAMTS-2) in vitro and in pericellular assays. The mutant pN-collagen which results is efficiently incorporated into matrix by cultured fibroblasts and osteoblasts and is prominently present in newly incorporated and immaturely cross-linked collagen. Dermal collagen fibrils have significantly reduced cross-sectional diameters, corroborating incorporation of pN-collagen into fibrils in vivo. Differential scanning calorimetry revealed that these mutant collagens are less stable than the corresponding procollagens, which is not seen with other type I collagen helical mutations. These mutations disrupt a distinct folding region of high thermal stability in the first 90 residues at the amino end of type I collagen and alter the secondary structure of the adjacent N-proteinase cleavage site. Thus, these OI/EDS collagen mutations are directly responsible for the bone fragility of OI and indirectly responsible for EDS symptoms, by interference with N-propeptide removal.

Cabral W. A., Makareeva E., Letocha A. D., Scribanu N., Fertala A., Steplewski A., Keene D. R., Persikov A. V., Leikin S., and Marini J. C. (2007) Y-position cysteine substitution in type I collagen (alpha1(I) R888C/p.R1066C) is associated with osteogenesis imperfecta/Ehlers-Danlos syndrome phenotype. *Hum Mutat* **28**, 396-405.

Abstract: The most common mutations in type I collagen causing types II-IV osteogenesis imperfecta (OI) result in substitution for glycine in a Gly-Xaa-Yaa triplet by another amino acid. We delineated a Y-position substitution in a small pedigree with a combined OI/Ehlers-Danlos Syndrome (EDS) phenotype, characterized by moderately decreased DEXA z-score (-1.3 to -2.6), long bone fractures, and large-joint hyperextensibility. Affected individuals have an alpha1(I)R888C (p.R1066C) substitution in one COL1A1 allele. Polyacrylamide gel electrophoresis (PAGE) of [(3)H]-proline labeled steady-state collagen reveals slight overmodification of the alpha1(I) monomer band, much less than expected for a substitution of a neighboring glycine residue, and a faint alpha1(I) dimer. Dimers form in about 10% of proband type I collagen. Dimer formation is inefficient compared to a possible 25%, probably because the SH-side chains have less proximity in this Y-position than when substituting for a glycine. Theoretical stability calculations, differential scanning calorimetry (DSC) thermograms, and thermal denaturation curves showed only weak local destabilization from the Y-position substitution in one or two chains of a collagen helix, but greater destabilization is seen in collagen containing dimers. Y-position collagen dimers cause kinking of the helix, resulting in a register shift that is propagated the full length of the helix and causes resistance to procollagen processing by N-proteinase. Collagen containing the Y-position substitution is incorporated into matrix deposited in culture, including immaturely and maturely cross-linked fractions. In vivo, proband dermal fibrils have decreased density and increased diameter compared to controls, with occasional aggregate formation. This report on Y-position substitutions in type I collagen extends the range of phenotypes caused by nonglycine substitutions and shows that, similar to X- and Y-position substitutions in types II and III collagen, the phenotypes resulting from nonglycine substitutions in type I collagen are distinct from those caused by glycine substitutions. *Hum Mutat* 0, 1-10, 2007. Published 2007 Wiley-Liss, Inc.

Castaneda L., Valle J., Yang N., Pluskat S. and Slowinska K. (2008) Collagen Cross-Linking with Au Nanoparticles. *Biomacromolecules* (epublication).

Abstract: Tiopronin (N-(2-mercaptopropionyl)glycine)-protected gold nanoparticles (TPAu) were cross-linked to collagen via EDC (1-ethyl-3-(3-dimethyl aminopropyl) carbodiimide) coupling. On average, each TPAu forms eight amide bonds with collagen lysine moieties. The resulting gels were studied with environmental SEM, TEM, micro-DSC, and TNBS assay. The porous structure of collagen was

significantly altered by cross-linking, resulting in the reduction of the pore size from ca. 140 to <1 μm depending on the concentration of nanoparticles. The collagenase biodegradation assay showed improved stability of cross-linked material. The cell viability assay, CellTiter96, indicates that the gold nanoparticles are not toxic at the concentrations used in gel synthesis. This new material has potential for the delivery of small molecule drugs as well as Au nanoparticles for photothermal therapies, imaging, and cell targeting

Chan B. P. and So K. F. (2005) Photochemical crosslinking improves the physicochemical properties of collagen scaffolds. *J Biomed Mater Res A* **75**, 689-701.

Abstract: Collagen is a natural biomaterial with excellent biocompatibility. However, unprocessed collagen has low stability and weak mechanical strength, which limits its application in tissue engineering. The current study aimed to improve the physicochemical properties of collagen scaffolds by using photochemical crosslinking. Collagen gel was reconstituted and photochemically crosslinked by using laser irradiation in the presence of a photosensitizer. Scanning electron microscope was used to characterize the surface and cross-sectional morphology. Stress-strain relationship and other mechanical properties were determined by uniaxial tensile tests. Thermostability and water-binding capacities also were analyzed by using differential scanning calorimetry and swelling ratio measurements, respectively. Photochemically crosslinked porous structures showed fine microstructure with interconnected micron-sized pores, whereas uncrosslinked controls only showed macrosheet-like structures. The stabilizing effect of photochemical crosslinking also was revealed by retaining the three-dimensional lamellae-like structures after thermal analysis in crosslinked membranes but not in the controls. Photochemical crosslinking also significantly reduced the swelling ratio, improved the stress-strain relationship, peak load, ultimate stress, rupture strain, and tangent modulus of collagen membranes. The current study showed that an innovative photochemical crosslinking process was able to produce collagen scaffolds with fine microstructures; to strengthen, stiffen, and stabilize collagen membranes; and to modify their swelling ratio. This may broaden the use of collagen-based scaffolds in tissue engineering, particularly for weight-bearing tissues. (c) 2005 Wiley Periodicals, Inc. *J Biomed Mater Res*, 2005.

Charbonneau C., Gautrot J. E., Hebert M. J., Zhu X. X. and Lerouge S. (2007) Chondroitin-4-sulfate: a bioactive macromolecule to foster vascular healing around stent-grafts after endovascular aneurysm repair. *Macromol. Biosci.* **7**, 746-752.

Abstract: Deficient healing after endovascular aneurysm repair with a stent-graft is thought to be related to pro-apoptotic environment in abdominal aortic aneurysms and inertness of graft materials. We developed a bioactive coating containing chondroitin-4-sulfate and assessed its potential to improve cell adhesion, viability and resistance to apoptosis on PET surfaces. Coatings of collagen type I and CS were prepared and characterized by DMMB, FT-IR, DSC, SEM and contact angle goniometry. Preliminary cell culture experiments with vascular smooth muscle cells showed increased adhesion and viability in serum-free medium on CS-coated surfaces compared to control PET films.

Choudhury S. D., DasGupta S. and Norris G. E. (2007) Unravelling the mechanism of the interactions of oxazolidine A and E with collagens in ovine skin. *Int J Biol Macromol.* **40**, 351-361.

Abstract: Cross-linking agents play an important part in the physical properties of collagen based biomaterials. Oxazolidines are novel aldehydic tanning agents that are widely used to stabilise collagens in the leather industry. The exact mechanism through which they cross-link collagens is, however, not well understood. When they are combined with vegetable tannins, it is thought that oxazolidines form carbocationic intermediates through ring opening, which are then able to interact with the amino acid side chains of collagens and flavonoid ring systems of vegetable tannins. In this study, the interactions of oxazolidines, with collagens, have been investigated using a number of analytical techniques. High pressure liquid chromatography (HPLC) analysis of oxazolidine tanned collagen samples showed that there is an irreversible reaction with tyrosine side chains. Mass spectrometry (MS) revealed the formation of a Schiff's base adduct with lysine residues, which was reversible in nature. MS analysis of reaction of oxazolidines with a model peptide Suc-Ala-Phe-Lys-AMC in presence of NaCNBH(3), indicated the formation of a product with an increase in molecular weight of 28 kD characteristic of the addition of two methyl groups to lysine. Differential scanning calorimetry showed a synergistic effect for combination tanning, with best results being obtained when vegetable tan was added prior to the aldehydic tanning agents. Circular dichroism (CD) studies of collagen in presence of the more reactive oxazolidine A showed that there was a loss in ellipticity simply because of aggregation of collagen molecules rather than a change

in the secondary structure. Based on the results obtained, a scheme has been proposed to explain the possible mechanism of action of oxazolidines with the collagen amino acid side chains.

Cote M. F., Laroche G., Gagnon E., Chevallier P., and Doillon C. J. (2004) Denatured collagen as support for a FGF-2 delivery system: physicochemical characterizations and in vitro release kinetics and bioactivity. *Biomaterials* **25**, 3761-3772.

Abstract: Collagen-based materials have scaffold properties to support bioactive molecules such as growth factor (GF). Gelatin, a denatured collagen, may have also some potential to interact with GF. An alternative process to denature collagen using trifluoroacetic acid (TFA) was investigated. Physicochemical characterization (XPS, DSC, isoelectric point, water uptake) of TFA-denatured collagen was comparable to regular gelatin, except a significant hydrophilicity and a pH sensitivity. FGF-2 was mixed with either regular gelatin or TFA-denatured collagen, then incorporated to a collagen sponge. Autoradiography revealed a relatively homogenous distribution of radiolabeled FGF-2 within the sponge. In vitro release kinetic of radiolabeled FGF-2 was investigated as well as the bioactivity of FGF-2 towards endothelial cell growth. The mixture was also sorbed to hydrogels made of ethylene vinyl acetate co-polymer and poly(2-hydroxyethyl methacrylate), and to cell culture insert membranes as control. Release of FGF-2 from collagen was progressive in the presence of TFA-denatured collagen, and cell growth was stimulated (significant peak at 8 and 10 days) by TFA-denatured collagen and FGF-2 eluted particularly from collagen sponges. Whereas control hydrogels, and those with regular gelatin showed a early stimulation of cell growth (1-5 days). Thus, the combination of both FGF-2 and an acid-denatured collagen in collagen sponges allows to sustain in vitro endothelial cell activity.

Damodaran G., Collighan R., Griffin M. and Pandit A. (2008) Tethering a laminin peptide to a crosslinked collagen scaffold for biofunctionality. *J Biomed. Mater. Res A*. (epublication)

Abstract: Cell adhesion peptide regulates various cellular functions like proliferation, attachment, and spreading. The cellular response to laminin peptide (PPFLMLLKGSTR), a motif of laminin-5 alpha3 chain, tethered to type I collagen, crosslinked using microbial transglutaminase (mTGase) was investigated. mTGase is an enzyme that initiates crosslinking by reacting with the glutamine and lysine residues on the collagen fibers stabilizing the molecular structure. In this study that tethering of the laminin peptide in a mTGase crosslinked collagen scaffold enhanced cell proliferation and attachment. Laminin peptide tethered crosslinked scaffold showed unaltered cell morphology of 3T3 fibroblasts when compared with collagen and crosslinked scaffold. The triple helical structure of collagen remained unaltered by the addition of laminin peptide. In addition a dose-dependent affinity of the laminin peptide towards collagen was seen. The degree of crosslinking was measured by amino acid analysis, differential scanning calorimeter and fourier transform infrared spectroscopy. Increased crosslinking was observed in mTGase crosslinked group. mTGase crosslinking showed higher shrinkage temperature. There was alteration in the fibrillar architecture due to the crosslinking activity of mTGase. Hence, the use of enzyme-mediated linking shows promise in tethering cell adhesive peptides through biodegradable scaffolds. (c) 2008 Wiley Periodicals, Inc. *J Biomed Mater Res*, 2008

Danilov N. A., Ignatieva N. Y., Iomdina E. N., Semenova S. A., Rudenskaya G. N., Grokhovskaya T. E. and Lunin V. V. (2008) Stabilization of scleral collagen by glycerol aldehyde cross-linking. *Biochim Biophys Acta* **1780**, 764-772.

Abstract: The paper aims at the evaluation of prospects for using glyceraldehyde as a cross-linking agent for the scleral tissue. Stability parameters (denaturation temperature, Young's modulus, ultimate tensile stress, proteolytic resistance) and analytical parameter (fluorescence intensity) were determined during the glycation process of isolated rabbit sclera. The analysis of fluorescence spectral characteristic provided information about some glycation products. The glyceraldehyde treatment was resulted in a significant increase in thermal stability, proteolytic resistance and improvement of biomechanical characteristics (Young's modulus, ultimate tensile stress). Unique properties of the reaction between scleral collagen and glyceraldehyde are observed at short cross-linking times. The appearance of intermediate collagen fraction with lowest thermal and proteolytic stability was detected

Du C., Wang M., Liu J., Pan M., Cai Y. and Yao J. (2008) Improvement of thermostability of recombinant collagen-like protein by incorporating a foldon sequence. *Appl. Microbiol Biotechnol.* **79**, 195-202.

Abstract: Collagen is a popular biomaterial in many specific biological interactions as well as a structural element. In this work, the recombinant collagen-like proteins were synthesized using Escherichia coli expression system. A foldon sequence, GYIPEAPRDGQAYVRKDG EWVLLSTFL, derived from the native T4 phage fibrin was incorporated at the C-terminal of collagen-like protein molecules to stabilize the triple helix formed in the proteins. The differential scanning calorimetry and thermogravimetric analysis measurements showed that the thermostability of the recombinant collagen-like proteins was significantly improved when compared with those without the foldon sequence at the C-terminal. Fourier transform infrared and scanning electron microscopy observations indicated that the collagen-like proteins forms the triple helix structure and prefer to aggregate as fibrils, same as the native collagen. Moreover, the mice fibroblasts L929 cells could attach and grew very well on the recombinant collagen-like proteins. 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay showed that the cell biocompatibility of collagen-like proteins produced in this work was even better than that of native collagen, suggesting that the collagen-like proteins may be a satisfactory candidate for the future applications as a biomaterial

Duan X. and Sheardown H. (2005) Crosslinking of collagen with dendrimers. *J Biomed Mater Res A* **75**, 510-518.

Abstract: Polypropyleneimine octaamine dendrimers were studied as an alternative means of generating highly crosslinked collagen. Crosslinking was effected by using the water-soluble carbodiimide 1-ethyl-3-(3-dimethyl aminopropyl) carbodiimide hydrochloride (EDC). The multifunctional dendrimers were introduced as novel crosslinkers after the activation of the carboxylic acid groups of glutamic and aspartic acid residues in collagen. The conventional crosslinker glutaraldehyde was used as a control. EDC, itself an alternative crosslinker, which forms zero-length crosslinks by directly covalently binding collagen molecules, as well as a low molecular weight diamine and a low molecular weight triamine, were also studied. All of the resultant gels were freeze-dried to obtain sponges for characterization. Water uptake of the gels decreased from 90% to 60% after dendrimer crosslinking compared with EDC crosslinking. DSC results showed an increase of denaturation temperature of collagen after crosslinking with the various methods. The generation 2 and 3 dendrimer-crosslinked collagen samples had the highest denaturation temperature, at up to 90 degrees C compared with 50 degrees C in the uncrosslinked collagen control. The dendrimer-crosslinked collagen also showed unique thermal characteristics, with multiple denaturation temperature peaks in contrast to the single peak noted with the other crosslinked collagens. This is thought to be due to the heterogeneous nature of dendrimer crosslinking. Collagenase results revealed that the dendrimer-crosslinked collagen had a comparative resistance to proteolysis to glutaraldehyde-crosslinked collagen. Measurement of activated carboxylic acid groups before and after crosslinking indicated that 40-70% of the activated carboxylic acid was consumed during crosslinking with dendrimers. The results suggest that dendrimer crosslinking of collagen produces stable gels. The presence of a large number of excess amine groups in the dendrimers may also be useful for subsequent modification with biologically relevant groups. (c) 2005 Wiley Periodicals, Inc. *J Biomed Mater Res*, 2005.

Fathima N. N., Bose M. C., Rao J. R., and Nair B. U. (2006) Stabilization of type I collagen against collagenases (type I) and thermal degradation using iron complex. *J Inorg Biochem* **100**, 1774-1780.

Abstract: The widespread application of collagen as a biomaterial warrants research in understanding the stabilization of the same. In this study, interaction of iron-tetrakis (hydroxymethyl) phosphonium (THP) complex with type I collagen has been investigated. DSC and hydrothermal measurement studies reveal that the shrinkage temperature of iron-THP treated rat tail tendon (RTT) collagen is 33 degrees C higher than that of native RTT collagen. Fe-THP complex also brings about high degree of enzymatic stability to type I collagen. The effect of Fe-THP on the conformation of collagen was studied using circular dichroism and it was found that no major alterations in the triple helical structure of collagen occur on treatment with Fe-THP. It is observed from viscosity experiment results that though Fe-THP complex is able to bring about long range ordering of collagen, as evident from the thermal and enzymatic stability imparted to collagen, this ordering does not lead to any aggregation of collagen. Since THPS is reducing in nature, it is expected to keep iron in the +2 state and if THP chelates to Fe(II), the hydrolytic behavior of iron can also be controlled.

Fernandes R. M., Couto Neto R. G., Paschoal C. W., Rohling J. H. and Bezerra C. W. (2008) Collagen films from swim bladders: preparation method and properties. *Colloids Surf B Biointerfaces* **62**, 17-21.

Abstract: This paper describes the preparation and characterization of collagen films extracted from swim bladders of three species of tropical fishes: *Arius parkeri* (Gurijuba), *Cynoscion acoupa* (Pescada Amarela) and *Cynoscion leiarchus* (Pescada Branca). Collagen was extracted under acidic conditions (CH₃COOH, 2.5 pH) and precipitated by the addition of NaCl up to 3.0 mol L⁻¹. The films were prepared in acrylic containers and dried in a vacuum atmosphere. The collagen films were characterized by hydroxyproline contents, thermal analysis, scanning electron microscopy and impedance spectroscopy. The determined values of 4-hydroxyproline and collagens in the films were: 105.23±4.48 and 873.2; 102.94±4.42 and 854.1; 100.65±4.80 and 835.8 mg g⁻¹ for *A. parkeri*, *C. acoupa* and *C. leiarchus*, respectively. Differential scanning calorimetry revealed high denaturation temperature peaks at temperatures ranging from 65.9 to 74.8 degrees C. The micrographs showed no fibrillar organization along the material, but spongy structure, with cavity diameters relatively uniform, at around 2 microm. The impedance spectroscopy presented a distributed relaxation process. *A. parkeri*'s films showed piezoelectricity

Figueiro S. D., Macedo A. A., Melo M. R., Freitas A. L., Moreira R. A., de Oliveira R. S., Goes J. C., and Sombra A. S. (2006) On the dielectric behaviour of collagen-algal sulfated polysaccharide blends: effect of glutaraldehyde crosslinking. *Biophys Chem* **120**, 154-159.

Abstract: In this paper, impedance measurements in the frequency range from 10⁻² to 10⁶ Hz are presented for collagen and algal sulfated polysaccharide crosslinked films. We are considering the development of new biomaterials which have potential applications in coating of cardiovascular prostheses, support for cellular growth and in systems for controlled drug delivery. The effect of crosslink sulfated polysaccharide on the physical chemical properties of collagen was studied using FT-infrared spectroscopy, differential scanning calorimetry (DSC), dielectric spectroscopy. The resulting films crosslinked with glutaraldehyde (GA) in concentrations of 0.001% and 0.05% when analysed by DSC, showed that the GA treatment not only left the thermal stability of the collagen unaffected, but it also decreased the thermal transition energy. Dielectric spectroscopy shows that the effect of the crosslink on the blend film was associated to the decrease and stabilization of the dielectric permittivity at low frequencies and decreased its conductivity.

Freudenberg U., Behrens S. H., Welzel P. B., Muller M., Grimmer M., Salchert K., Taeger T., Schmidt K., Pompe W. and Werner C. (2007) Electrostatic interactions modulate the conformation of collagen I. *Biophys J* **92**, 2108-2119.

Abstract: The pH- and electrolyte-dependent charging of collagen I fibrils was analyzed by streaming potential/streaming current experiments using the Microslit Electrokinetic Setup. Differential scanning calorimetry and circular dichroism spectroscopy were applied in similar electrolyte solutions to characterize the influence of electrostatic interactions on the conformational stability of the protein. The acid base behavior of collagen I was found to be strongly influenced by the ionic strength in KCl as well as in CaCl₂ solutions. An increase of the ionic strength with KCl from 10⁻⁴ M to 10⁻² M shifts the isoelectric point (IEP) of the protein from pH 7.5 to 5.3. However, a similar increase of the ionic strength in CaCl₂ solutions shifts the IEP from 7.5 to above pH 9. Enhanced thermal stability with increasing ionic strength was observed by differential scanning calorimetry in both electrolyte systems. In line with this, circular dichroism spectroscopy results show an increase of the helicity with increasing ionic strength. Better screening of charged residues and the formation of salt bridges are assumed to cause the stabilization of collagen I with increasing ionic strength in both electrolyte systems. Preferential adsorption of hydroxide ions onto intrinsically uncharged sites in KCl solutions and calcium binding to negatively charged carboxylic acid moieties in CaCl₂ solutions are concluded to shift the IEP and influence the conformational stability of the protein.

He C., Wang Y., Yang L., Pan J., Xia L., and Zhang J. (2005) [Preparation and characterization of bovine bone collagen matrix]. *Sheng Wu Yi Xue Gong Cheng Xue Za Zhi* **22**, 698-703.

Abstract: A process of preparing bovine cortical bone in order to form materials suitable for biomedical xenograft implants was described. Fresh bone samples cut from the middiaphyseal region of bovine femora were obtained from a local slaughterhouse. The bovine bone collagen matrix (BBCM) of various shapes fabricated from bovine bone by defatting and deproteination procedure may be implanted surgically for various purposes. The bone cubes were first defatted in a mixture of defatting agent; subsequently, the samples were extracted to release noncollagenous proteins, followed by digestion using a proteolytic enzyme to remove the telopeptide portions of collagen and residual noncollagenous proteins. Finally, the

samples were dried in vacuum, packed and sterilized by gamma irradiation. The bone specimens were characterized by a suite of analytical techniques involving FTIR spectroscopy, X-ray diffraction spectroscopy, differential scanning calorimetry (DSC), uniaxial tension mechanical tests and scanning electron microscopy (SEM). The result showed that BBCM occurred as a white structure with suitable porosity. It contains reasonable proportion of mineral and organic components in the original osseous architecture of the bovine bone, which is beneficial to keeping the mechanic property and weaker immunogenicity; therefore, it can serve as a potential bone implantable material and extracellular matrix material in bone tissue engineering.

Henkel W., Vogl T., Echner H., Voelter W., Urbanke C., Schleuder D., and Rauterberg J. (1999) Synthesis and folding of native collagen III model peptides. *Biochemistry* **38**, 13610-13622.

Abstract: Solid-phase synthesis of triple-helical peptides, including native collagen III sequences, was started with a trimeric branch, based upon the lysine dipeptide [Fields, C. G., Mickelson, D. J., Drake, S. L., McCarthy, J. B., and Fields, G. B. (1993) *J. Biol. Chem.* 268, 14153-14160]. Branch synthesis was modified, using TentaGel R as resin, p-hydroxybenzyl alcohol (HMP) as linker, Dde as N(epsilon)-protective group, and HATU/HOAT as coupling reagent. Three homotrimeric sequences, each containing the Gly 606-Gly 618 portion of human type III collagen, were added to the amino functions of the branch. The final incorporation of GlyProHyp triplets, stabilizing the collagen III triple helix, was performed by using protected GlyProHyp tripeptides, each containing tert-butylated hydroxyproline [P(tBu)] instead of hydroxyproline (P). Among the protected tripeptides FmocP(tBu)PG, FmocPP(tBu)G, and FmocGPP(tBu), prepared manually on a chlorotrityl resin, incorporation of FmocPP(tBu)Gly was best suited for synthesis of large and stable peptides, such as PPG(8), containing 8 (PPG)(3) trimers (115 residues, 10 610 Da). The structures of five peptides, differing from each other by the type and number of the triplets incorporated, were verified by MALDI-TOF-MS. Their conformations and thermodynamic data were studied by circular dichroism and differential scanning calorimetry. Except for PPG(8), containing 8 (PPG)(3) trimers with hydroxyproline in the X position and adopting a polyproline II structure, all peptides were triple-helical in 0.1 M acetic acid and their thermal stabilities ranged from $t(1/2) = 39.4$ to $t(1/2) = 62.5$ degrees C, depending on the identity and number of the triplets used. Similar values of the van't Hoff enthalpy, $\Delta H(vH)$, derived from melting curves, and the calorimetric enthalpy, $\Delta H(cal)$, obtained from heat capacity curves, indicate a cooperative ratio of $CR = \Delta H(vH)/\Delta H(cal) = 1$, establishing a two-state process for unfolding of THP(III) peptides. The independence of the transition temperatures $t(1/2)$ on peptide concentration as well as equilibrium centrifugation data indicate monomolecular dimer(f) to dimer(u) ($F(2) \leftrightarrow U(2)$) transitions and, in addition, bimolecular dimer(f) to monomer(u) transitions ($F(2) \leftrightarrow 2U$). The dominance of the concentration-independent monomolecular reaction over the concentration-dependent bimolecular reaction makes thermal unfolding of THP(III) peptides appear to be monomolecular. If one designates the molecularity described by the term pseudomonomolecular, unfolding of the dimeric peptides PPG(6-8) follows a two-state, pseudomonomolecular reaction.

Kar K., Amin P., Bryan M. A., Persikov A. V., Mohs A., Wang Y. H., and Brodsky B. (2006) Self-association of collagen triple helix peptides into higher order structures. *J Biol Chem* **281**, 33283-33290.

Abstract: Interest in self-association of peptides and proteins is motivated by an interest in the mechanism of physiologically higher order assembly of proteins such as collagen as well as the mechanism of pathological aggregation such as beta-amyloid formation. The triple helical form of (Pro-Hyp-Gly)(10), a peptide that has proved a useful model for molecular features of collagen, was found to self-associate, and its association properties are reported here. Turbidity experiments indicate that the triple helical peptide self-assembles at neutral pH via a nucleation-growth mechanism, with a critical concentration near 1 mM. The associated form is more stable than individual molecules by about 25 degrees C, and the association is reversible. The rate of self-association increases with temperature, supporting an entropically favored process. After self-association, (Pro-Hyp-Gly)(10) forms branched filamentous structures, in contrast with the highly ordered axially periodic structure of collagen fibrils. Yet a number of characteristics of triple helix assembly for the peptide resemble those of collagen fibril formation. These include promotion of fibril formation by neutral pH and increasing temperature; inhibition by sugars; and a requirement for hydroxyproline. It is suggested that these similar features for peptide and collagen self-association are based on common lateral underlying interactions between triple helical molecules mediated by hydrogen-bonded hydration networks involving hydroxyproline.

Komsa-Penkova R., Koynova R., Kostov G., and Tenchov B. G. (1996) Thermal stability of calf skin collagen type I in salt solutions. *Biochim Biophys Acta* **1297**, 171-181.

Abstract: The thermal stability of acid-soluble collagen type I from calf skin in salt solutions is studied by high-sensitivity differential scanning calorimetry. Three concentration ranges have been clearly distinguished in the dependence of collagen thermal stability on ion concentration. At concentrations below 20 mM, all studied salts reduce the temperature of collagen denaturation with a factor of about 0.2 degree C per 1 mM. This effect is attributed to screening of electrostatic interactions leading to collagen stabilisation. At higher concentrations, roughly in the range 20-500 mM, the different salts either slightly stabilise or further destabilise the collagen molecule in salt-specific way that correlates with their position in the lyotropic series. The effect of anions is dominating and follows the order H.

Komsa-Penkova R., Koynova R., Kostov G., and Tenchov B. (2000) Discrete reduction of type I collagen thermal stability upon oxidation. *Biophys Chem* **83**, 185-195.

Abstract: The oxidation of acid-soluble calf skin collagen type I caused by metal-dependent free radical generating systems, Fe(II)/H₂O₂ and Cu(II)/H₂O₂, was found to bring down in a specific, discrete way the collagen thermal stability, as determined by microcalorimetry and scanning densitometry. Initial oxidation results in splitting of the collagen denaturational transition into two components. Along with the endotherm at 41 degrees C typical for non-oxidized collagen, a second, similarly cooperative endotherm appears at 35 degrees C and increases in enthalpy with the oxidant concentration and exposure time, while the first peak correspondingly decreases. The two transitions at 35 and 41 degrees C were registered by densitometry as stepwise increases of the collagen-specific volume. Further oxidation results in massive collagen destruction manifested as abolishment of both denaturational transitions. The two oxidative systems used produce identical effects on the collagen stability but at higher concentrations of Cu(II) in comparison to Fe(II). The discrete reduction of the protein thermal stability is accompanied by a decrease of the free amino groups, suggestive of an oxidation attack of the side chains of lysine residues. Since the denaturation temperature of collagen shifts from above to below body temperature (41 degrees C-35 degrees C) upon oxidation, it appears important to account for this effect in a context of the possible physiological implications of collagen oxidation.

Kotch F. W. and Raines R. T. (2006) Self-assembly of synthetic collagen triple helices. *Proc Natl Acad Sci U S A* **103**, 3028-3033.

Abstract: Collagen is the most abundant protein in animals and the major component of connective tissues. Although collagen isolated from natural sources has long served as the basis for some biomaterials, natural collagen is difficult to modify and can engender pathogenic and immunological side effects. Collagen comprises a helix of three strands. Triple helices derived from synthetic peptides are much shorter (<10 nm) than natural collagen (approximately 300 nm), limiting their utility. Here, we describe the synthesis of short collagen fragments in which the three strands are held in a staggered array by disulfide bonds. Data from CD spectroscopy, dynamic light scattering, analytical ultracentrifugation, atomic force microscopy, and transmission electron microscopy indicate that these "sticky-ended" fragments self-assemble via intermolecular triple-helix formation. The resulting fibrils resemble natural collagen, and some are longer (>400 nm) than any known collagen. We anticipate that our self-assembly strategy can provide synthetic collagen-mimetic materials for a variety of applications.

Kumar T. R., Shanmugasundaram N., and Babu M. (2003) Biocompatible collagen scaffolds from a human amniotic membrane: physicochemical and in vitro culture characteristics. *J Biomater Sci Polym Ed* **14**, 689-706.

Abstract: A reconstituted collagen membrane from human amnion has been investigated as a source of collagen matrix, which could be used as a substratum for culturing human fibroblasts. The suitability of pepsin-solubilized reconstituted human amniotic membrane, before and after cross-linking with chitosan, as a dermal matrix for culturing fibroblast was assessed by morphologic, physicochemical, cytotoxic and histochemical methods. Measurement of thermodynamic behaviour, by differential scanning calorimetric (DSC) and thermogravimetric analysis (TGA), and tensile strength suggested that the cross-linked membrane had sufficient elasticity to serve as an efficient dermal substrate for in vitro culture of fibroblasts. Fibroblasts cultured on the chitosan cross-linked collagen membrane had good adherence, retaining their morphology as indicated by microscopic analysis. Proliferation of fibroblasts. observed on

this membrane affirms its non-toxic nature. These results support the application of reconstituted human amniotic collagen membrane as collagenous scaffolds to culture fibroblasts in vitro.

Kuznetsova N. V., McBride D. J., and Leikin S. (2003) Changes in thermal stability and microunfolded pattern of collagen helix resulting from the loss of alpha2(I) chain in osteogenesis imperfecta murine. *J Mol Biol* **331**, 191-200.

Abstract: Homozygous mutations resulting in formation of alpha1(I)(3) homotrimers instead of normal type I collagen cause mild to severe osteogenesis imperfecta (OI) in humans and mice. Limited studies of changes in thermal stability of type I homotrimers were reported previously, but the results were not fully consistent. We revisited this question in more detail using purified tendon collagen from wild-type (alpha1(I)(2)alpha2(I) heterotrimers) and oim (alpha1(I)(3)) mice as well as artificial alpha1(I)(3) homotrimers obtained by refolding of rat-tail-tendon collagen. We found that at the same heating rate oim homotrimers completely denature at approximately 2.5deg.C higher temperature than wild-type heterotrimers, as determined by differential scanning calorimetry. At the same, constant temperature, homotrimers denature approximately 100 times slower than heterotrimers, as determined by circular dichroism. Detailed analysis of proteolytic cleavage at different temperatures revealed that microunfolded of oim homotrimers and wild-type heterotrimers occurs at similar rate but within a number of different sites. In particular, the weakest spot on the oim triple helix is located approximately 100 amino acid residues from the C-terminal end within the cyanogen bromide peptide CB6. The same microunfolded site is also present in wild-type collagen, but the weakest spot of the latter is located close to the N-terminal end of CB8. Amino acid analysis and differential gel electrophoresis showed virtually no posttranslational overmodification of oim mouse tendon collagen. Moreover, thermal stability and microunfolded of artificial rat-tail-tendon homotrimers were similar to oim homotrimers. Thus, the observed changes are associated with difference in the amino acid composition of alpha1(I) and alpha2(I) chains rather than posttranslational overmodification.

Lazarev I., Lazareva A. V., Khromova T. B., and Grechishko V. S. (1997) [Thermodynamic studies of triple-helical structures of the collagen type in oligotripeptides during study of molecular chain elongation]. *Biofizika* **42**, 326-333.

Abstract: The conformational transition collagen-like triple helix in equilibrium with chains of oligotripeptides Z-(Gly-Pro-Pro)_n-OMe with n = 6, 7, 8 in water by variation of solution temperature and sample concentration has been studied using IR-, CD-spectroscopy and microcalorimetry methods. The straight line correlation between the obtained value of the transition enthalpy and entropy and the number of the triplets (3n - 2), involved in the interpeptide set of hydrogen bonds was revealed. Evidently the effect of terminal groups is really weak in this case, and the interpeptide bonds of the triple helix may be regarded as equivalent one another. The estimated cooperative block of nucleation corresponds in length to the one full turn of the superhelix. The state diagrams of the oligotripeptides with n = 6, 7, 8 in aqueous solution are presented.

Leikina E., Merts M. V., Kuznetsova N., and Leikin S. (2002) Type I collagen is thermally unstable at body temperature. *Proc Natl Acad Sci U S A* **99**, 1314-1318.

Abstract: Measured by ultra-slow scanning calorimetry and isothermal circular dichroism, human lung collagen monomers denature at 37 degrees C within a couple of days. Their unfolding rate decreases exponentially at lower temperature, but complete unfolding is observed even below 36 degrees C. Refolding of full-length, native collagen triple helices does occur, but only below 30 degrees C. Thus, contrary to the widely held belief, the energetically preferred conformation of the main protein of bone and skin in physiological solution is a random coil rather than a triple helix. These observations suggest that once secreted from cells collagen helices would begin to unfold. We argue that initial microunfolded of their least stable domains would trigger self-assembly of fibers where the helices are protected from complete unfolding. Our data support an earlier hypothesis that in fibers collagen helices may melt and refold locally when needed, giving fibers their strength and elasticity. Apparently, Nature adjusts collagen hydroxyproline content to ensure that the melting temperature of triple helical monomers is several degrees below rather than above body temperature.

Makareeva E., Cabral W. A., Marini J. C., and Leikin S. (2006) Molecular mechanism of alpha 1(I)-osteogenesis imperfecta/Ehlers-Danlos syndrome: unfolding of an N-anchor domain at the N-terminal end

of the type I collagen triple helix. *J Biol Chem* **281**, 6463-6470.

Abstract: We demonstrate that 85 N-terminal amino acids of the alpha1(I) chain participate in a highly stable folding domain, acting as the stabilizing anchor for the amino end of the type I collagen triple helix. This anchor region is bordered by a microunfolded region, 15 amino acids in each chain, which include no proline or hydroxyproline residues and contain a chymotrypsin cleavage site. Glycine substitutions and amino acid deletions within the N-anchor domain induce its reversible unfolding above 34 degrees C. The overall triple helix denaturation temperature is reduced by 5-6 degrees C, similar to complete N-anchor removal. N-propeptide partially restores the stability of mutant procollagen but not sufficiently to prevent N-anchor unfolding and a conformational change at the N-propeptide cleavage site. The ensuing failure of N-proteinase to cleave at the misfolded site leads to incorporation of pN-collagen into fibrils. Similar, but weaker, effects are caused by G88E substitution in the adjacent triplet, which appears to alter N-anchor structure as well. As in Ehlers-Danlos syndrome (EDS) VIIA/B, fibrils containing pN-collagen are thinner and weaker causing EDS-like laxity of large and small joints and paraspinal ligaments. However, distinct structural consequences of N-anchor destabilization result in a distinct alpha1(I)-osteogenesis imperfecta (OI)/EDS phenotype.

Makareeva E., Mertz E. L., Kuznetsova N. V., Sutter M. B., DeRidder A. M., Cabral W. A., Barnes A. M., McBride D. J., Marini J. C. and Leikin S. (2008) Structural heterogeneity of type I collagen triple helix and its role in osteogenesis imperfecta. *J Biol Chem* **283**, 4787-4798.

Abstract: We investigated regions of different helical stability within human type I collagen and discussed their role in intermolecular interactions and osteogenesis imperfecta (OI). By differential scanning calorimetry and circular dichroism, we measured and mapped changes in the collagen melting temperature (DeltaTm) for 41 different Gly substitutions from 47 OI patients. In contrast to peptides, we found no correlations of DeltaTm with the identity of the substituting residue. Instead, we observed regular variations in DeltaTm with the substitution location in different triple helix regions. To relate the DeltaTm map to peptide-based stability predictions, we extracted the activation energy of local helix unfolding (DeltaG) from the reported peptide data. We constructed the DeltaG map and tested it by measuring the H-D exchange rate for glycine NH residues involved in interchain hydrogen bonds. Based on the DeltaTm and DeltaG maps, we delineated regional variations in the collagen triple helix stability. Two large, flexible regions deduced from the DeltaTm map aligned with the regions important for collagen fibril assembly and ligand binding. One of these regions also aligned with a lethal region for Gly substitutions in the alpha1(I) chain

Mesropian I., Burdzhaniadze T. V., Barbakadze S., Esipova N. G., and Monaselidze D. R. (2006) [A microcalorimetric study of collagen hydrated water in a temperature range 20-100 degrees C]. *Biofizika* **51**, 151-152.

Abstract: A new method for determining bound water was developed, which is based on precise measurements of the enthalpy of water evaporation from a sample using differential scanning calorimetry.

Metreveli N. O., Hamicheishvili L. O., Dzhariashvili K. K., Chikvaidze E. N., and Mrevlishvili G. M. (2006) [Microcalorimetric and electron spin resonance study of the influence of UV radiation on collagen]. *Biofizika* **51**, 39-43.

Abstract: It has been shown by microcalorimetry that UV-irradiation cardinally alters the temperature dependence of heat capacity of a collagen solution and decreases the enthalpy of collagen heat denaturation. By using the method of electron spin resonance (ESR), it was found that the primary products of UV-irradiated acid-soluble collagen are the atomic hydrogen and the anion radical of acetic acid. The latter, under the influence of long-wavelength UV light, is transformed into the methyl radical, which interacts with acetic acid to produce acetic acid radical. The above free radicals interact with the collagen molecule, as a result of which seven superfine components with the split of $\Delta H = 1.13$ mT are obtained in the ESR spectrum. It is assumed that this spectrum is related to the free radical that occurred in the proline residue of the collagen molecule. In this particular case, this is a major structural defect in the triple helix of collagen, which results in instability of the macromolecule.

Miles C. A., Burjanadze T. V., and Bailey A. J. (1995) The kinetics of the thermal denaturation of collagen in unrestrained rat tail tendon determined by differential scanning calorimetry. *J Mol Biol* **245**, 437-446.

Abstract: This paper shows that the position and shape of the denaturation endotherm of collagen fibrils are

governed by the kinetics of an irreversible rate process. This was proved by measuring the rate of denaturation in rat tail tendons held isothermally at different temperatures, thereby determining rate constant characteristics such as the activation enthalpy and entropy and predicting endotherm position and shape therefrom. Comparison with actual scanning results showed good correspondence. Isothermal measurements of the rate of collagen denaturation, measured continuously using a calorimetric method, were used to determine rate constants for collagen denaturation in tendons immersed in water and 0.5 M acetic acid. The temperature dependence of the rate constants were fitted to the three rate process models, previously examined theoretically: the D and z formulation, the Arrhenius equation and the absolute rate theory. For example, in water the activation enthalpy was 0.518 (+/- 0.016) Mj mol⁻¹ and the activation entropy 1.485 (+/- 0.049) kj mol⁻¹ K⁻¹, while in acetic acid the corresponding figures were 1.306 (+/- 0.099) Mj mol⁻¹ and 4.142 (+/- 0.323) kj mol⁻¹ K⁻¹. These characteristics are discussed in terms of the thermal activation of a region of the molecule, the co-operative unit. The ratio of the activation enthalpy to the calorimetry enthalpy of denaturation indicated a co-operative unit that was 66 (+/- 5) residues long when fibrils were swollen in acetic and the collagen molecules acted essentially independently. On the other hand the intact fibrils in water gave a co-operative unit of 26 (+/- 1) residues long. The reason for the reduction in size of the co-operative unit is that it is surrounded, and therefore stabilized by other molecules in the fibre. It is interesting to note that the suggested co-operative unit lies almost entirely within the "gap" zone of the collagen fibril in its quarter-staggered arrangement of molecules. We believe that the co-operative unit would be represented by a domain that is free of stabilising hydroxyproline residues. Indeed such a domain exists near the C terminus of the triple helix from Gly877 to Pro941, i.e. 65 residues. In acetic acid, activation is similar to that of collagen molecules in solution. All the inter alpha-chain hydrogen bonds in the co-operative unit are broken and the separate chains in this short region are free to flail around under the action of thermal collisions relatively unimpeded by intermolecular interactions.(ABSTRACT TRUNCATED AT 400 WORDS).

Miles C. A. and Ghelashvili M. (1999) Polymer-in-a-box mechanism for the thermal stabilization of collagen molecules in fibers. *Biophys J* **76**, 3243-3252.

Abstract: Collagen molecules in solution unfold close to the maximum body temperature of the species of animal from which the molecules are extracted. It is therefore vital that collagen is stabilized during fiber formation. In this paper, our concept that the collagen molecule is thermally stabilized by loss of configurational entropy of the molecule in the fiber lattice, is refined by examining the process theoretically. Combining an equation for the entropy of a polymer-in-a-box with our previously published rate theory analysis of collagen denaturation, we have derived a hyperbolic relationship between the denaturation temperature, T_m , and the volume fraction, ϵ , of water in the fiber. DSC data were consistent with the model for water volume fractions greater than 0.2. At a water volume fraction of about 0.2, there was an abrupt change in the slope of the linear relationship between $1/T_m$ and ϵ . This may have been caused by a collapse of the gap-overlap fiber structure at low hydrations. At more than 6 moles water per tripeptide, the enthalpy of denaturation on a dry tendon basis was independent of hydration at 58.55 +/- 0.59 J g⁻¹. Between about 6 and 1 moles water per tripeptide, dehydration caused a substantial loss of enthalpy of denaturation, caused by a loss of water bridges from the hydration network surrounding the triple helix. At very low hydrations (less than 1 mole of water per tripeptide), where there was not enough water to form bridges and only sufficient to hydrogen bond to primary binding sites on the peptide chains, the enthalpy was approximately constant at 11.6 +/- 0.69 J g⁻¹. This was assigned mainly to the breaking of the direct hydrogen bonds between the alpha chains.

Miles C. A., Sionkowska A., Hulin S. L., Sims T. J., Avery N. C., and Bailey A. J. (2000) Identification of an intermediate state in the helix-coil degradation of collagen by ultraviolet light. *J Biol Chem* **275**, 33014-33020.

Abstract: Differential scanning calorimetry has revealed the presence of a new denaturation endotherm at 32 degrees C following UV irradiation of collagen, compared with 39 degrees C for the native triple helix. Kinetic analyses showed that the new peak was a previously unknown intermediate state in the collagen helix-coil transition induced by UV light, and at least 80% of the total collagen was transformed to random chains via this state. Its rate of formation was increased by hydrogen peroxide and inhibited by free radical scavengers. SDS-polyacrylamide gels showed evidence of competing reactions of cross-linking and random primary chain scission. The cross-linking was evident from initial gelling of the collagen solution, but there was no evidence for a dityrosine cross-link. Primary chain scission was confirmed by end group

analysis using fluorescamine. Electron microscopy showed that the segment long spacing crystallites formed from the intermediate state were identical to the native molecules. Clearly, collagen can undergo quite extensive damage by cleavage of peptide bonds without disorganizing the triple helical structure. This leads to the formation of a damaged intermediate state prior to degradation of the molecules to short random chains.

Miles C. A. and Burjanadze T. V. (2001) Thermal stability of collagen fibers in ethylene glycol. *Biophys J* **80**, 1480-1486.

Abstract: The mechanism that renders collagen molecules more stable when precipitated as fibers than the same molecules in solution is controversial. According to the polymer-melting mechanism the presence of a solvent depresses the melting point of the polymer due to a thermodynamic mechanism resembling the depression of the freezing point of a solvent due to the presence of a solute. On the other hand, according to the polymer-in-a-box mechanism, the change in configurational entropy of the collagen molecule on denaturation is reduced by its confinement by surrounding molecules in the fiber. Both mechanisms predict an approximately linear increase in the reciprocal of the denaturation temperature with the volume fraction (epsilon) of solvent, but the polymer-melting mechanism predicts that the slope is inversely proportional to the molecular mass of the solvent (M), whereas the polymer-in-a-box mechanism predicts a slope that is independent of M. Differential scanning calorimetry was used to measure the denaturation temperature of collagen in different concentrations of ethylene glycol (M = 62) and the slope found to be $(7.29 \pm 0.37) \times 10^{-4} \text{ K}^{-1}$, compared with $(7.31 \pm 0.42) \times 10^{-4} \text{ K}^{-1}$ for water (M = 18). This behavior was consistent with the polymer-in-a-box mechanism but conflicts with the polymer-melting mechanism. Calorimetry showed that the enthalpy of denaturation of collagen fibers in ethylene glycol was high, varied only slowly within the glycol volume fraction range 0.2 to 1, and fell rapidly at low epsilon. That this was caused by the disruption of a network of hydrogen-bonded glycol molecules surrounding the collagen is the most likely explanation.

Miles C. A., Avery N. C., Rodin V. V., and Bailey A. J. (2005) The increase in denaturation temperature following cross-linking of collagen is caused by dehydration of the fibres. *J Mol Biol* **346**, 551-556.

Abstract: Differential scanning calorimetry (DSC) was used to study the thermal stability of native and synthetically cross-linked rat-tail tendon at different levels of hydration, and the results compared with native rat-tail tendon. Three cross-linking agents of different length between functional groups were used: malondialdehyde (MDA), glutaraldehyde and hexamethylene diisocyanate (HMDC). Each yielded the same linear relation between the reciprocal of the denaturation temperature in Kelvin, $T(\text{max})$, and the water volume fraction, epsilon ($1/T(\text{max}) = 0.000731\epsilon + 0.002451$) up to a critical hydration level, the volume fraction of water in the fully hydrated fibre. Thereafter, water was in excess, $T(\text{max})$ was constant and the fibre remained unchanged, no matter how much excess water was added. This $T(\text{max})$ value and the corresponding intrafibrillar volume fraction of water were as follows: 84.1 degrees C and 0.48 for glutaraldehyde treated fibres, 74.1 degrees C and 0.59 for HMDC treated fibres, 69.3 degrees C and 0.64 for MDA treated fibres, and 65.1 degrees C and 0.69 for untreated native fibres. Borohydride reduction of the native enzymic aldimines did not increase the denaturation temperature of the fibres. As all samples yielded the same temperature at the same hydration, the temperature could not be affected by the nature of the cross-link other than through its effect on hydration. Cross-linking therefore caused dehydration of the fibres by drawing the collagen molecules closer together and it was the reduced hydration that caused the increased temperature stability. The cross-linking studied here only reduced the quantity of water between the molecules and did not affect the water in intimate contact with, or bound to, the molecule itself. The enthalpy of denaturation was therefore unaffected by cross-linking. Thus, the "polymer-in-a-box" mechanism of stabilization, previously proposed to explain the effect of dehydration on the thermal properties of native tendon, explained the new data also. In this mechanism, the configurational entropy of the unfolding molecule is reduced by its confinement in the fibre lattice, which shrinks on cross-linking.

Miles C. A. and Bailey A. J. (2001) Thermally labile domains in the collagen molecule. *Micron* **32**, 325-332.

Abstract: We have proposed that the denaturation kinetics of the characteristic sharp melting point of the collagen molecules is an irreversible rate rather than an equilibrium process as previously believed. This leads to the concept of domains of variable thermal stability along the length of the molecule. We have identified the major thermally labile domains from which the denaturation process is initiated as hydroxyproline deficient sequences of 65, 65 and 59 residues near the carboxy terminus in fibrillar collagen

types I, II and III, respectively. These domains differ in that there is a single hydroxyproline in the type II domain and two hydroxyprolines in the type III domain. Similar sized domains are conserved in these collagen types across species including amphibians and invertebrates. The effective size of the domain is reduced in the fibrillar aggregates to 26 residues due to the interaction with adjacent molecules and because of the precise quarter-staggered alignment of the molecules the domains are located in the gap region. This spatial confinement within the lattice of the fibre leads to the significant increase in denaturation temperature of the fibre compared to the molecule. These labile domains have also been located in molecules that form the non-fibrillar type IV basement membrane collagens and the fibril-associated aggregates such as type IX. Based on the location of the different domains in type IX we have proposed a different arrangement of the type IX on the type II fibril. The model stresses the importance of hydroxyproline in stabilising the triple helix and supports the concept of hydrogen-bonded water-bridges originally proposed from X-ray diffraction studies in contrast to other studies indicating water-bridges do not play a role in stabilising the collagen molecule.

Miles C. A., Sims T. J., Camacho N. P., and Bailey A. J. (2002) The role of the alpha2 chain in the stabilization of the collagen type I heterotrimer: a study of the type I homotrimer in oim mouse tissues. *J Mol Biol* **321**, 797-805.

Abstract: We have previously reported that the fragility of skin, tendon and bone from the oim mouse is related to a significant reduction in the intermolecular cross-linking. The oim mutation is unlikely to affect the efficacy of the lysyl oxidase, suggesting that the defect is in the molecule and fibre. We have therefore investigated the integrity of both the oim collagen molecules and the fibre by differential scanning calorimetry. The denaturation temperature of the oim molecule in solution and the fibre from tail tendon were found to be higher than the wild-type by 2.6deg.C and 1.9deg.C, respectively. With the loss of the alpha2 chain, the hydroxyproline content of the homotrimer is higher than the heterotrimer, which may account for the increase. There is a small decrease in the enthalpy of the oim fibres but it is not significant, suggesting that the amount of disorder of the triple-helical molecules and of the fibres is small and involves only a small part of the total bond energy holding the helical structure together. The difference in denaturation temperature of the skin collagen molecules ($t(m)$) and fibres ($t(d)$) is significantly lower for the oim tissues, 19.9deg.C against 23.1deg.C, indicating reduced molecular interactions and hence packing of the molecules in the fibre. Computation of the volume fraction of the water revealed that the interaxial separation of the oim fibres was indeed greater, increasing from 19.6Å to 21.0Å. This difference of 1.4Å, equivalent to a C-C bond, would certainly decrease the ability of the telopeptide aldehyde to interact with the epsilon -amino group from an adjacent molecule and form a cross-link. We suggest, therefore, that the reduction of the cross-linking is due to increased water content of the fibre rather than a distortion of the molecular structure. The higher hydrophobicity of the alpha2 chain appears to play a role in the stabilisation of heterotrimeric type I collagen, possibly by increasing the hydrophobic interactions between the heterotrimeric molecules, thereby reducing the water content and increasing the binding of the molecules in the fibre.

Miles C. A. and Bailey A. J. (2004) Studies of the collagen-like peptide (Pro-Pro-Gly)(10) confirm that the shape and position of the type I collagen denaturation endotherm is governed by the rate of helix unfolding. *J Mol Biol* **337**, 917-931.

Abstract: The kinetics of unfolding of a collagen-like peptide, (Pro-Pro-Gly)(10), has been studied under isothermal conditions to gain a better understanding of the stabilization of the collagen triple helix. The formation process was third-order and relatively insensitive to temperature at concentrations of 1 mg/ml and below, while the unfolding process was first-order and highly temperature-dependent. The helix-coil transition was studied over a range of scanning rates and polymer concentrations, using differential scanning calorimetry and the observations were compared with solutions of an approximate differential equation governing the process. At high concentrations (24 mg/ml) and very low scanning rates (0.025 degrees C min⁻¹), the helicity, F , approached a quasistatic state in which it reached its equilibrium value at all temperatures. Under these conditions, the temperature at which the endotherm peaked, T_{max} , increased with chain concentration but was independent of scanning rate, while $(dF/dT)_{max}$ was dependent on the van't Hoff enthalpy and on the order of the formation process. On scanning from a low to a high temperature (up-scanning) at low concentrations (0.25-1.0 mg/ml) and higher scanning rates (0.1 degrees C min⁻¹ and above), the peak in dF/dT was taller and narrower than for slow quasistatic scanning. T_{max} increased linearly with the logarithm of the scanning rate, and was independent of concentration, while $(dF/dT)_{max}$

was governed by the temperature-dependence of the rate of unfolding. At intermediate scanning rates, two peaks in dF/dT were apparent. One peak was a nascent "quasistatic peak"; the other was a nascent "rate peak". Comparison of this peptide data with the properties of the collagen denaturation endotherm showed that the collagen denaturation endotherm was determined only by the rate of unfolding, and not by an unobserved equilibrium.

Miles C. A. (2007) Kinetics of the helix/coil transition of the collagen-like peptide (Pro-Hyp-Gly)₁₀. *Biopolymers* **87**, 51-67.

Abstract: This article measures the rates of folding and unfolding of the collagen-like peptide (Pro-Hyp-Gly)₁₀ over overlapping concentration and temperature ranges. The data allow calculation of the orders of the folding and the unfolding reactions, the effective Arrhenius activation energies, and numerical solution of the differential equation controlling the helix/coil transition during temperature scanning. The resulting predictions of helicity closely followed DSC measurements of the peptide in both up- and down-scanning modes, confirming the validity of the theoretical equations governing the kinetics of the folding/unfolding process. In both up- and down-scanning, three regions were apparent: "quasistatic," "rate," and "mixed." At very low scanning rates, a quasistatic region revealed a broad, short endotherm that was independent of scanning rate, but dependent on concentration and equal to the equilibrium endotherm. At high up-scanning rates, the "rate region" endotherm was sharp and tall and T(max) increased with scanning rate. In down-scanning, the "rate peak" was very broad and very short and T(max) decreased with scanning rate. The "mixed region" showed nascent "rate" and nascent "quasistatic" peaks, which were evident in the same up-scan under certain conditions. Comparison of (Pro-Hyp-Gly)₁₀ and (Pro-Pro-Gly)₁₀ showed that the higher temperature stability of (Pro-Hyp-Gly)₁₀ is due mainly to its slower rate of unfolding and higher activation energy.

Misenheimer T. M., Huwiler K. G., Annis D. S., and Mosher D. F. (2000) Physical characterization of the procollagen module of human thrombospondin 1 expressed in insect cells. *J Biol Chem* **275**, 40938-40945.

Abstract: Thrombospondin 1 (TSP1) is a homotrimeric glycoprotein composed of 150-kDa subunits connected by disulfide bridges. The procollagen module of thrombospondin 1 has been implicated in antiangiogenic activity. Procollagen modules are found in a number of extracellular proteins and are identifiable by 10 cysteines with characteristic spacing. We expressed and studied the procollagen module (C) of human TSP1, both by itself and in the context of the adjoining oligomerization sequence (o) and N-terminal module (N). The coding sequences were introduced into baculoviruses along with an N-terminal signal sequence and C-terminal polyhistidine tag. Proteins were purified from conditioned medium of infected insect cells by nickel-chelate chromatography. NoC is a disulfide bonded trimer and cleaves readily at a site of preferential proteolysis to yield monomeric N and trimeric oC. These are known properties of full-length TSP1. Mass spectroscopy indicated that C is N-glycosylated, and all 10 cysteine residues of C are in disulfides. By equilibrium ultracentrifugation, C is a monomer in physiological salt solution. Circular dichroism, intrinsic fluorescence, and differential scanning calorimetry experiments suggest that the stability of C is determined by the disulfides. The two tryptophans of C are in a polar, exposed environment as assessed by iodide fluorescence quenching and solvent perturbation. The oC far UV circular dichroism spectrum could be modeled as the sum of C and a coiled-coil oligomerization domain. The results indicate that the recombinant C folds autonomously into its native structure, and trimerization of the modules in TSP1 does not perturb their structures.

Mizuno K., Hayashi T., Peyton D. H., and Bachinger H. P. (2004) Hydroxylation-induced stabilization of the collagen triple helix. Acetyl-(glycyl-4(R)-hydroxyprolyl-4(R)-hydroxyprolyl)₁₀-NH₂ forms a highly stable triple helix. *J Biol Chem* **279**, 38072-38078.

Abstract: The collagen triple helix is one of the most abundant protein motifs in animals. The structural motif of collagen is the triple helix formed by the repeated sequence of -Gly-Xaa-Yaa-. Previous reports showed that H-(Pro-4(R)Hyp-Gly)₁₀-OH (where '4(R)Hyp' is (2S,4R)-4-hydroxyproline) forms a trimeric structure, whereas H-(4(R)Hyp-Pro-Gly)₁₀-OH does not form a triple helix. Compared with H-(Pro-Pro-Gly)₁₀-OH, the melting temperature of H-(Pro-4(R)Hyp-Gly)₁₀-OH is higher, suggesting that 4(R)Hyp in the Yaa position has a stabilizing effect. The inability of triple helix formation of H-(4(R)Hyp-Pro-Gly)₁₀-OH has been explained by a stereoelectronic effect, but the details are unknown. In this study, we synthesized a peptide that contains 4(R)Hyp in both the Xaa and the Yaa positions, that is, Ac-(Gly-4(R)Hyp-4(R)Hyp)₁₀-NH₂ and compared it to Ac-(Gly-Pro-4(R)Hyp)₁₀-NH₂, and Ac-(Gly-4(R)Hyp-Pro)₁₀-

NH₂. Ac-(Gly-4(R)Hyp-4(R)Hyp)₁₀-NH₂ showed a polyproline II-like circular dichroic spectrum in water. The thermal transition temperatures measured by circular dichroism and differential scanning calorimetry were slightly higher than the values measured for Ac-(Gly-Pro-4(R)Hyp)₁₀-NH₂ under the same conditions. For Ac-(Gly-4(R)Hyp-4(R)Hyp)₁₀-NH₂ the calorimetric and the van't Hoff transition enthalpy ΔH were significantly smaller than that of Ac-(Gly-Pro-4(R)Hyp)₁₀-NH₂. We postulate that the denatured states of the two peptides are significantly different, with Ac-(Gly-4(R)Hyp-4(R)Hyp)₁₀-NH₂ forming a more polyproline II-like structure instead of a random coil. Two-dimensional nuclear Overhauser effect spectroscopy suggests that the triple helical structure of Ac-(Gly-4(R)Hyp-4(R)Hyp)₁₀-NH₂ is more flexible than that of Ac-(Gly-Pro-4(R)Hyp)₁₀-NH₂. This is confirmed by the kinetics of amide (1)H exchange with solvent deuterium of Ac-(Gly-4(R)Hyp-4(R)Hyp)₁₀-NH₂, which is faster than that of Ac-(Gly-Pro-4(R)Hyp)₁₀-NH₂. The higher transition temperature of Ac-(Gly-4(R)Hyp-4(R)Hyp)₁₀-NH₂, can be explained by the higher trans/cis ratio of the Gly-4(R)Hyp peptide bonds than that of the Gly-Pro bonds, and this ratio compensates for the weaker interchain hydrogen bonds.

Mizuno K., Peyton D. H., Hayashi T., Engel J. and Bachinger H. P. (2008) Effect of the -Gly-3(S)-hydroxyprolyl-4(R)-hydroxyprolyl- tripeptide unit on the stability of collagen model peptides. *FEBS J* **275**, 5830-5840.

Abstract: In order to evaluate the role of 3(S)-hydroxyproline [3(S)-Hyp] in the triple-helical structure, we produced a series of model peptides with nine tripeptide units including 0-9 3(S)-hydroxyproline residues. The sequences are H-(Gly-Pro-4(R)Hyp)(l)-(Gly-3(S)Hyp-4(R)Hyp)(m)-(Gly-Pro-4(R)Hyp)(n)-OH, where (l, m, n) = (9, 0, 0), (4, 1, 4), (3, 2, 4), (3, 3, 3), (1, 7, 1) and (0, 9, 0). All peptides showed triple-helical CD spectra at room temperature and thermal transition curves. Sedimentation equilibrium analysis showed that peptide H-(Gly-3(S)Hyp-4(R)Hyp)(9)-OH is a trimer. Differential scanning calorimetry showed that replacement of Pro residues with 3(S)Hyp residues decreased the transition enthalpy, and the transition temperature increases by 4.5 degrees C from 52.0 degrees C for the peptide with no 3(S)Hyp residues to 56.5 degrees C for the peptide with nine 3(S)Hyp residues. The refolding kinetics of peptides H-(Gly-3(S)Hyp-4(R)Hyp)(9)-OH, H-(Gly-Pro-4(R)Hyp)(9)-OH and H-(Gly-4(R)Hyp-4(R)Hyp)(9)-OH were compared, and the apparent reaction orders of refolding at 10 degrees C were $n = 1.5, 1.3$ and 1.2 , respectively. Replacement of Pro with 3(S)Hyp or 4(R)Hyp has little effect on the refolding kinetics. This result suggests that the refolding kinetics of collagen model peptides are influenced mainly by the residue in the Yaa position of the -Gly-Xaa-Yaa- repeated sequence. The experiments indicate that replacement of a Pro residue by a 3(S)Hyp residue in the Xaa position of the -Gly-Xaa-4(R)Hyp- repeat of collagen model peptides increases the stability, mainly due to entropic factors

Mohs A., Li Y., Doss-Pepe E., Baum J., and Brodsky B. (2005) Stability junction at a common mutation site in the collagenous domain of the mannose binding lectin. *Biochemistry* **44**, 1793-1799.

Abstract: Missense mutations in the collagen triple-helix that replace one of the required Gly residues in the (Gly-Xaa-Yaa)(n)(l) repeating sequence have been implicated in various disorders. Although most hereditary collagen disorders are rare, a common occurrence of a Gly replacement mutation is found in the collagenous domain of mannose binding lectin (MBL). A Gly --> Asp mutation at position 54 in MBL is found at a frequency as high as 30% in certain populations and leads to increased susceptibility to infections. The structural and energetic consequences of this mutation are investigated by comparing a triple-helical peptide containing the N-terminal Gly-X-Y units of MBL with the homologous peptide containing the Gly to Asp replacement. The mutation leads to a loss of triple-helix content but only a small decrease in the stability of the triple-helix ($\Delta T(m)$ approximately 2 degrees C) and no change in the calorimetric enthalpy. NMR studies on specifically labeled residues indicate the portion of the peptide C-terminal to residue 54 is in a highly ordered triple-helix in both peptides, while residues N-terminal to the mutation site have a weak triple-helical signal in the parent peptide and are completely disordered in the mutant peptide. These results suggest that the N-terminal triplet residues are contributing little to the stability of this peptide, a hypothesis confirmed by the stability and enthalpy of shorter peptides containing only the region C-terminal to the mutation site. The Gly to Asp replacement at position 54 in MBL occurs at the boundary of a highly stable triple-helix region and a very unstable sequence. The junctional position of this mutation minimizes its destabilizing effect, in contrast with the significant destabilization seen for Gly replacements in peptides modeling collagen diseases.

Mohs A., Popiel M., Li Y., Baum J., and Brodsky B. (2006) Conformational features of a natural break in the type IV collagen Gly-X-Y repeat. *J Biol Chem* **281**, 17197-17202.

Abstract: Fibrillar collagens have an absolute requirement for Gly as every 3rd residue, whereas breaks in the Gly-X-Y repeating pattern are found normally in the triple helix domains of non-fibrillar collagens, such as type IV collagen in basement membranes. In this study, a model 30-mer peptide is designed to include the interruption GPOGAAVMGPOGPO found in the alpha5 chain of type IV collagen. The GAAVM peptide forms a stable triple helix, with $T_m = 29$ degrees C. When compared with a control peptide with Gly as every 3rd residue, the GAAVM peptide has a marked decrease in the 225 nm maximum of its CD spectrum and a 10 degrees C drop in stability. A 50% decrease in calorimetric enthalpy is observed, which may result from disruption of ordered water structure anchored by regularly placed backbone carbonyls. NMR studies on specific ^{15}N -labeled residues within the GAAVM peptide indicate a normal triple helical structure for Gly-Pro-Hyp residues flanking the break. The sequence within the break is not disordered but shows altered hydrogen exchange rates and an abnormal Val chemical shift. It was previously reported that a peptide designed to model a similar kind of interruption in the peptide (Pro-Hyp-Gly) $_{10}$, (GPOGPOPOGPO), is unable to form a stable triple helix, and replacement of GAA by GPO or VM by PO within the GAAVM break decreases the stability. Thus, rigid imino acids are unfavorable within a break, despite their favorable stabilization of the triple helix itself. These results suggest some non-random structure typical of this category of breaks in the Gly-X-Y repeat of the triple helix.

Mohs A., Silva T., Yoshida T., Amin R., Lukomski S., Inouye M. and Brodsky B. (2007) Mechanism of stabilization of a bacterial collagen triple helix in the absence of hydroxyproline. *J Biol Chem* **282**, 29757-29765.

Abstract: The *Streptococcus pyogenes* cell-surface protein Scl2 contains a globular N-terminal domain and a collagen-like domain, (Gly-Xaa-X'aa) $_{79}$, which forms a triple helix with a thermal stability close to that seen for mammalian collagens. Hyp is a major contributor to triple-helix stability in animal collagens, but is not present in bacteria, which lack prolyl hydroxylase. To explore the basis of bacterial collagen triple-helix stability in the absence of Hyp, biophysical studies were carried out on recombinant Scl2 protein, the isolated collagen-like domain from Scl2, and a set of peptides modeling the Scl2 highly charged repetitive (Gly-Xaa-X'aa) $_{n}$ sequences. At pH 7, CD spectroscopy, dynamic light scattering, and differential scanning calorimetry of the Scl2 protein all showed a very sharp thermal transition near 36 degrees C, indicating a highly cooperative unfolding of both the globular and triple-helix domains. The collagen-like domain isolated by trypsin digestion showed a sharp transition at the same temperature, with an enthalpy of 12.5 kJ/mol of tripeptide. At low pH, Scl2 and its isolated collagen-like domain showed substantial destabilization from the neutral pH value, with two thermal transitions at 24 and 27 degrees C. A similar destabilization at low pH was seen for Scl2 charged model peptides, and the degree of destabilization was consistent with the strong pH dependence arising from the GKD tripeptide unit. The Scl2 protein contained twice as much charge as human fibril-forming collagens, and the degree of electrostatic stabilization observed for Scl2 was similar to the contribution Hyp makes to the stability of mammalian collagens. The high enthalpic contribution to the stability of the Scl2 collagenous domain supports the presence of a hydration network in the absence of Hyp.

Mrevlishvili G. M., Metreveli N. O., and Mdzinarashvili T. D. (1997) [Denaturation increment of heat capacity in diluted aqueous solutions of collagen]. *Biofizika* **42**, 78-81.

Abstract: The experimental values of the denaturation increment of collagen heat capacity in diluted aqueous solutions, obtained at different scanning rates, are presented. It is shown that the dependences of the "equilibrium" enthalpy and entropy of collagen denaturation on denaturation-induced variation in heat capacity do not obey the empiric law of the linear correlation of the thermodynamic parameters of denaturation at 25 degrees C for globular proteins, indicating that the stabilization of the triple collagen helix proceeds by a special mechanism with the participation of water molecules.

Mu C., Li D., Lin W., Ding Y. and Zhang G. (2007) Temperature induced denaturation of collagen in acidic solution. *Biopolymers* **86**, 282-287.

Abstract: The denaturation of collagen solution in acetic acid has been investigated by using ultra-sensitive differential scanning calorimetry (US-DSC), circular dichroism (CD), and laser light scattering (LLS). US-DSC measurements reveal that the collagen exhibits a bimodal transition, i.e., there exists a shoulder transition before the major transition. Such a shoulder transition can recover from a cooling when

the collagen is heated to a temperature below 35 degrees C. However, when the heating temperature is above 37 degrees C, both the shoulder and major transitions are irreversible. CD measurements demonstrate the content of triple helix slowly decreases with temperature at a temperature below 35 degrees C, but it drastically decreases at a higher temperature. Our experiments suggest that the shoulder transition and major transition arise from the defibrillation and denaturation of collagen, respectively. LLS measurements show the average hydrodynamic radius $R(h)$, radius of gyration $R(g)$ of the collagen gradually decrease before a sharp decrease at a higher temperature. Meanwhile, the ratio $R(g)/R(h)$ gradually increases at a temperature below approximately 34 degrees C and drastically increases in the range 34-40 degrees C, further indicating the defibrillation of collagen before the denaturation.

Nezu T., Nishiyama N., Nemoto K., and Terada Y. (2005) The effect of hydrophilic adhesive monomers on the stability of type I collagen. *Biomaterials* **26**, 3801-3808.

Abstract: The adsorption effects of adhesive monomers on the structural stability of type I collagen were studied at an acid pH condition for two monomers: 2-hydroxyethyl methacrylate (HEMA), a neutral monomer and N-methacryloyl glycine (NMGly), an acidic monomer. Differential scanning calorimetry (DSC) measurements were done to assess the denaturation temperature (T_d), which is a measure of the structural stability of the proteins, including the bovine tendon collagen (BTC). While HEMA lowered the T_d of the BTC linearly with HEMA concentrations, NMGly exhibited a two-step decrease of the T_d . The rate of decrease in the T_d by the NMGly was by far greater than the rate of decrease with the HEMA. The first step had a larger slope than the second step in the T_d vs. CNMGly plot. The degree of adsorption of these two monomers to the BTC was estimated from infrared absorption measurements on the monomer solutions of various concentrations, before and after the immersion of the BTC. Both the adsorption of HEMA to the BTC and the T_d of the BTC were linearly dependent on HEMA concentrations. Conversely, NMGly was adsorbed to the BTC, again, in a two-step decrease similar to the T_d vs. CNMGly profile. An enhanced adsorption of NMGly, which might be attributed to a strong electrostatic interaction, was observed below 0.013 mol%. Circular dichroism measurements of the collagen of the same type as the BTC, in the absence and in the presence of the monomers, revealed that the native collagen helix structure was scarcely affected by the monomers. From these observations, it was concluded that (1) both of the monomers were adsorbed onto the BTC, which thus destabilized the triple helical collagen structure, and that (2) the effect was higher for NMGly in which the electrostatic attraction with the oppositely charged collagen might be effective at a pH of 3. If compared to HEMA, an acidic NMGly is a potential monomer that binds strongly to collagen and one that is hardly hydrolyzed.

Nishad F. N., Balaraman M., Raghava R. J., and Unni N. B. (2003) Effect of zirconium(IV) complexes on the thermal and enzymatic stability of type I collagen. *J Inorg Biochem* **95**, 47-54.

Abstract: Understanding the mechanism of stabilization of collagen is an important area of research. Metal ions are known to interact with collagen and bring about the stability of the same. In the present investigation, the interaction of zirconium(IV) complexes with collagen was studied. The effect of zirconium(IV) complexes, namely zirconium oxychloride and zirconium oxalate on the enzymatic and thermal stability of collagen was investigated. Zirconium has been found to increase the hydrothermal stability of the rat tail tendon (RTT) collagen fibers to about 8-10 degrees C more than that of the native collagen. The order of stabilization of zirconium(IV) complexes is zirconium oxychloride > zirconium oxalate. This could be due to the differences in the type of interaction with collagen, which is also reflected in the differences in the conformational changes of collagen brought about by the two complexes. Zirconium oxychloride, which forms tetrameric species in solution, has been shown to have better crosslinking with collagen as seen from viscometry studies and hence provides better enzymatic stability to collagen than zirconium oxalate, which largely forms monomeric species in solution.

Nishi Y., Uchiyama S., Doi M., Nishiuchi Y., Nakazawa T., Ohkubo T., and Kobayashi Y. (2005) Different effects of 4-hydroxyproline and 4-fluoroproline on the stability of collagen triple helix. *Biochemistry* **44**, 6034-6042.

Abstract: Differential scanning calorimetry (DSC) analyses of a series of collagen model peptides suggest that 4-hydroxyproline (Hyp) and 4-fluoroproline (fPro) have different effects on the stability of the collagen triple helices according to the sequence of amino acids and stereochemistry at the 4 positions of these imino acids. The thermodynamic parameters indicate that the enhanced stabilities are classified into two different types: the enthalpy term is primarily responsible for the enhanced stability of the triple helix

of (Pro-Hyp(R)-Gly)(10), whereas the entropy term dominates the enhanced stability of (Pro-fPro(R)-Gly)(10). The difference between the molecular volumes observed in solution and intrinsic molecular volumes calculated from the crystal structure indicates the different hydration states of these peptides. (Pro-Hyp(R)-Gly)(10) is highly hydrated compared to (Pro-Pro-Gly)(10), which contributes to the larger enthalpy. In contrast, the volume of (Pro-fPro(R)-Gly)(10) shows a smaller degree of hydration than that of (Pro-Pro-Gly)(10). The entropic cost of forming the triple helix of the fPro-containing peptides is compensated by a decrease in an ordered structure of water molecules surrounding the peptide molecule, although the contribution of enthalpy originating from the hydration is reduced. These arguments about the different contribution of entropic and enthalpic terms were successfully applied to interpret the stability of the triple helix of (fPro(S)-Pro-Gly)(10) as well.

Nomura Y., Toki S., Ishii Y., and Shirai K. (2000) Improvement of the material property of shark type I collagen by composing with pig type I collagen. *J Agric Food Chem* **48**, 6332-6336.

Abstract: Fibril reconstruction process, that is, the nucleation and growth of mixed type I collagen fibril of shark and pig, progressed faster than that of the individual collagen species of shark or pig. The reconstructed mixed collagen fibril had a greater resistance to return to the solution or to melt into gelatin in comparison with the counterpart consisting solely of shark collagen. The denaturation temperature of the mixed collagen gel was about 10 degrees C higher than that of shark, and about 5 degrees C lower than that of pig. By scanning electron microscopy, the diameter of mixed collagen fibril showed an intermediate range between shark and pig collagen fibril. The breaking strength of the mixed collagen gel was tougher than that of pig, but weaker than that of shark. Other physicochemical properties of the mixed type I collagen gel were observed to be at intermediate positions between those of shark and pig type I collagen gels.

Nomura Y., Toki S., Ishii Y., and Shirai K. (2000) The physicochemical property of shark type I collagen gel and membrane. *J Agric Food Chem* **48**, 2028-2032.

Abstract: The physicochemical properties of shark type I collagen gel and membrane were not same as those of pig type I collagen. The denaturation temperature of shark collagen gel was about 15 degrees C lower. According to scanning electronic micrography, the diameter of shark collagen fibril was relatively thin and more homogeneous. The breaking strength of shark collagen gel was greater, and shark collagen membrane had a greater mechanical strength and a higher water vapor sorption.

Nomura Y., Toki S., Ishii Y., and Shirai K. (2001) Effect of transglutaminase on reconstruction and physicochemical properties of collagen gel from shark type I collagen. *Biomacromolecules* **2**, 105-110.

Abstract: The effects of microbial transglutaminase (MTGase) on type I collagen self-assembly and properties of reconstructed collagen fibrils from shark were investigated. Collagen self-assembly was accelerated with the addition of MTGase in dependence on that concentration. The relative amount of reconstructed collagen slightly decreased with MTGase. The diffusion coefficient of collagen gel was reduced by treatment with MTGase, and that suggested the reduction of mobility of the whole collagen network. At a high temperature, used to denature the collagen, MTGase-treated collagen gel remained as aggregates. By differential scanning calorimetry, the denaturation temperature of MTGase-treated collagen gel was about 2 degrees C higher than that of nontreated collagen gel. Treatment with MTGase yielded thermally stable cross-links in collagen molecules.

Nymalm Y., Puranen J. S., Nyholm T. K., Kapyla J., Kidron H., Pentikainen O. T., Airene T. T., Heino J., Slotte J. P., Johnson M. S., and Salminen T. A. (2004) Jararhagin-derived "RKKH1-peptides induce structural changes in alpha 1 I domain of human integrin alpha 1beta 1. *J Biol Chem.* **279**, 7962-7670.

Abstract: Integrin alpha1beta1 is one of four collagen-binding integrins in human. Collagens bind to the alpha1 domain and in the case of alpha2I collagen binding is competitively inhibited by peptides containing the RKKH sequence and derived from the metalloproteinase jararhagin of snake venom from Bothrops jararaca. In alpha2I these peptides bind near the metal ion dependent adhesion site (MIDAS) where a collagen (I)-like peptide is known to bind; magnesium is required for binding. Published structures of the ligand-bound open conformation of alpha2I differs significantly from the closed conformation seen in the structure of apo-alpha2I near MIDAS. Here we show that two peptides, CTRKKHDC and CARKKHDC, derived from jararhagin also bind to alpha1I and competitively inhibit collagen I binding. Furthermore, calorimetric and fluorimetric measurements show that the structure of the complex of alpha1I with Mg(2+)

and CTRKKHDC differs from structure in the absence of peptide. A comparison of the X-ray structure of apo-alpha1I (closed conformation) and a model structure of the alpha1I (open conformation) based on the closely-related structure of alpha2I reveals that the binding site is partially blocked to ligands by Glu255 and Tyr285 in the closed structure, while in the open structure helix C is unwound and these residues are shifted, and the RKKH peptides fit well when docked. The open conformation of alpha2I resulting from binding a collagen (I)-like peptide leads to exposure of hydrophobic surface, also seen in the model of alpha1I and shown experimentally for alpha1I using a fluorescent hydrophobic probe.

Persikov A. V., Xu Y., and Brodsky B. (2004) Equilibrium thermal transitions of collagen model peptides. *Protein Sci* **13**, 893-902.

Abstract: The folding of collagen in vitro is very slow and presents difficulties in reaching equilibrium, a feature that may have implications for in vivo collagen function. Peptides serve as good model systems for examining equilibrium thermal transitions in the collagen triple helix. Investigations were carried out to ascertain whether a range of synthetic triple-helical peptides of varying sequences can reach equilibrium, and whether the triple helix to unfolded monomer transition approximates a two-state model. The thermal transitions for all peptides studied are fully reversible given sufficient time. Isothermal experiments were carried out to obtain relaxation times at different temperatures. The slowest relaxation times, on the order of 10-15 h, were observed at the beginning of transitions, and were shown to result from self-association limited by the low concentration of free monomers, rather than cis-trans isomerization. Although the fit of the CD equilibrium transition curves and the concentration dependence of T_m values support a two-state model, the more rigorous comparison of the calorimetric enthalpy to the van't Hoff enthalpy indicates the two-state approximation is not ideal. Previous reports of melting curves of triple-helical host-guest peptides are shown to be a two-state kinetic transition, rather than an equilibrium transition.

Pietrucha K. (2005) Changes in denaturation and rheological properties of collagen-hyaluronic acid scaffolds as a result of temperature dependencies. *Int J Biol Macromol* **36**, 299-304.

Abstract: This report describes the effect of temperature on the mechanical viscoelastic properties such as storage modulus (E'), loss modulus (E''), and loss tangent ($\tan\delta$) of the collagen sponges modified with hyaluronic acid (HA). In order to detect collagen-HA copolymer denaturation and to assess its thermal stability, the differential scanning calorimetry (DSC) supplemented by thermogravimetric (TG) measurements was used. The denaturation temperature ($T(d)$) of unmodified collagen samples increased from 69 to 86 degrees C for cross-linked samples, respectively. These temperature dependencies show remarkable changes in E' and E'' at selected temperature up to 226 degrees C for all samples due to the release of loosely and strongly bound water. The influence of HA on the viscoelastic behavior of collagen is manifested by a shift of the $\tan\delta$ peak associated with the process of decomposition towards higher temperatures resulting in a higher thermo-stability of the modified scaffolds.

Prathiba V. and Suryanarayanan M. (1999) Biochemical and dynamic studies of collagen from human normal skin and keloid tissue. *Indian J Biochem Biophys* **36**, 158-164.

Abstract: Keloids are exuberant scars, in which collagen, fibronectin and glycosaminoglycans are overdeposited. Biochemical analysis of the collagen isolated from normal skin and keloid tissue by pepsin treatment, indicated an increase in the type III and GAG content. Viscosity measurements of collagen from normal skin and keloid tissue were used in the present study to establish the interaction between collagen and GAG. Physico-chemical properties such as intrinsic viscosity, reduced viscosity and hydrated volume were computed from viscosity measurements. These measurements were also used to determine the denaturation temperature of collagen which was further confirmed by DSC measurements. Chondroitinase has been used in this study to probe the influence of GAG on the physico-chemical characteristic of keloid collagen.

Rochdi A., Foucat L., and Renou J. P. (1999) Effect of thermal denaturation on water-collagen interactions: NMR relaxation and differential scanning calorimetry analysis. *Biopolymers* **50**, 690-696.

Abstract: The dependence of the proton spin-lattice relaxation rate, and of the enthalpy and temperature of denaturation on water content, were studied by nmr and differential scanning calorimetry (DSC) in native and denatured collagen. Collagen was first heated at four different temperatures ranging from 40 to 70 degrees C. The percentage of denatured collagen induced by these preheating treatments was determined from DSC measurements. The DSC results are discussed in terms of heat-induced structural changes. A

two-exponential behavior for the spin-lattice relaxation was observed with the appearance of denatured collagen. This was attributed to the presence of a noncollagen protein fraction. The variations in the different longitudinal relaxation rates as a function of the moisture content and of the denatured collagen percentage are described within the multiphase water proton exchange model. This study highlights the complementarity of the information obtained from the two analytical tools used.

Rose C. and Mandal A. B. (1996) The interaction of sodium dodecyl sulfate and urea with cat-fish collagen solutions in acetate buffer: hydrodynamic and thermodynamic studies. *Int J Biol Macromol* **18**, 41-53.

Abstract: Cat-fish collagen was extracted and characterized. Shrinkage temperature of cat-fish collagen is 54.5 degrees C. SDS-PAGE pattern indicated that the cat-fish collagen is Type I in nature. The ratio of proline and hydroxyproline is 1:2 and it suggests cat-fish collagen is vertebrate. The molecular weight of cat-fish collagen was determined by using molecular sieve chromatography and it was found to be 3 20,000 Da. The mutual interaction of cat-fish collagen with SDS and urea was studied at various temperatures. The results suggest that the aggregation of collagen is facilitated by the presence of SDS, whereas hindered by urea. The various thermodynamic parameters were estimated from viscosity measurements and the transfer of collagen into SDS micelles, urea and the reverse phenomenon was analysed. These transfer properties are temperature-dependent. Our thermodynamic results are also able to predict the exact denaturation temperature as well as the structural order of water in the collagen in various environments. The hydrated volumes, V_h of collagen in buffer, SDS, and urea environments using Simha-Einstein equation and intrinsic viscosity were also calculated. The low intrinsic viscosity $[\eta]$ and high V_h value of collagen in an SDS environment compared to buffer and other environments suggested a more workable system in cosmetic and dermatological preparations. The one and two-hydrogen-bonded models of this collagen in various environments have been analysed. The calculated thermodynamic parameters varied with the concentration of collagen as well as concentration of additives. The change of thermodynamic parameters from coiled-coil to random-coil conformation upon denaturation of collagen were calculated from the amount of proline and hydroxyproline residues and compared with viscometric results. Denaturation enthalpy of the catfish collagen in buffer, SDS and urea environments has also been determined by differential scanning calorimetric (DSC) measurements, and the results are in good agreement with the viscosity-derived values. The asymmetry and molecular geometry of this collagen in buffer, SDS and urea environments are also computed. Overall, our hydrodynamic and thermodynamic results suggest that the stability of the collagen in the additive environments is in the following order: SDS > buffer > urea.

Sacca B., Renner C., and Moroder L. (2002) The chain register in heterotrimeric collagen peptides affects triple helix stability and folding kinetics. *J Mol Biol* **324**, 309-318.

Abstract: Collagen type IV is a highly specialized form of collagen found only in basement membranes, where it provides mechanical stability and structural integrity to tissues and organs, and binding sites for cell adhesion. In its ubiquitous form, collagen type IV consists of two alpha1 chains and one alpha2 chain, whose internal alignment within the triple helix seems to exert a strong influence on the binding affinity to alpha1beta1 integrin receptor. This has been assessed recently using two synthetic collagen peptides that contain the cell adhesion epitope of collagen type IV and are assembled into the most plausible alpha1alpha2alpha1' and alpha2alpha1alpha1' registers. In the present study, the effects of the chain register on the stability of the triple helix and the folding kinetics of these collagen peptides were investigated by CD spectroscopy and microcalorimetry. The results revealed a multi-domain structural organization for both trimers, with an unexpected strong effect of the chain alignment on the conformational stability. Molecular dynamics simulations served to rationalize more properly the experimental results.

Sacca B., Fiori S., and Moroder L. (2003) Studies of the local conformational properties of the cell-adhesion domain of collagen type IV in synthetic heterotrimeric peptides. *Biochemistry* **42**, 3429-3436.

Abstract: Collagen type IV is a specialized form of collagen that is found only in basement membranes. It is involved in integrin-mediated cell-adhesion processes, and the responsible binding sites for the alpha1beta1 integrin cell receptor have been identified as Asp461 of the two alpha1 chains and Arg461 of the alpha2 chain. In the most plausible stagger of native collagen type IV the alpha2 chain is the tailing one. This has recently been confirmed by the differentiated binding affinities of synthetic heterotrimeric collagen peptides in which the chains were staggered in this native register as well as in the less plausible alpha1alpha2alpha1' register with an artificial cystine knot. In the present work, two heterotrimeric collagen peptides with chain registers identical to the previous ones were synthesized for fluorescence resonance

energy transfer and emission anisotropy measurements, exploiting the native Phe⁴⁶⁴ in the alpha₂ chain as donor and an Ile⁴⁶⁷Tyr mutation in the alpha₁' chain as acceptor fluorophore. This fluorophore pair allowed extraction of more detailed information on the conformational properties of the cell-adhesion epitope incorporated into the central part of the trimeric collagen model peptides. A comparison of the experimentally derived values of the interfluorophore distance and of the orientation factor $\kappa(2)$ with the values extracted from the molecular model of the trimer in the native stagger confirmed a triple-helical structure of the adhesion-site portion at low temperature. The thermal unfolding of this central domain was specifically monitored by emission anisotropy, allowing unambiguous assignment of the three structural domains of the trimeric collagen molecules detected by microcalorimetry, with the integrin binding site as the portion of weakest triple-helical stability flanked by two more stable triple-helical regions. The results are consistent with the picture of a conformational microheterogeneity as the responsible property for selective recognition of collagens by interacting proteins.

Samouillan V., Dandurand J., Lacabanne C., Thoma R. J., Adams A., and Moore M. (2003) Comparison of chemical treatments on the chain dynamics and thermal stability of bovine pericardium collagen. *J Biomed Mater Res* **64A**, 330-338.

Abstract: A new approach for the replacement of heart valves consists of obtaining an acellular matrix from animal aortic valves that performs mechanically, is nonantigenic, and is free from calcification and fibroblast proliferation. Novel biochemical treatments must be developed for this purpose. In this work, we focus on the characterization of collagen in acellular bovine cardiovascular tissues, fresh or glutaraldehyde treated, and stored in different solutions [phosphate-buffered saline (PBS), ethanol, octanol, and glutaraldehyde], to determine whether the resulting fibrous material is structurally preserved. The preservation of the triple helical structure of collagen is checked by differential scanning calorimetry (DSC), which is a well suited technique to analyze thermal transitions in proteins, such as denaturation. To get insight into the molecular dynamics of collagen in the nanometric range, we used thermally stimulated currents, a dielectric technique running at low frequency, that measure the dipolar reorientations in proteins submitted to a static electrical field. The combined use of these two techniques allowed us to evaluate the physical structure and conformation of collagen after the different chemical treatments. We have found that the glutaraldehyde treatment followed by octanol storage preserves the triple helical conformation of the polypeptidic chains of collagen, contrary to the ethanol and PBS storage that induce drastic changes in the thermal and dielectric behavior of the protein. Moreover, this particular chemical treatment stabilizes the collagen structure (shift toward high temperature of the collagen denaturation and stiffening of the chains by a cross-linking action) when compared to the control sample, and so could provide interesting fibrous material for the conception of bioprosthetic heart valve.

Schumacher M. A., Mizuno K., and Bachinger H. P. (2006) The crystal structure of a collagen-like polypeptide with 3(S)-hydroxyproline residues in the Xaa position forms a standard 7/2 collagen triple helix. *J Biol Chem* **281**, 27566-27574.

Abstract: Collagen has a triple helical structure comprising strands with a repeating Xaa-Yaa-Gly sequence. L-Proline (Pro) and 4(R)-hydroxyl-L-proline (4(R)Hyp) residues are found most frequently in the Xaa and Yaa positions. However, in natural collagen, 3(S)-hydroxyl-L-proline (3(S)Hyp) occurs in the Xaa positions to varying extents and is most common in collagen types IV and V. Although 4(R)Hyp residues in the Yaa positions have been shown to be critical for the formation of a stable triple helix, the role of 3(S)Hyp residues in the Xaa position is not well understood. Indeed, recent studies have demonstrated that the presence of 3(S)Hyp in the Xaa positions of collagen-like peptides actually has a destabilizing effect relative to peptides with Pro in these locations. Whether this destabilization is reflected in a local unfolding or in other structural alterations of the collagen triple helix is unknown. Thus, to determine what effect the presence of 3(S)Hyp residues in the Xaa positions has on the overall conformation of the collagen triple helix, we determined the crystal structure of the polypeptide H-(Gly-Pro-4(R)Hyp)₃-(Gly-3(S)Hyp-4(R)Hyp)₂-(Gly-Pro-4(R)Hyp)₄-OH to 1.80 Å resolution. The structure shows that, despite the presence of the 3(S)Hyp residues, the peptide still adopts a typical 7/2 superhelical symmetry similar to that observed in other collagen structures. The puckering of the Xaa position 3(S)Hyp residues, which are all down (C_{gamma}-endo), and the varphi/psi dihedral angles of the Xaa 3(S)Hyp residues are also similar to those of typical collagen Pro Xaa residues. Thus, the presence of 3(S)Hyp in the Xaa positions does not lead to large structural alterations in the collagen triple helix.

Shanmugasundaram N., Ravikumar T., and Babu M. (2004) Comparative physico-chemical and in vitro properties of fibrillated collagen scaffolds from different sources. *J Biomater Appl* **18**, 247-264.

Abstract: Collagen from different sources was isolated and designed as scaffolds to act as a three-dimensional substrate for culturing human skin fibroblasts, which can be used as dermal substitutes. The thermodynamic behavior of the scaffolds developed was analyzed through Differential Scanning Calorimetric (DSC) and Thermogravimetric analysis (TGA). Analysis by Fourier Transform Infrared Spectroscopy (FTIR) revealed the functional groups in the scaffolds and the mechanical stability of various scaffolds was assessed through tensile strength analysis. Human skin fibroblasts were cultured on the developed scaffolds to assess their cellular interaction and behavior, and the morphological characteristics of the cultured fibroblasts were evaluated using Scanning Electron Microscopy (SEM). The collagen scaffold exhibited unique features when developed from various sources and it was observed that cells could grow and proliferate well and spread as a monolayer in the reconstituted collagen scaffold.

Shanmugasundaram N., Ravichandran P., Reddy P. N., Ramamurty N., Pal S., and Rao K. P. (2001) Collagen-chitosan polymeric scaffolds for the in vitro culture of human epidermoid carcinoma cells. *Biomaterials* **22**, 1943-1951.

Abstract: A biodegradable polymer scaffold was developed using collagen and chitosan, in the form of interpenetrating polymeric network (IPN), for in vitro culture of human epidermoid carcinoma cells (HEp-2, Cincinnati). Glutaraldehyde was used as cross-linking agent for the development of scaffold. Various types of scaffolds were prepared using different proportionate mixtures of collagen and chitosan solutions in the ratio of 3:7, 4:6, 5:5, 6:4 and 7:3 (collagen:chitosan). These scaffolds were fully characterized by Fourier transform infrared spectroscopy (FT-IR), differential scanning calorimetry (DSC) and Thermogravimetric analysis (TGA). Equilibrium swelling studies were carried out in phosphate buffer of physiological pH (7.4) to study its swelling characteristics at slightly alkaline pH. The scaffold that showed optimum swelling property was selected as the best scaffold for performing in vitro culture studies. In vitro culture studies were carried out using HEp-2 cells, over the selected scaffold and its growth morphology was determined through optical photographs taken at different magnifications at various days of culture. The results of the above studies suggest that the scaffolds prepared from collagen and chitosan can be utilized as a substrate to culture HEp-2 cells and can also be used as an in vitro model to test anticancerous drugs.

Sionkowska A. (2005) Thermal stability of UV-irradiated collagen in bovine lens capsules and in bovine cornea. *J Photochem Photobiol B* **80**, 87-92.

Abstract: The thermal stability of UVB irradiated collagen in bovine lens capsules and in bovine cornea has been investigated by differential scanning calorimetry (DSC). During UVB irradiation the lens capsules and cornea were immersed in water to keep the collagen in a fully hydrated condition at all times. UV irradiation induced changes in collagen which caused both stabilization and destabilization of the collagen structure. The helix-coil transition for non-irradiated collagen in cornea occurred near 66 degrees C, instead for the irradiated one for 3h it occurred at 69 degrees C. After irradiating for longer times (20-96h) the helix-coil transition peak occurred at much lower temperatures. The peak was very broad and suggested that collagen was reduced by UV to different polypeptides of different molecular weight and different lower thermal stabilities. The irradiation of lens capsules with UVB light in vitro resulted in changes in the thermal properties of type-IV collagen consistent with increased cross-linking. DSC of lens capsules showed two major peaks at melting temperatures at 54 degrees C Tm1 and 78 degrees C Tm2, which can be attributed to the denaturation of the triple helix and 7S domains, respectively. UVB irradiation of lens capsules in vitro for 6 h caused an increase in Tm1 from 54 to 57 degrees C. The higher temperature required to denature the type-IV collagen after irradiation in vitro suggested an increase of intermolecular cross-linking.

Sionkowska A. (2005) Thermal denaturation of UV-irradiated wet rat tail tendon collagen. *Int J Biol Macromol* **35**, 145-149.

Abstract: The thermal helix-coil transition of UV irradiated collagen in rat tail tendon has been investigated by differential scanning calorimetry. During UVB irradiation the tendons were immersed in water to keep the collagen fibers in a fully hydrated condition at all times. UV irradiation induced changes in collagen which caused both stabilization and destabilization of the triple helix in fibers. The helix-coil transition for non-irradiated collagen occurred near 64 degrees C, for irradiated 1 and 3 h at 66 and 67

degrees C, respectively. After irradiating for longer times (20-66 h) the helix-coil transition peak occurred at much lower temperatures. The peak was very broad and suggested that collagen was reduced by UV to different polypeptides of different molecular weight and different lower thermal stabilities. It was caused by the disruption of a network of hydrogen-bonded water molecules surrounding the collagen macromolecule.

Sionkowska A. and Kaminska A. (1999) Thermal helix-coil transition in UV irradiated collagen from rat tail tendon. *Int J Biol Macromol* **24**, 337-340.

Abstract: The thermal helix-coil transition in UV irradiated collagen solution, collagen film and pieces of rat tail tendon (RTT) were compared. Their thermal stability's were determined by differential scanning calorimeter (DSC) and by viscometric measurements. The denaturation temperatures of collagen solution, film and pieces of RTT were different. The helix-coil transition occur near 40 degrees C in collagen solution, near 112 degrees C in collagen film, and near 101 degrees C in pieces of RTT. After UV irradiation the thermal helix-coil transition of collagen samples were changed. These changes depend on the degree of hydration.

Slatter D. A., Miles C. A., and Bailey A. J. (2003) Asymmetry in the triple helix of collagen-like heterotrimers confirms that external bonds stabilize collagen structure. *J Mol Biol* **329**, 175-183.

Abstract: Heating and subsequent cooling mixtures of (Pro-Pro-Gly)(10) and (Pro-Hyp-Gly)(10) peptides leads to formation of model heterotrimeric collagen helices that can be isolated by HPLC. These heterotrimeric collagen peptide helices are shown to be fundamentally unstable as denaturing then renaturing experiments result in heterotrimeric/homotrimeric mixtures. As the proportion of hydroxyproline-containing chains in the trimers increases, differential scanning calorimetry shows that the helix melting temperatures and denaturation enthalpies increasing non-linearly. Three types of Rich-Crick hydrogen bonds observed by NMR allow modelling of heterotrimeric structures based on published homotrimeric X-ray data. This revealed a small axial movement of (Pro-Hyp-Gly)(10) chains towards the C-terminal of the helix, demonstrating heterotrimeric asymmetry.

Suarez E., Diaz N. and Suarez D. (2008) Entropic Control of the Relative Stability of Triple-helical Collagen Peptide Models. *J Phys. Chem B*. (epublication)

Abstract: Herein, we show that current methodologies in atomistic simulations can yield reliable standard free energy values in aqueous solution for the transition from the dissociated monomeric form to the triple-helix state of collagen model peptides. The calculations are performed on a prototypical highly stable triple-helical peptide, [(Pro-Hyp-Gly) 10] 3 (POG10), and on the so-called T3-785 triple-helix mimicking a fragment from the type III human collagen, which is more thermally labile. On the basis of extensive MD simulations in explicit solvent followed by molecular-mechanical and electrostatic Poisson-Boltzmann calculations complemented with an accurate estimation of the nonpolar contributions to solvation, the computed free energy change for the aggregation processes of the POG10 and T3-785 peptides leading to their triple-helices is -6.6 and -6.1 kcal/mol, respectively. For POG10, this value is in agreement with differential scanning calorimetric data. However, it is shown that conformational entropy, which is estimated by means of an expansion of mutual information functions, preferentially destabilizes the triple-helical state of T3-785 by around 4.6 kcal/mol, thus explaining its lower thermal stability. Altogether, our computational results allow us to ascertain, for the first time, the actual thermodynamic forces controlling the absolute and relative stability of collagen model peptides

Tang H. R., Covington A. D., and Hancock R. A. (2003) Use of DSC to detect the heterogeneity of hydrothermal stability in the polyphenol-treated collagen matrix. *J Agric Food Chem* **51**, 6652-6656.

Abstract: The hydrothermal stability of the collagen matrixes treated with plant polyphenols (tannins) depends on not only the strength of the polyphenol-collagen interactions but also the distribution uniformity of polyphenolic molecules within the collagen fibrils. Traditional methods of uniformity tests rely heavily on the expertise of workers and are thus subjective. This paper describes a differential scanning calorimetry (DSC) study of the sheepskin collagen samples treated with hydrolyzable tannins, including two commercial tannins' extracts (chestnut and valonea), two pure ellagitannins (vescalagin and castalagin), and six synthetic gallotannins (di-galloyl-ethylene glycol (DGE), tri-galloyl-glycerol, tetra-galloyl-meso-erythritol, penta-galloyl-adonitol, penta-galloyl-glucose, and hexa-galloyl-ducitol). The collagen sample without polyphenol treatment and the sample treated with DGE showed a single sharp peak in their DSC

thermogram with a full peak width at half height (fwhh) of 3-4 degrees C. The samples treated with other tannins all showed multiple peak DSC profiles with the fwhh of each peak at about 3-4 degrees C. These multiple peak profiles imply that in these polyphenol-treated samples, there is a distribution of collagen molecules having different hydrothermal stability. The results have demonstrated that DSC offers an objective method to detect the stability heterogeneity of collagen matrixes in the solid state, providing a useful tool for the leather industry to evaluate the uniformity of leather tanning.

Tiktopulo E. I. and Kajava A. V. (1998) Denaturation of type I collagen fibrils is an endothermic process accompanied by a noticeable change in the partial heat capacity. *Biochemistry* **37**, 8147-8152.

Abstract: Thermal transitions of type I collagen fibrils were investigated by differential scanning calorimetry and spectrophotometry of turbidity within a wide range of external conditions. The advanced microcalorimeter allowed us to carry out the measurements at low concentrations of collagen (0.15-0.3 mg/mL). At these concentrations of collagen and under fibril-forming conditions, the melting curves display two pronounced heat adsorption peaks (at 40 and 55 degreesC). The low-temperature peak was assigned to the melting of monomeric collagen, while the high-temperature peak was assigned to the denaturation of collagen fibrils. It was shown that the denaturation of fibrils, in contrast to the monomeric collagen, is accompanied by a noticeable change in the partial specific heat capacity. Surprisingly, comparison of the collagen calorimetric curves in the fibril-forming and nonforming conditions revealed that ΔC_p of fibril denaturation is caused by a decrease in the C_p of collagen at premelting temperatures. This suggests the existence of an intermediate structural state of collagen in a transparent solution preceding fibril formation. Our study also shows that collagen fibrils formed prior to heating have thermodynamic parameters different from those of fibrils formed and denatured during heating in the calorimeter. Analysis of the data allowed us to determine the denaturation enthalpy of the mature fibrils and to conclude that the enthalpy plays a more important role in fibril stabilization than was previously assumed. The observed large ΔC_p value of fibril denaturation as well as the difference between thermodynamic parameters of the mature and newly formed fibrils is readily explained by the presence of water molecules in the fibril structure.

Tiong W. H., Damodaran G., Naik H., Kelly J. L. and Pandit A. (2008) Enhancing amine terminals in an amine-deprived collagen matrix. *Langmuir* **24**, 11752-11761.

Abstract: Collagen, though widely used as a core biomaterial in many clinical applications, is often limited by its rapid degradability which prevents full exploitation of its potential in vivo. Polyamidoamine (PAMAM) dendrimer, a highly branched macromolecule, possesses versatile multiterminal amine surface groups that enable them to be tethered to collagen molecules and enhance their potential. In this study, we hypothesized that incorporation of PAMAM dendrimer in a collagen matrix through cross-linking will result in a durable, cross-linked collagen biomaterial with free $-NH_2$ groups available for further multi-biomolecular tethering. The aim of this study was to assess the physicochemical properties of a G1 PAMAM cross-linked collagen matrix and its cellular sustainability in vitro. Different amounts of G1 PAMAM dendrimer (5 or 10 mg) were integrated into bovine-derived collagen matrices through a cross-linking process, mediated by 5 or 25 mM 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC) in 5 mM N-hydroxysuccinimide (NHS) and 50 mM 2-morpholinoethane sulfonic acid buffer at pH 5.5. The physicochemical properties of resultant matrices were investigated with scanning electron microscopy (SEM), collagenase degradation assay, differential scanning calorimetry (DSC), Fourier transform infrared (FTIR) spectra, and ninhydrin assay. Cellular sustainability of the matrices was assessed with Alamar Blue assay and SEM. There was no significant difference in cellular behavior between the treated and nontreated groups. However, the benefit of incorporating PAMAM in the cross-linking reaction was limited when higher concentrations of either agent were used. These results confirm the hypothesis that PAMAM dendrimer can be incorporated in the collagen cross-linking process in order to modulate the properties of the resulting cross-linked collagen biomaterial with free $-NH_2$ groups available for multi-biomolecular tethering.

Tsereteli G. I. and Belopol'skaia T. V. (1994) [New data on heat denaturation of collagen fibrils]. *Biofizika* **39**, 793-794.

Abstract: Using the differential scanning calorimetry we have found that the structural changes due to the aging have the influence on the temperatures of denaturation and the values of denaturation heats of the

intact collagen (rat tail tendon). The magnitude of the specific heat jump at denaturation of the collagen fibrills differs from that for the isolated molecules.

Tsereteli G. I., Belopol'skaia T. V., and Anisimov V. N. (1996) [Parameters of heat disruption of native collagen structures and their connection with biological growth]. *Biofizika* **41**, 658-664.

Abstract: By means of differential scanning calorimetry we have studied the influence of structural changes induced by the biological aging on the processes of thermal denaturation of collagen tissue (rat tail tendon, age from 14 days to 24 months). We have found that some parameters of the thermal denaturation process, namely, the denaturation temperature T_d and the denaturation heat Q_d are sensitive to the biological age of the tissue. Both T_d and Q_d are increasing with the age. However, this increase takes place only on early stages of aging. When the denaturation heat Q_d of the tissue is normalized on the content of collagen in it, one obtains the independent on the age value of 75 J/g. So the increase of Q_d with aging reflects the increase of the collagen content in tissue. We have found that the half-widths of the denaturation curves $\Delta T_{1/2}$, as well as the heat capacity increment at denaturation ΔC_p do not depend on the tissue age. Both for fibrills of collagen and for its solution the measured value of ΔC_p is equal 0.42 +/- 0.004 J/g degrees C which differs considerably from the earlier published data.

Tsereteli G. I., Belopol'skaia T. V., and Mel'nik T. N. (1997) [Thermal properties of collagen-water system. I. Increments of heat capacity during denaturation and glass transition]. *Biofizika* **42**, 68-74.

Abstract: The absolute values of heat capacity of the collagen-water systems with different relative content of the components in both native and denatured state were studied by the method of differential scanning calorimetry in a wide temperature range (-40 +/- 140 degrees C) which includes the region of the denaturation phase transition as well as the region of the relaxation glass transition. From the experimental data the values of denaturation increment ΔC_{pnd} -0.42 +/- 0.04 J/(g.K) at the collagen content 10-50% and the values of glass transition increment ΔC_{pg} -0.54 +/- 0.12 J/(g.K) for moist denatured protein were calculated. Different processes influencing the increment values are analysed. The nonequilibrium character of the glass-like state of moist proteins was clearly demonstrated in the study of glass transition.

Tsereteli G. I., Belopol'skaia T. V., and Mel'nik T. N. (1997) [Thermal characteristics of a collagen-water system. II. Conformational mobility of macromolecules in native and denatured states]. *Biofizika* **42**, 584-590.

Abstract: By calorimetric study of the collagen-water systems with 10-100% content of protein in the temperature range 20 + 90 degrees C we have measured the proper heat capacity of protein in native and denatured state. It is shown that S-like dependence of heat capacity on the water content for both native and denatured samples is caused by glass transition. At temperatures above the glass transition in moist collagen or above the denaturation of native collagen the translational mobility of segments in protein molecules appears. This mobility is most probably the cause of the increments in the temperature dependence of heat capacity. According to our results, for the denatured collagen the value of heat capacity in solution exceeds that for dry samples at least by the magnitude of heat capacity increment at glass transition.

Vyazovkin S., Vincent L. and Sbirrazzuoli N. (2007) Thermal denaturation of collagen analyzed by isoconversional method. *Macromol. Biosci.* **7**, 1181-1186.

Abstract: An isoconversional method is proposed to be used for evaluating activation energy of protein denaturation. Applied to DSC data on collagen denaturation, the method yields an activation energy that decreases throughout the process. The Lumry-Eyring model gives an explanation for this decrease and affords estimates for the enthalpy of the reversible step and the activation energy of the irreversible step of denaturation. The reversible unfolding is detectable by multi-frequency temperature-modulated DSC.

Zhang L., Hum M., Wang M., Li Y., Chen H., Chu C., and Jiang H. (2005) Evaluation of modifying collagen matrix with RGD peptide through periodate oxidation. *J Biomed Mater Res A* **73**, 468-475.

Abstract: The aim of the study is to evaluate the effect of modifying collagen matrices with Arg-Gly-Asp (RGD) peptide through periodate oxidation. The collagen matrices were modified with RGD peptide, by periodate activation. The modified collagen matrices and unmodified matrices were characterized by scanning electron microscopy (SEM), differential scanning calorimetry (DSC), and electron spectroscopy for chemical analysis (ESCA). Mesenchymal stem cells (MSCs) were used to evaluate the cell

compatibility of collagen matrices. In terms of cell growth, the MSCs attached much better on the modified matrix than on the unmodified one. But there was no significant difference between two groups regarding the MSC proliferation. Compared to the unmodified matrices, the mechanical strength of the modified matrix decreased sharply, and its 3D structure was destroyed. Introducing specific RGD receptor-mediated adhesion sites on matrices obviously enhanced the MSC adhesion on collagen matrices, but the coupled method of periodate oxidation would likely result in the declination of the mechanical strength of the matrix, as well as the destruction of the matrix structure. This would affect the cell growth on the matrix, and decrease the histocompatibility of the matrices.

Zhang L., Ma D., Wang F., and Zhang Q. (2002) The modification of scaffold material in building artificial dermis. *Artif Cells Blood Substit Immobil Biotechnol* **30**, 319-332.

Abstract: Type X collagen is principal extracellular matrix (ECM) in natural dermis. To prepare artificial dermis, collagen is traditional, and most superior biomaterial. But beside collagen, the dermis also contains many other ECM. Among them, glycosaminoglycan (GAG) is another important substance. To imitate the natural dermis, and modificate the scaffold materials, two types of scaffolds were prepared: one is traditional type X collagen spongy scaffold, the other is collagen-chondroitin sulfate (CS) spongy scaffold. Collagen was blended with CS, one kind of GAG, and cross-linked by 1-ethyl-3-(3-dimethyl aminopropyl) carbodiimide (EDC). Dermis fibroblast was isolated from neonate prepuce, and dermis fibroblasts were cultured on the scaffolds. The physical and chemical properties of the scaffolds were tested, including SEM, DSC, H&E staining, immunohistochemical staining and CS content analysis and so on. The results indicated that EDC is an effective and non-cytotoxic cross-link reagent, and attaching CS into collagen scaffold could improve the stability and histocompatibility of scaffold.