



Link-Lock: an explanation of the chemical stabilisation of collagen

Anthony D. Covington, Lijiang Song, Ono Suparno

Applied Collagen Research Group, The University of Northampton
and

Hannah E. C. Koon, Matthew J. Collins,

BioArch, Depts. of Biology and Archaeology, The University of York

Chemical modifications of collagen, of the type known in the leather tanning industry, can raise the denaturation temperature, from 60°C in its natural state, up to 130°C. There are only a few chemical reactions known to be capable of achieving the highest values and these have long been assumed to be unrelated.

In this paper it is shown that all the stabilising mechanisms are fundamentally the same, regardless of chemical type. Any single component of a stabilising reaction has the effect of linking part of the collagen structure into the surrounding matrix of water: the outcome is always to confer moderate hydrothermal stability, up to 85°C. The effect is merely to hinder the shrinking/denaturation transition, so no single component reaction can exceed this moderate result.

However, in addition, a second reaction component can be applied in the process, which may have the ability to lock the linked structure together, creating a macromolecular structure around the triple helices. The effect of the concerted interaction with collagen is to prevent more effectively the unravelling of the triple helices and thereby to raise the hydrothermal stability to much higher values of denaturation temperature.

This proposed 'link-lock' mechanism opens up the possibility of achieving high collagen stability in new ways, which will contribute to the development of new collagenic biomaterials.

Collagen has received much attention, regarding the origin of its high hydrothermal stability relative to other proteins. The stability of intact, native collagen can be attributed in the first instance to its hierarchy of structure, within which hydrogen bonding¹ and inductive effects² are likely to operate. In addition, it has been postulated that the quinary structure, namely the packing of the triple helices into fibrils, constitutes a 'polymer-in-a-box' model³, providing a further level of stabilisation. Recent studies on unmodified and chemically modified collagen confirmed that the observed hydrothermal stability is dependent on the moisture content; reducing the water content causes the fibres to approach more closely, preventing them from collapsing into the interstices and this effect is correlated with elevated denaturation temperature⁴. Therefore, a reduced ability to shrink is the same as increased hydrothermal stability. Consequently, chemical modification may reduce the ability of collagen to shrink, which results in higher observed denaturation temperature.

It has been demonstrated that the triple helix of collagen is surrounded by a supramolecular water sheath nucleated at the hydroxyproline sidechains⁵, in which the water molecules binding the sheath to the triple helix are labile, but constitute a stable structure⁶. In studies of the effects of basic chromium(III) sulfate and tara extract (hydrolysable plant polyphenol) on skin collagen, dynamic mechanical thermal analysis of leather during drying indicates that the amount of structural water is independent of the nature of the stabilising chemistry⁷.

When collagen is wet, the matrix can be degraded at elevated temperature, at the same time hydrogen bonds in the triple helix are broken, allowing the alpha helices to become detached from

the triple helix structure, observed as shrinking, leading to gelatinisation. If the supramolecular structure is only made of water, the process is facilitated to the greatest degree, causing the lowest denaturation temperature. However, if the supramolecular water sheath is chemically modified, resistance to shrinking is introduced, observed as an elevation of the denaturation temperature, that is higher hydrothermal stability.

The hydrothermal stability of collagen can be altered by many different chemical reactions, well known in the fields of histology, leather tanning and other industrial applications of collagen⁸. The effects of some of these chemical modifications can be summarised as shown in *Panel I*, where the denaturation temperature is typically measured by the perceptible onset of shrinking.

From these selected data, well known in the art of leather making but which do not represent the complete extent of the options available, it appears that the stabilising effects fall into two groups: moderate increase or large increase in hydrothermal stability. This has been rationalised in terms of the entropic and enthalpic contributions to the modified collagen structure⁹, when the shrinking/denaturation kinetics are controlled by the enthalpy of activation, moderated by the entropy of activation⁸. In this way, most chemical modifications confer only the moderate shrinkage temperature rise observed in the majority of cases.

This begs the question: if most chemical modifications can only raise the denaturation temperature by up to 20°C, what is the mechanism by which collagen can achieve high hydrothermal stability, when the denaturation temperature is raised by over 60°C?



Panel 1: Typically observed effects of some chemical modifications on denaturation temperature ranges of collagen.

Chemical modification	Denaturation temperature (°C)
None	65
Metal salts: eg Al(III), Ti(IV), Zr(IV) etc.	70-85
Plant polyphenol: gallotannin or ellagitannin	75-80
Plant polyphenol: flavonoid	80-85
Synthetic tanning agent: polymerised phenols	75-85
Aldehyde: formaldehyde or glutaraldehyde	80-85
Aldehydic: phosphonium salt or oxazolidine	80-85
Basic chromium(III) sulfate	105-115
Combination: gallotannin + Al(III)	105-115
Combination: flavonoid polyphenol + oxazolidine	105-115

To put this into context, assuming an activation energy for the shrinking/denaturation reaction of 518 kJ/mol¹⁰, elevating the shrinkage temperature from 65°C to 85°C enhances the hydrothermal stability by four orders of magnitude, but the rise from 85°C to 110°C represents a further five.

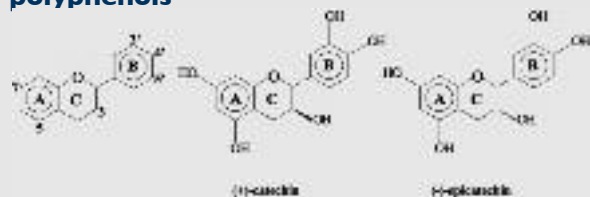
This paper proposes an answer to that question by synthesising an explanation, a mechanism of tanning, based on recent thinking in leather making science, the observation of known tanning reactions and experimental investigations of novel reactions for stabilising collagen. This new idea, designated 'link-lock', explains all known stabilising reactions and predicts the requirements for new reactions.

High stability vegetable tanning combination reactions

The combination reactions in *Panel 1* have some features in common. The primary reaction between hydrolysable plant polyphenols (the gallotannins and ellagitannins), and collagen is through multiple hydrogen bonding. The subsequent reaction is to crosslink the tannin molecules together, via the pyrogallol moieties locked together by the complexation reaction with metal ions^{11,12}. There is a similar primary reaction between collagen and the flavonoid tannins, illustrated in *Panel 2*, which is weaker in terms of the availability of phenolic hydroxyl reaction sites on the tannin, but which also includes some covalent bonding between the lysine sidechains of the collagen and the aromatic A-ring of the flavonoid, via quinoid reactions.

The next reaction involves crosslinking the tannin molecules by oxazolidine, an aldehydic reactant: this occurs at the 6- and 8-positions on the A-rings of procyanidin or prorobinetinidin

Panel 2: The flavonoid ring system of plant polyphenols

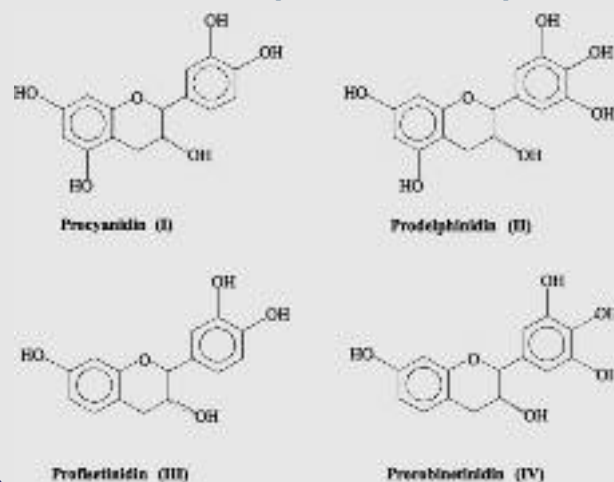


polyphenols¹³, additionally at the 2'- and 6'-positions of the B-rings of prodelphinidin or profisetinidin polyphenols¹⁴, when the latter polyphenolic reactions yield higher denaturation temperatures. The structures of these polyphenols are illustrated in *Panel 3*.

In each case, the synergistic combination reactions create matrices of crosslinked polyphenolic species, which means the polyphenolic compounds act in concert, effectively working as a single chemical moiety. In the case of the flavonoid combination stabilisation reaction, it has been shown that an important feature of the reaction is the linking of the polyphenol species to collagen via the aldehydic crosslinking reaction¹⁵.

Similar high stability combination reactions have been observed with melamine-formaldehyde polymer as primary reactant, with tetrakis hydroxymethyl phosphonium sulfate as the aldehydic crosslinker¹⁶ and with low molecular weight phenolic compounds crosslinked with aldehydic compounds or laccase, polyphenol oxidase¹⁵.

Panel 3: Hydroxylation patterns in condensed tannins: illustrated by the monomeric precursors



Chromium III and the sulfate counterion in high stability reactions

In the group of high stability reactions, the process involving chromium(III) salt might appear to be a chemical exception, having little in common with the organic (vegetable tanning) combination reactions. This is deceptive.

It is known that the chromium(III) species are covalently bound at carboxyl sidechains. Extended X-ray absorption fine structure (EXAFS) studies¹⁷ of chromium(III) bound to collagen showed that the dominating bound species are linear tetrachromium compounds, but the counterion, in this case sulfate, is not directly bound to the chromium as a ligand. Furthermore, if the counterion is different, for example chloride or perchlorate¹⁸, the effect of the stabilisation is only moderate, producing denaturation temperatures of about 85°C.

So, the chromium(III) species and the counterion must create a matrix with the supramolecular water structure, in a system analogous to the combination reactions. In the case of the inorganic reaction, the effect is a combination of the metal ions and the counterions and the water. The overall synergistic nature of the combined reactions depends on the functioning of the counterion as a structure maker in water: sulfate is known to act in this way, but chloride is a water structure breaker¹⁹, shown by circular dichroism to be capable of destabilising the collagen structure²⁰.



Insight into the structure of modified collagen

It is also a feature of these high hydrothermal stability reactions that they exhibit a strong reaction with collagen: in some cases of multiple hydrogen bonding and in others there is some covalent reaction. In this way, the matrix is firmly bound to the collagen structure. It has been proposed that this aspect of the combination reaction is an important element in the overall requirements for matrix stabilisation of collagen that leads to high shrinkage temperature¹⁶.

The results of combination reactions involving flavonoid compounds with oxazolidine are presented in *Panel 4*¹⁴, in which green tea (low molecular weight prodelphinidin), pecan (high molecular weight prodelphinidin), Myrica esculenta (high molecular weight prodelphinidin), mimosa (high molecular weight profisetinidin and prorobinetinidin) and quebracho (high molecular weight profisetinidin) are plant polyphenol extracts.

The reaction is illustrated in *Panel 5*, showing the structure of the crosslinker and the sites of reaction on the flavonoid²¹.

A simple calculation of the additive effect of the two components of the combination reaction reveals the extent of the synergy of the reaction, which measures the influence of the matrix:

$$\text{Synergy} = \Delta T_{\text{observed}} - \Delta T_{\text{polyphenol}} - \Delta T_{\text{oxazolidine}}$$

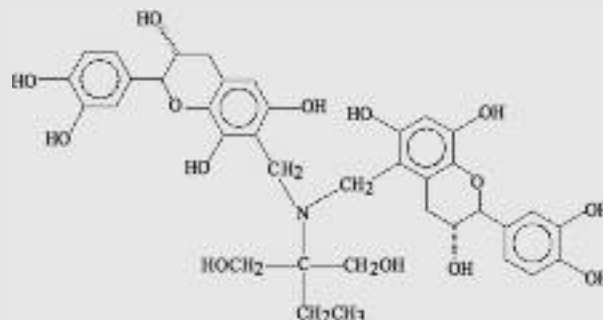
A useful method for gaining insight into the structure of modified collagen is hydrothermal isometric tension (HIT), when samples are constrained against shrinking and the forces generated during transitions are recorded. The HIT curves of unmodified and chemically modified collagen are shown in *Panels 6, 7 and 8*.

The traces can be divided into three parts:

- 1) the tension increasing process from zero to maximum tension,
- 2) followed by a relatively constant tension process, if present;
- 3) finally, the tension will be constant or a relaxation process will occur, due to the gradual destruction of collagen structure or rupture of some crosslinking bonds.

The slope of the curve in the tension increasing process accounts for the collagen fibre rigidity, caused by crosslinks: the steeper the slope of the contraction curve, the more crosslinks

Panel 5: Illustration of the reaction between catechin and oxazolidine



should be present in the collagen materials. Relaxation represents the stability of these connecting elements (crosslinking bonds): the steeper the rate of the relaxation curve, the more unstable is the crosslinking. In the case of collagen, the crosslinking does not refer to direct links between triple helices, but to the structure of the intervening matrix.

To date, no mathematical or physical model has been proposed for a full analysis of hydrothermal isometric tension curves obtained under linear heating conditions²². In 1987, Kopp and Bonnet proposed a tentative model for the development of isometric tension in collagen²³. Unfortunately, these equations are probably only suited to very limited conditions (medium, pH, ionic strength *et al.*) and cannot be applied to these experiments. But we still can get some useful information about the relative crosslink density and stability from the shapes of the HIT curves. The calculated results are shown in *Panel 9*²⁴.

Chromium tanning: a chemical matrix securely bound to collagen

Stabilisation by polyphenol reactions yields results that can be understood in terms of the availability of hydrogen bonding and subsequent localised crosslinking. However, the rate of tension increase during the shrinking transition of chromium(III) stabilised collagen exhibits a result similar to that of raw collagen.

The natural conclusion is that chromium does not crosslink collagen by joining adjacent sidechains.

This runs counter to the traditional view of collagen stabilisation, in which the sidechains of the collagen structure are supposed to be linked by single species, such as chromium(III) molecular ions; however, the only quantitative analysis in the literature leading to such a conclusion, the Gustavson model²⁵, has been shown to be flawed²⁶. Therefore, the traditional view of crosslinking does not have to be invoked in explaining chemical stabilisation reactions. The explanation lies in the creation of a chemical matrix, securely bound to the collagen.

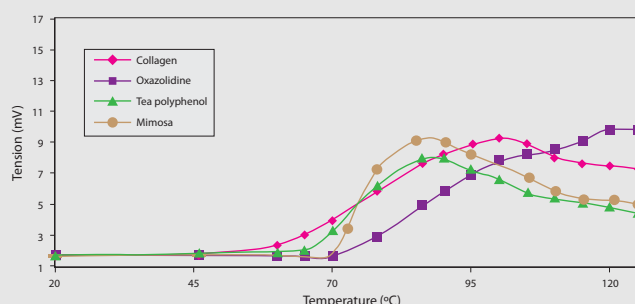
Supporting evidence of the part played by fixation of the matrix to collagen comes from the work of Holmes²⁷: he showed that modifying collagen by binding chelating pairs of carboxyl groups to the amino groups of lysine can enhance the weak stabilising effect of aluminium(III) salts, to the extent of increasing the denaturation

Panel 4: The effects of crosslinking polyphenol with oxazolidine on the denaturation temperature, the synergy of the reaction and the effect on the hydrothermal stability of acetone washing to break hydrogen bonding (ΔT_s) ($^{\circ}\text{C}$).

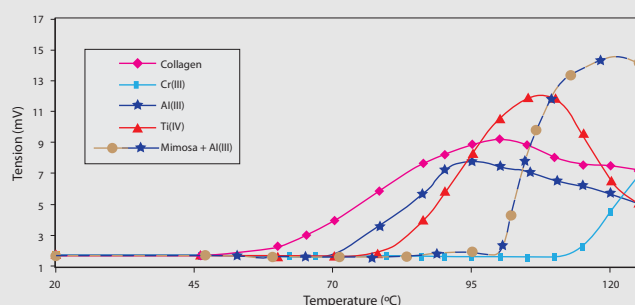
Polyphenol offer	Polyphenol alone	Oxazolidine crosslinked	Synergy	Acetone wash: ΔT_s
Control: no treatment	60			
Control: 8% oxazolidine		83		0
5% phloroglucinol	60	95	+12	-2
5% tea polyphenol	68	101	+10	-5
20% pecan	83	112	+6	-8
20% myrica	85	113	+5	-8
20% mimosa	82	110	+5	-6
20% quebracho	80	98	-5	-5



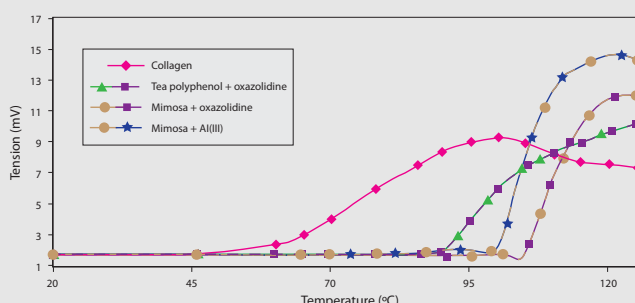
Panel 6: Hydrothermal isometric tension tests on skin treated with single organic stabilising agents.



Panel 7: Hydrothermal isometric tension tests on skin treated with mineral stabilising agents.



Panel 8: Hydrothermal isometric tension tests on skin treated with combinations of stabilising agents.



temperature from 75°C to 95°C. It is noteworthy that the only way to create stabilising reactions from which the denaturation temperature outcomes are positioned between those of the moderate stabilising reactions and the high stability reactions is by modifying the substrate.

Alternative matrixes providing high thermal stability

The requirements for high hydrothermal stability from an organic reaction do not include complexity of reagent. Indeed, using simple reactants that allow a matrix to be created can result in high hydrothermal stability. This is exemplified in *Panel 10* in which dihydroxy naphthalenes are the primary reactant, the linking agent, and oxazolidine is the locking agent²⁸.

Bone is an example of a situation where the stability of collagen clearly comes from the presence of a matrix. Here, the matrix is polymeric hydroxyapatite, not thought of as a typical crosslinker, but which can raise the shrinkage temperature of the constrained collagen to upwards of 155°C^{29,30}. In terms of the 'polymer-in-a-box'³, this is a very strong 'box', an extreme case

and probably the upper limit to collagen stabilisation, without dehydrating the protein. Indeed, it is not yet clear that the mechanism of denaturation of collagen in bone is the same as for free collagen, because at this elevated temperature the melting of the triple helix will be competing with hydrolysis of the peptide backbone, which has a lower activation energy, 173kJ/mol³¹. This would explain the surprisingly low activation energies (~350kJ/mol) observed by Trębacz and Wójtowicz for the denaturation of mineralised collagen³⁰.

Locking the matrices components together

The stability of unmodified or chemically modified collagen must depend on its ability to collapse or shrink by unravelling its chains into the available space between the chains. The ease with which this can happen depends on the constraints applied to the chains or the ease with which the intervening molecules can be displaced as the collagen shrinks. If the molecules are water, this matrix can be broken down and displaced relatively easily, since the reaction is the breaking of the hydrogen bonds in bulk water. If the matrix is stabilised by the inclusion of species bound to collagen, also by substituting some of the supramolecular water

Panel 9: Relative rates of increase in tension after shrinking transition is initiated and relative rates of decrease in tension after the shrinking transition.

Stabilising agent	Slope of contraction	Slope of relaxation
None (raw collagen)	0.25	-0.13
Aluminium(III)	0.27	-0.20
Oxazolidine	0.28	0.00
Chromium(III)	0.29	Assumed zero
Green tea polyphenol	0.37	-0.13
Titanium(IV)	0.50	-0.52
Mimosa	0.67	-0.20
Mimosa + oxazolidine	0.91	Assumed zero
Mimosa + aluminium(III)	1.23	-0.05

Panel 10: The stabilisation of collagen (as hide powder) by the combination of dihydroxy naphthalene and oxazolidine.

Stabilising	Denaturation temperature (°C)	Amount of crosslinked DHN removed by acetone, H-bond breaker (%)
None	57	
Oxazolidine (oxaz)	75	
1,5-DHN + oxaz	85	85
1,6-DHN + oxaz	90	50
2,6-DHN + oxaz	110	51
2,7-DHN + oxaz	79	65



and interacting with the remaining water, the matrix is less easily displaced, observed as a rise in denaturation temperature.

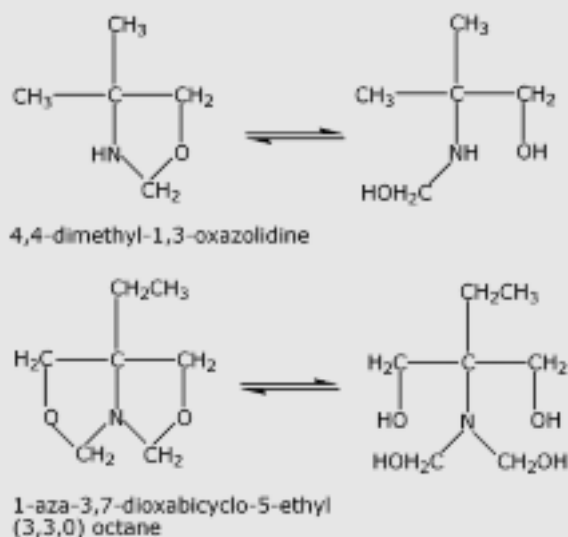
If the notion of direct crosslinking between triple helices is dismissed as the source of stabilisation, the matrix displacement model explains the similarity of outcome for the stabilising reactions already known. Some of the more compelling evidence comes from Das Gupta³², in his studies of oxazolidine tanning, illustrated in *Panel 11*. In comparing a mono-functional with a bi-functional derivative, it was shown that the outcome of solo tanning of sheepskin was the same, the shrinkage temperature was about 80°C; the potential ability to crosslink did not affect the linking reaction. However, when used to retan vegetable tanned leather, the mono-functional oxazolidine conferred only a small increase in T_s , but the bi-functional derivative produced a big increase in T_s .

Therefore, the maximum effect of a single reagent, even with the potential to crosslink, is due to interference in the shrinking process, i.e. the bound species merely hinder the process by getting in the way. This view is confirmed by the HIT data and the specific case of chromium(III): if the crosslinking reaction was paramount, the stabilising effect of chromium(III) salts would be independent of the counterion – but it is demonstrably true that shrinkage/denaturation temperature is highly dependent on the nature of the counterion³³.

Even the relationship between the linking agent and the supramolecular water does not control the outcome of the stabilisation reaction. *Panel 1* indicates the range of stabilising reactions which yield only moderate hydrothermal stability: all have different relationships with water, in terms of their hydrophilicity or hydrophobicity, but the results are the same. The small variations in hydrothermal stability achievable by linking reagents appear to be related to the nature of their primary interaction with collagen: the weaker the interaction, the lower the denaturation temperature. The order of increasing hydrothermal stability depends on the chemistry of the bonding:

electrostatic < hydrogen bonded < covalent
exemplified by the following selection of reactions:
Al(III) < hydrolysable polyphenol < aldehyde.

Panel 11: The tanning chemistry of oxazolidines: N-methylol derivatives.



It has been assumed that the shrinking reaction is independent of the stabilising reaction, because the enthalpy of denaturation is independent of the stabilising chemistry³⁴: the assumption was that the process is controlled only by the breaking of hydrogen bonds within the triple helix. If collagen stabilisation is modelled in these terms³⁵, the number of crosslinks required to elevate the melting temperature is unacceptably high. However, it is more satisfactory to regard the shrinking process of collagen, unmodified or modified, as controlled by the breaking of hydrogen bonds of the water in the matrix. This is clear, since the relationship between denaturation temperature and moisture content is broadly similar (although different in detail) for unmodified collagen and highly stabilised, chromium(III) tanned collagen³⁶, where the difference in the denaturation temperatures of the wet materials is about 40°C, but the denaturation/decomposition temperature for both the dry materials is about 200°C.

As an approach to stabilising collagen, merely loading the structure with molecular species is not sufficient to confer high hydrothermal stability. For example, plant polyphenols confer only moderate stability, limited to about 85°C, even when present at 30% on dry weight of collagen. Like most other stabilising reactions, the chemical reactions are limited to linking elements of the collagen structure to a relatively unstable matrix: in this case, a packed array of unlinked molecules, but with some water still in the interstices. Therefore, any linking step of collagen stabilisation confers only moderate hydrothermal stability because the matrix can only hinder the process of helical structure to random coil conversion.

In those chemical processes which result in high hydrothermal stability, the linking step must be combined with an additional step that locks the components of the matrix together. In this way, the matrix acts more like a single chemical compound, which is much less easily displaced. The higher energy required to achieve breakdown of the structure is observed as higher temperature transition. It is an important aspect of the matrix stabilising mechanism that the matrix should be bound to the collagen in a stable way, so that displacement of the interaction, which might lead to allowing shrinking, is prevented. This element of the high hydrothermal stability requirements may be conferred during the linking reaction or it may be conferred as a side effect, an additional aspect of the locking reaction.

Alternative routes for new high performance leathers

This proposal, that chemical modifications of collagen are actually modifications of the matrix around and bound to collagen, is already finding acceptance in the field of leather science³⁷.

This explanation of the stabilisation of collagen is simpler and more powerful than the currently accepted view, whereby chemical stabilisation is derived from specific linking reactions by single chemical species, between specific sidechains on the collagen triple helices. There is no evidence in the literature to support the old assumed view, but much evidence to indicate the new, matrix-based view is right.

This new understanding of how high hydrothermal stability is conferred to collagen - 'link-lock' - has profound implications for the development of alternative processes for all those industries exploiting the unique properties of collagen. Hitherto, it has been assumed that high hydrothermal stability of modified collagen is the property of a few unrelated chemical reactions. Here we have demonstrated that the mechanism is, in fact, general and that high hydrothermal stability may be achieved



merely by virtue of the reaction conforming to the requirements of a stable, bound matrix, as defined here.

The global leather industry currently relies on chromium(III) salts to confer the properties required for modern applications. Other reactions capable of achieving high hydrothermal stability can offer alternative routes to modern leathers, with their many high performance applications. More generally, the development of new chemistries for stabilising collagen to different degrees, based on this clearer understanding of the origin of hydrothermal stability, will be important in the fields of new biomaterials and new composites. ●

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