We are IntechOpen, the world's leading publisher of Open Access books Built by scientists, for scientists

6,900
Open access books available

185,000
International authors and editors

200M
Downloads

Our authors are among the

154
Countries delivered to

TOP 1%
most cited scientists

12.2%
Contributors from top 500 universities



WEB OF SCIENCE™

Selection of our books indexed in the Book Citation Index in Web of Science™ Core Collection (BKCI)

Interested in publishing with us?
Contact book.department@intechopen.com

Numbers displayed above are based on latest data collected.
For more information visit www.intechopen.com



11

Preparation and Characterization Urea-Solubilized Sol-Gel Type I Collagen and Its Possible Use in Applications

Xin Xiong[1,2], Herwig Brunner[1,2] and Robin Ghosh[3]
[1]*Institute of Interfacial Engineering, University of Stuttgart, Stuttgart,*
[2]*Fraunhofer Institute of Interfacial Engineering and Biotechnology, Stuttgart,*
[3]*Department of Bioenergetics, Institute of Biology, University of Stuttgart, Stuttgart*
Germany

1. Introduction

The extracellular matrix (ECM) is composed of a mixture of glue-like macromolecules in the extracellular spaces which give a tissue its characteristic form and maintains its integrity [Exposito, 2002; Gelse, 2003]. The ECM also regulates a variety of cellular behaviors such as proliferation, differentiation, motion and communication [Kadler, et al., 2007; Tsuchiya, et al., 2007]. Collagens are the most abundant proteins in the animal kingdom and have been highly conserved in evolution [Exposito, 2002; Kadler, et al., 2007]. These proteins constitute one third of the human proteome and contribute three-quarters of dry weight of human skin [Kotch & Raines, 2006]. Increasingly, this family of proteins is gaining attention from scientists in different fields, ranging from biochemistry to tissue engineering.

In the last century, collagen has been studied extensively by researchers from different disciplines including chemistry, physics, medicine and biology. Despite of this intensity, the understanding of many collagen properties is still limited and the number of collagen types or collagen-like proteins (CLP) discovered are increasing continuously. Type I collagen is the most abundant protein in the animal body and located in all ECM of connective tissues like bone, skin, tendon and blood vessels. The primary function of Type I collagen is to give mechanical support for the structural organization of the ECM of connective tissues, therefore giving the tissues their characteristic form and maintaining the cellular integrity in the ECM. Possibly there are still many unknown functions to be found. Type I collagen represents the characteristic structure very well and has the characteristic large domains comprised of peptides with varied length of [Gly-X-Y], where X and Y are generally proline (Pro), repeats with a length about 300 nm and folded into unique triple helix structure as its native form [Prockop, & Kivirikko, 1995]. Every chain from the three polypeptide-chains $(\alpha_1)_2(\alpha_2)$ form a left-handed helix then fold together to form a right-handed triple helix.

1.1 Self-assembly of collagen Type I and role of proline hydroxylation in collagen aggregation

After the biosynthesis of collagen, it is post-translationally modified by different enzymes, including some modifications (particularly hydroxylation) which are unique to this class of proteins. Enzyme-mediated hydroxylation introduces a hydroxyl (–OH) group into prolyl or lysyl residues, thereby oxidizing them to 4-hydroxyproline (4-Hyp), 3-hydroxyproline (3-Hyp) and hydroxylysine (Hyl). The function of 4-Hyp is generally accepted to stabilize the collagen triple helix structure, while the function of 3-Hyp is still not clearly known [Jenkins, et al., 2003; Berg & Prockop, 1973; Wick, et al., 1978]. Hydroxyproline can be determined by chemical methods, as described by [Jamall et al., 1981]. Whereas many studies have suggested that the 4-Hyps enhance the stability of collagen strongly, in contrast, 3-Hyp has been suggested to exhibit a *destabilizing* function [Jenkins, et al., 2003].

Since collagen contains very few aliphatic or aromatic residues, it is unlikely that the assembly of the collagen chains is entropy-mediated as a consequence of the hydrophobic association of amino acids. In fact, extensive calorimetric studies by Privalov in the early 1980s (see [Privalov, 1982]) showed that the thermodynamic behavior of Type I collagen melting was exceptional. In particular, the melting transition was exceptionally sharp compared with those observed for other proteins, and the magnitude of the specific heat change for the melting transition was also significantly larger than for most proteins. Privalov [Privalov, 1982] also concluded, from a limited data set, that 4–Hyp *at the third position* of the characteristic amino acid triplet made the largest contribution to Hyp-mediated collagen stabilization. Finally, Privalov concluded that the every large enthalpy change upon collagen denaturation was due to extensive hydrogen bonding, and that the evidence strongly favored one of the original Ramachandran models [Ramachandran et al., 1973], where an additional water bridge can be formed between Hyp at the third position of the triplet and a strongly-bound water molecule. This unusual bonding leads to an extensive hydrogen-bonding network, which strongly stabilizes the structure. From the thermodynamic data, and also the exceptionally high value (determined experimentally) for the strongly bound water, about 7-8 water molecules must be tightly bound per collagen triplet. Brodsky and Persikov [Brodsky, & Persikov, 2005] have also noted that 4-Hyp on the Y position can accept a hydrogen bond from the neighboring Gly, therefore contributing to the conformational stability of the triple helix. Reports also indicate that the Hyp is rarely found to be at the X position thus preventing steric hindrance, which would lead to structural destabilization.

Collagen fibril formation is a self-assembly process that has been investigated *in vitro* for over four decades. Although it is now clear that the major driving force of the fibril building is from the amino acids residing in the sequence, many studies showed other factors could affect or participate in this process [Kadler, et al., 1986; Ioozo, 1998]. Not only the kinetics of fibril formation but also the diameter of the fibril are controlled by these factors. Studies have shown that proteoglycan is involved in control of the shape and size of Type I collagen in echinoderm. The presence of the protein decorin has also been suggested to influence the fibril fusion [Kadler, et al., 1996]. Although different assembly forms were observed, the aggregation of collagen molecules into unbranched fibrils has been conserved in evolution. The fibril formation *in vivo* in vertebrates is either bipolar or unipolar, thus the spatial arrangement of the fibrils determine the mechanical and physical properties of the tissues.

Fibril-forming collagens including Type I-III, V, XI, XXIV, and XXVII are all similar in size and share the same basic sequence in that they contain a large uninterrupted triple-helical

(collagenous) region. The collagenous region is flanked by N- and C-terminal non-collagenous domains and both propeptides [Khoshnoodi, et al., 2006]. The propeptides are synthesized first in the cytoplasm as the collagen precursor, procollagen, and then processed to collagen in the extracellular space. At this step, the N- and C-terminal propeptides are cleaved off to yield mature collagen. However, the assembly to triple helical structure begins already in the ER, with collagen single chains forming homo- or heterotrimers, even though the homotrimer of $(\alpha_1)_3$ is the thermodynamically preferred form of Type I collagen assembly. *In vitro* studies suggest that collagen trimerization is controlled by the non-collagenous (NC) regions [Kuznetsova, et al., 2003; Leikina, et al., 2002]. It has been shown that the homotrimer of α_1 is the default form which assembles in absence of the α_2 chain, whereas the homotrimer of α_2 is only formed if the C-terminal non-collagenous domain is replaced with synthetic peptides. Thus the C-NC-domain of α_2 is recognized as a key domain for the heterotrimer formation. Early studies have also shown that the disulfide bridges in the C-NC domain of the α_2 are crucial for heterotrimer assembly and folding [Koivu, 1987]. Energy minimization and molecular dynamic simulations have indicated that the trimerization of both α_1 C-NC domains with the α_2 C-NC domains is a key step in folding, docking and assembly of the three heterotrimer chains [Malone, et al., 2005]. Thus, folding of collagen is initiated from the C-termini by association of α_2 and $(\alpha_1)_2$ and then proceeds sequentially toward the N-termini. Trimerization begins after the interchain disulfide bonds have been formed between α_2 and $(\alpha_1)_2$ C-NC domains.

At the same time, new recognition sites, encoded by the sequence information, allow formation of oligomers and fibril according to the tissue specific requirements [Khoshnoodi, et al., 2006]. These recognition sites determine the enzymatic cleavage sites, connections of collagen molecules, lateral association and interaction with other macromolecules from cells such as integrin and proteoglycans in the ECM. Although the understanding of the mechanisms for the trimerization and self-assembly has been increased significantly in last decades, several features are still open for future studies, e.g. the recognition motif for trimerization, differential regulation of different C-NC domains. These could be very useful for engineering proteins with a superstructure which is more suitable for application as a biomaterial. Furthermore, the understanding of N-NC domain is still limited [Bornstein, 2002]. It is known that the N-NC peptides in procollagen α_1 are involved in numerous processes, including the maturation of collagen and function of Type I collagen. Strangely, however, the deletion of these peptides appears to have no significant phenotype in the mouse. Interestingly, the N-NC domains are cysteine-rich peptides (10 Cys in N-NC of α_1) and are very well-conserved in almost all known procollagen types and all animals. No other amino acids in the N-NC domains are seen to be conserved so strongly as cysteine. However, the function of the disulfide bonds in this region is still an open question [Bornstein, 2002].

Whether purified collagen can refold to triple-helical structure after the denaturation or unfolding is still unclear. Classical results in the last 40 years have indicated that unfolded collagen cannot assemble to form native triple helix. In these studies, although UV-CD-spectroscopy showed a "significant" positive peak around 225 nm, which is characteristic for triple helix, the electron microscopic images revealed that only misaligned helices were present. Some studies have also shown so-called segment-long-spacing aggregations. This conclusion is supported by the above theory that the C-NC domain is a key regulator for triple helix folding. Surprisingly, all studies so far have indicated that collagen at body temperature (37 °C) is only marginally stable [Privalov, 1982]. For instance, the most

modern studies show RTT Type I collagen to be only about 30% in the folded (fibrillar) state at 37 °C [Privalov, 1982]. Possibly, collagens are a subgroup of the class of "unfolded proteins" which maintain a high degree of conformational flexibility at physiological temperatures. It is also reasonable to suppose that the presence of proteoglycans, glycosylation, heat shock proteins (HSP) and Hyps serve to stabilize the collagen structure.
Recent studies have introduced some new results to understand the folding of the collagen which are based upon model peptides. Interestingly, these model peptides are from the collagenous region consisting only of [GPP] repeats. These researchers observed that the Hyp is not essential in stabilization of the collagen model *in vitro* [Mohs, et al., 2007], which is contradictory to the other studies. The question how collagen molecules are stabilized *in vivo* is still unresolved.

1.2 Experimental studies using synthetic peptides

There are two major methods to study collagen: using isolated natural collagen [Kadler, et al., 1988] and synthetic model peptide/protein [Kotch & Raines, 2006; Malone, et al., 2005]. Due to its high complexity and high molecular weight, most structural studies have relied on model peptides. Unfortunately, most model peptides studies have focused on the trimer repeats of [Gly-X-Y] and have been mostly limited to $[Gly\text{-}Pro\text{-}Hyp]_{10}$ [Mohs, et al., 2002], which is only a short peptide much shorter than natural collagen (≈300 nm). Although these model peptides yield much information about the dynamic process of the assembly and triple helix building, the information is just part of that necessary for the understanding of real collagen. Studies have shown that polymerization of these model peptides yield the triple helix form but with a high polydispersity [Kotch & Raines, 2006; Paramonov, et al., 2005]. Furthermore, it must be taken into account, the α_1 and α_2 chains, containing about 1000 amino acids each, show only 43 and 28 [Gly-Pro-Hyp] (rat Type I collagen, SP:02454 and 02466) repeats, respectively. For this reason, an understanding of the structure of the whole collagen protein is still required [Orgel, et al., 2001]. However, this task is always hampered by the low quality of the collagen derived from conventional procedures, including neutral salt and acetic acid-extraction [Becker, & Timpl, 1972; Miller, et al., 1982; Chandrakasan, et al., 1976; Rajan, et al., 2007] (A comparison of the three procedures is shown in Fig. 1).

1.3 Laboratory scale preparation of highly purified natural Type I collagen, using urea-extraction, and its characterization

In our previous study [Xiong, et al., 2009], we reported a new procedure for obtaining highly purified native like Type I collagen from rat tail tendon with a high yield. This urea-extracted collagen (UC) exhibits a very high "solubility" in water, and thus, using current physicochemical terminology, can be considered to be a sol-gel preparation. The collagen sol-gel can be directly employed as matrix for cell culture without any pre-treatment such as neutralization and gelling. Another new finding we reported concerned the differential hydroxylation pattern of the proline residues at the X positions, and the existence of the differential hydroxylation among the population of collagen α chains. Studies of the population structure of differential hydroxylations, may contribute to an understanding of the tissue-specific differences of Type I collagen as well as the different states of the aging process.
Finally, we further characterized the UC. In particular, we improved the ESI-MS/MS technique by means of combinational digestion, resulting in strongly increased sequence coverage from an initial α_1-chain (95.8%)/α_2-chain (88.1%) coverage to 99% coverage for both

α-chains. Moreover, we developed a new method based on UV-spectroscopy for fast determination of the urea collagen concentration facilitating the application of the UC. A special focus was also directed to understand the reversible self-assembly of the UC, which differed strongly from other collagen isolates. We hypothesized, and later proved experimentally, that the specific carbamylation of the lysine residues in the C-terminus of the α_1 chains might play a decisive role.

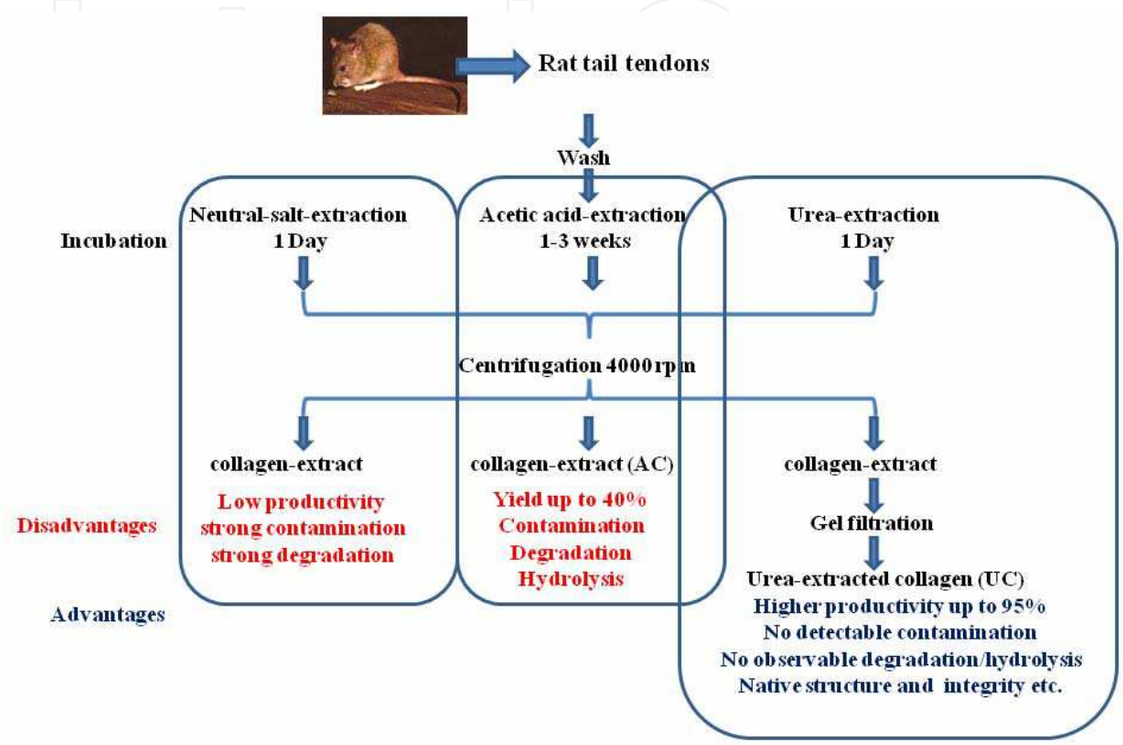


Fig. 1. Brief chart of major differences between the conventional method and newly established urea-extraction

Summary and comparison of the conventional procedures for collagen purification to urea extraction. In all three procedures, the tail tendons were solubilized into respective solutions within the indicated time frames. After centrifugation, the crude extracts are either stored as final products (neutral/acidic extracted collagen) or further purified (urea-extracted collagen). As the neutral salt extract has some known drawbacks, such as low yield and high contamination with other proteins, as well as often showing significant degradation, in our studies, only AC and UC have been directly compared using either commercially available AC or AC produced in our lab. Yield of the UC is calculated from independent experiments from dry weight of the tendons and isolated purified urea extracted collagen. The yield of the acetic acid procedure has been calculated using the wet weight of the tendons and the dried weight of the acetic collagen [Kadler, et al., 1988; Paramonov, et al., 2005; Becker, & Timpl, 1972; Orgel, et al., 2001; Miller & Gay, 1987]

In 2010, Eyre and coworkers published two articles describing a hypothesis based on the MS/MS studies of different types of collagens from a variety of tissues including rat tail tendon, human skin and bone. Thereby, they identified unusual 3-hydroxylations on the first Pro-residues in the C-terminal [GPP] repeats while the second Pro-residues are

conventionally 4-hydroxylated [Eyre, et al., 2011; Weis, et al., 2010]. Further, they speculated that the 3-Hyp is a tissue-specific feature and can be very important for defining the structure and function as well as assembly status of the Type I collagen in different tissues. This hypothesis is consistent with observations of our previous studies, where we demonstrated an unusual differential hydroxylation pattern in the RTT-Type I collagen. This aspect has not been studied previously in great detail, largely due to the severe limitations of the classical procedures for protein sequence analysis. The new findings are based on more modern and highly discerning mass spectrometric methods, in combination with our newly established UC preparation method. We believe that the combination of these two factors opens the door for a new understanding of this class of protein.

1.4 Goal of this chapter

In the following part, we will show some major results from the urea extraction procedure and the characterization which involved the AFM, SEM, UV-CD-spectroscopy and MS/MS as well as cell culture techniques. We also compare our data to those published recently by other groups.

2. Methods and results

2.1 Comparison of the isolation methods and the yield

Generally, the neutral salt and acetic methods have been established since 1960s. Later many groups modified the methods to improve the quality of the isolated collagens and to reduce the working time [Miller, & Rhodes, 1982; Miller, & Gay, 1987; Bailey, et al., 2011]. Briefly, different tissues such as rat tail tendons, skin from human, porcine and bovine sources are defatted and disinfected to germ free (e.g. with an ethanol wash) before collagen isolation. To increase the solubility of the collagen and avoid potential immunogenicity, the tissue is often treated with pepsin so that the telopeptides can be removed from the collagen molecules. The pre-treated tissues are then incubated in either neutral salt or acetic acid solution for several days to weeks, until a gel-like solution is formed. Finally, the raw extract is clarified by centrifugation to remove the non-soluble tissue residue.

In our newly-established urea-extraction method [Xiong, et al., 2009], we demonstrated that soluble sol-gel collagen can be obtained using a simple two step purification procedure: (1) PBS-washed rat tail tendons are solubilized in 10 M urea solution for up to 16 h at 25 °C (room temperature), and (2) followed by Sepharose 12 gel filtration. The collagen-containing fractions are then dialyzed against water, and the highly purified Type I collagen can be stored in water or concentrated by lyophilization. The most interesting aspect with this method is that, Type I collagen in different formats (solution, sol-gel-like preparation and powder) as well as different concentrations is now readily available. Up to 100 mg/ml UC can be redissolved in water or culture medium as required.

2.2 Alternative digestion of UC for mass spectrometric characterization

In order to increase the sequence coverage of the Type I collagen using the MS/MS technique, we applied an alternative digestion approach whereby the samples were digested separately with CNBr, trypsin and GluC. We also compared the results from the MS/MS analysis with those obtained by amino-acid composition analysis.

Lyophilized UC from gel filtration was dissolved in formic acid containing 30% ethanol (v/v) at a collagen concentration of 3 mM, as determined by the ΔA220 value. For the

cleavage of methionine, cyanogen bromide (CNBr) in a 75-fold molar excess of the His amount (which can be estimated from the ΔA220 (unpublished data)) was added to the solution in a Reacti-Vial (Pierce) and the reaction was performed for 6 h in the dark at room temperature under stirring. The reaction mix was centrifuged at 16,000 xg for 10 min and then the supernatant was transferred to an Eppendorf tube for drying in a Speed-Vac (Univapo-100H, Uniequip GmbH, Germany) for 2 h.
About 0.5 mg/ml of protein was dissolved in a solution containing 8 M urea and 50 mM NH_4HCO_3, then reduced and alkylated using standard protocols. The sample was diluted and tryptic digestion was carried out at 37 °C overnight. After digestion the solution was adjusted to a pH of 2–3 with concentrated formic acid.

2.3 UV-spectroscopy and UV-CD-spectroscopy

UV-spectra were obtained using a JASCO V-560 spectrometer with a scanning speed of 200 nm/min and slit width of 2 nm. Generally, 2 mm path-length quartz cuvettes were used for the measurements. The whole measurement was performed at 25 °C using a thermostatted cuvette holder.
Further, for determination of the UC concentration, we developed a rapid UV-spectroscopic measurement. As mature Type I collagen lacks the aromatic residues Trp, Phe and Tyr, but contains large amounts of His, we employed this amino acid as a spectroscopic indicator. In control experiments, 1 mM His (Serva) was dissolved in 10 mM sodium phosphate buffer adjusted to either pH 5.5 (protonated, charged form of His) or pH 7.5, and measured in both the presence and absence of 1 M urea. The same measurement was performed using mature Type I collagen dissolved in the same buffers. A comparison of the molar concentration of UC collagen obtained after a dry weight determination following lyophilization with that determined from the His extinction coefficient obtained above, showed the two measurements to be in close agreement.
To study the refolding of the UC after dialysis, UV-CD spectra were acquired with a JASCO J-715 CD spectrometer (scanning speed of 50 nm/min, 2 mm cuvette, slit width 2 nm, averaging over 9 scans). Spectra were obtained in the range of 320 nm to 190 nm. For the spectroscopic measurements, all buffers were degassed under vacuum prior to use.

2.4 Atomic force microscopy (AFM) of the UC-matrix and comparison to native rat tail tendon

The dialyzed urea extract was characterized by AFM. Images were captured using a CP microscope (Park Scientific Instrument Auto probe microscope CP/Veeco) in the tapping mode by using silicon-etched RTESPA cantilevers (Veeco Nanoprobes), which have a nominal tip radius smaller than 10 nm and a spring constant of 20-80 N/m. The drive frequency was set at 200-400 kHz with integral and proportional gains of 0.5-1.5. The scan rate was 1.0 Hz and the scan size was 2-5 and 20-50 μm for collection of heights. All images were flattened and zoomed off-line with ProScan software.

2.5 Cell culture assays for applications as a biomatrix for tissue engineering

The goal here was to characterize the UC collagen and compare its properties with the more conventional AC. The fibroblast 3T3 cell line was grown in Dulbecco's modified Eagle medium (DMEM) (GIBCO) in tissue culture flask (Greiner) at 37 °C, 5% CO2, until reaching confluence. This DMEM contains 10% fetal calf serum (FCS) (Clonetics), 2 mM L-glutamine,

0.1% gentamicin, 0.45% glucose and 0.1% penicillin-streptomycin. The same amount of UC and AC were dissolved in DMEM (1 mg/ml) and dropped into a 24 well-plate with inserts for expression analysis and into a 6-well plate with cover slips for phenotypical studies by SEM. The collagen solutions were incubated under a sterile bench for 2 h to allow gelling. Then, 1 x 10^4 and 2.5 x 10^4 cells in 100 µl DMEM were dropped onto the collagen surface in the inserts. For the collagen-coated cover slips only 5000 cells were employed. For both studies, the cells were cultivated for 14 days with changes of medium every 2 days. Cell vitality was judged by the live/dead cytotoxicity test. The samples were washed twice in phosphate-buffered saline (PBS) and stained for 60 min with 200 µl calcein/ethidium homodimer-1 solution. Subsequently, the samples were washed in PBS and fixed in 4% glutaraldehyde. To study the difference in cell behavior on different collagens, specific genes were chosen to be compared at a transcriptional level using real-time polymerase chain reactions (RT-PCR). Three culture conditions were compared cells growing on a plastic surface used as reference culture. Cells grew on UC and AC were compared to the reference culture.

3. Results and discussion

3.1 Comparison of the preparation methods for Type I collagen

As mentioned above and shown in Fig. 1, in the conventional isolation procedures, there are several drawbacks that cannot be ignored: (1) the contamination from other tissue proteins such as albumin and other type of collagens; (2) uncontrolled degradation of the collagens during pepsin treatment and the extraction, as the tissue own enzyme cannot be totally removed by washing the tissue prior to the extraction; (3) the acid treatment also causes some partial hydrolysis during the extraction, which is largely uncontrollable (Fig. 2); (4) the longer the tissue is incubated in the acid solution and the more collagen is solubilized, the higher the viscosity of the solution, which reduces the further solubilization of the remaining tissue collagen and complicates further purification steps e.g. chromatography. The last point creates severe limitations for up-scaling of the procedure for industrial high-throughput production; (5) the isolated collagen is normally kept in acidic solution which limits the shelf life and handicaps the delivery and application in the laboratory or hospital. To obviate these difficulties, some sources provide freeze-dried collagen products. However, as mentioned above, the collagen powder cannot be redissolved rapidly, and in the normal case, only part of the dried powder is soluble. Several other problems with the AC, such as the pH adjustment prior to usage in cell culture with medium and buffers, which is also batch-dependent, makes the application of such collagen very time- and labor-consuming. most importantly, this reduced the reproducibility of the collagen based experiments as indeed, the collagens produced from the neutral salt or acidic procedure are mixture from differentially hydrolyzed/degraded collagen peptides and intact collagen [Miller, et al., 1982; Chandrakasan, et al., 1976; Bailey, et al., 2011; Bann, et al., 2003; Mizuno, et al., 2004; Rajan, et al., 2006]. In Fig. 2, the isolated UC was analyzed by a 4% SDS-PAGE gel and the characteristic bands for α_1 and α_2 polypeptides are indicated with arrows. During the incubation in 9 M urea several samples were taken after 1 h, 3 h, 5 h, and 24 h respectively to test for degradation. As shown in Fig. 2A, the longer the incubation the more collagen could be dissolved in the 9 M urea. The amounts of the high molecular weight fractions were increased during the incubation. A smear across by the high molecular weight fractions was observed in all samples (Fig. 2A). Even though tendons were incubated

for 2 weeks in 9 M urea solution, no degradation was observed. There was no difference in the SDS-PAGE if the extracted collagen was treated with DTT prior to loading, indicating that the occurrence high molecular weight fractions is not due to the formation of disulfide bridges (Fig. 2B). Furthermore, the UC was treated in 0.5 M acetic acid for 7 days. The collagen was degraded continuously, indicating the obvious advantage of UC-process if compared to the acidic procedures with the regard to the integrity of the isolated collagen.

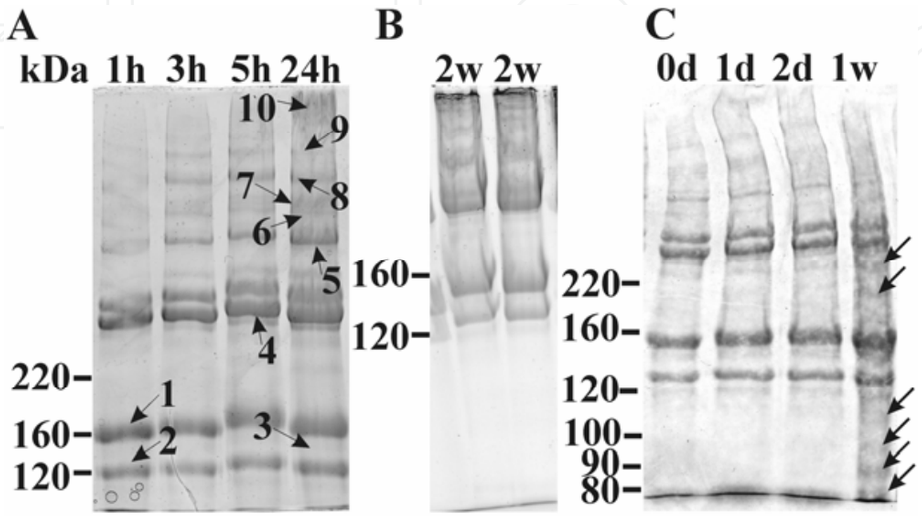


Fig. 2. Urea-extraction and acetic acid partial degradation of UC.
(A) Extraction of collagen from rat tail tendons. Lanes 1 to 4 were the samples taken at 1h, 3h, 5h and 24h during the incubation with 9 M urea. Arrows numbered from 1 to 10 correspond to bands excised for ESI-MS/MS protein identification. The bands 1 and 2 in lane 1 correspond to the α_1 and α_2 chains of the Type I collagen, respectively. All the protein bands indicated by arrows were identified using ESI-MS/MS and these bands contain only Type I collagen. (B) Tendons were incubated in 9 M urea for 2 weeks. Lane 1 shows the untreated sample (no DTT or heating prior to loading). Lane 2 shows the DTT-reduced and heated (at 100 °C for 5 min) sample. (C) The lyophilized extract from urea extraction was incubated in 0.5 M acetic acid. Lane 1 shows the input sample at the beginning, Lane 2 and 3 are the sample taken after one and two days respectively; Lane 4 is sample after 1 week incubation. The arrows indicate the bands which appeared during the incubation

In our lab, we have demonstrated that the yield of UC from the starting tissue is about 93%, significantly higher than the value we obtain for AC (compared to a value of about 60%, see [Xiong, et al., 2009] for a more detailed discussion)

3.2 UV-determination of the UC concentration

Surprisingly, a continuing problem is the need for a fast and accurate method for the determination of collagen protein concentration. Due to the low amount of aromatic amino acid residues present in Type I collagen, widely-used protein assays such as the Lowry-Ciocalteau reaction[Lowry, et al., 1951; Peterson, et al., 1977], or UV-Vis determinations at 280 nm, and the Bradford-type determinations [Bradford, 1976], which rely the binding of a chromophore to hydrophobic binding sites (almost not present in collagen), are largely useless in this context. In addition, for these latter methods, the collagen should be highly

purified and strongly diluted to prevent precipitation. This is often achieved by purification using acid extraction, and storing in dilute acid. The acetic acid storage method often limits the accuracy of these protein assays. The most accurate method is dry weight determination, whereby the collagen in solution is directly dried in oven or lyophilized to constant water content then weighed. The disadvantage here is that lyophilization requires long drying times (typically longer than 1 h), which is very inconvenient for routine measurements. Other groups use chemical or immunological reactions, which are based either upon a chemical reaction followed with photometric detection [Shormanov, & Bulatnikov, 2006] or anti-collagen antibodies. In principle, the dry weight methods are the most accurate but time-consuming whereas photometric methods are complicated due to the chemical reaction, which need an optimum condition for detection, and antibody reactions are only accurate when purified monoclonal antibodies are employed. Unfortunately, most commercial antibodies against Type I collagen show high cross-reactivity (up to 10%) to other fibril collagens such as Type III, so that their use in quantitative immunoblotting is often not accurate and also time- consuming.

Histidine (His) is present in a high quantity in collagen molecules. In the course of recent work, we examined the possibility of using UV-VIS spectrum of His to determine collagen protein concentration. His absorption spectroscopy has rarely, if ever, been used to determine protein concentration, as its extinction is much weaker than those of the aromatic amino acids. However, Type I collagen contains large amounts of His and very low amounts of aromatic amino acids. From a total of 30 His residues/mol (pro-α_1)$_2$(pro-α_2) procollagen (SP: P02454 and P02466, respectively), 13 are located in the "core" collagen sequences (2 in α_1 at position 256 and 1099; 9 His in α_2 at positions 152, 188, 518, 950, 972, 981, 1031, 1044 and 1076). In contrast to core region, 7 His are found in the pro-α_1 propeptides and only 3 His in pro-α_2 propeptides region. Of particular interest was whether the His spectrum could be observed in the presence of 1-4 M urea, which is often employed as a solubilizing agent, and whether the ionization state (His has pK_a 6.0) affects the measurement.

To test our procedure, we employed Type I UC. The final preparation is homogenous and corresponds to the "core" sequences described above, as indicated by mass spectrometry (data not shown). This UC can be dissolved completely in water following urea removal (by dialysis) and lyophilization, and provides an ideal test object for testing the His determination method here. We showed that 1 mM His dissolved in 4 M urea yielded a differential absorbance value at 220 nm (ΔA_{220}) of 0.64. 1.3 mg dried collagen was dissolved in 1 ml buffer containing 4 M urea and from this sample 200 µl was added to 2 ml for spectroscopic determination.

We determined the amount of His using UV-spectroscopy. In the α_1 chain contains 2 His residues, whereas the α_2 chain, contains 9 His residues. Assuming an (α_1)$_2$ α_2 stoichiometry, these values imply that a single native mature collagen molecule contains 13 His residues. Using the extinction coefficient at 220 nm, we calculated the predicted concentration of weighed 1.3 mg UC sample (4.6 nmol collagen) to be 1.6 mg (5.6 nmol collagen). Although the 20% error margin is still significant, the method is very accurate compared to those mentioned above. This measurement is also in good agreement with the data from amino acid composition analysis and the ESI-MS/MS data, which identified all the His present in UC. This measurement is highly reproducible and can be used even in presence of different salts and urea. Although amounts of phenylalanine are also very high in Type I collagen (26 in α_1, 22 in α_2), these were not visible in a UV-absorption spectrum, indicating that these phenylalanine residues are most likely modified.

We found out that the His absorption at 220 nm is very useful and reliable for this purpose even in presence of urea at high concentrations (up to 8 M). A full description of the method will be reported elsewhere.

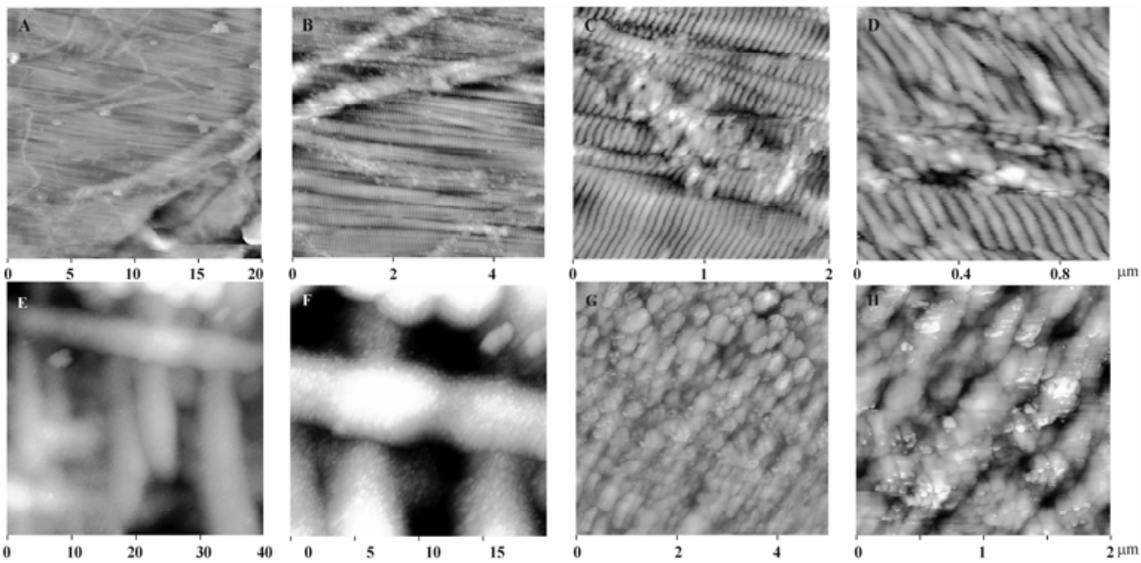


Fig. 3. AFM image comparison of RTT and the UC samples
(A-D) are the images taken directly from the lyophilized RTTs without any further purification steps to show the native fibril structure of the RTT in different scales from 20 to 0.4 μm. (E-H) are the correspondent scaled images from the UC samples after dialysis against water without lyophilization. The sol-gel-like UC, showed significant random fibril formation at this stage, which could be seen to be highly similar to native RTT fibrils shown in (E). However, the fibril has another dimension (large diameter) if compared to native RTT. Additionally, the sol-gel UC has a high water content and no fine D-periods structure

3.3 Discovery of differential hydroxylations of proline residues and its application as identifying feature for collagen

The differential hydroxylation of collagen is a topical subject, which until only recently, has been very difficult to address experimentally. An illuminating example of the use of this phenomenon is illustrated by recent work by Mary Schweitzer and colleagues, reported recently in two seminal Science papers, who have demonstrated that soft tissue, purported to be collagen, can be isolated from fossils which are between 68-80 million years old [Schweitzer, et al., 2007; Schweitzer, et al., 2009]. In both works, Schweitzer and co-workers were able to extract and sequence several small polypeptides from the fossil material, some of which was assigned to arise from collagen. If correct, the Schweitzer work would represent a huge breakthrough in molecular paleontology. However, the first paper, where only 4 small collagen fragments could be isolated from a 68 million year-old *Tyranosaurus rex* fossil, was heavily criticized. In particular, the mass spectrometric sequence determination was only slightly above the noise level, and at least one of the sequences may have arisen from bacterial contamination (see [Schweitzer, et al., 2009] for a detailed discussion). In the latest paper by Schweitzer and collaborators, however, 8 collagen fragments (six arising from the Type I collagen α_1 chain, and two arising from the Type I

collagen α_2 chain), extracted from soft tissue arising from an 80 million year-old hadrosaur (*Brachylophosaurus canadiensis*) fossil, could be obtained and sequenced with a high level of precision. In this work, significant effort was taken to minimize contamination problems, redressing many of the criticisms of the *T. rex* work. Furthermore, in an impressive technical *tour de force*, Schweitzer and colleagues were able to determine the hydroxylation profile of the isolated polypeptides, confirming the assignments by comparison to synthetic hydroxylated polypeptides.

However, an unsatisfying aspect of the *B. canadiensis* work is the exclusive reliance upon synthetic polypeptides. The confirmation of hydroxylated amino acids at the same homologous positions of a modern-day native collagen would support the *B.canadiesis* assignments significantly. So far, this has been difficult to perform, since the databases contain only fragmentary sequence information, much of which is rather old and based upon the contiguous assembly of CNBr fragments sequenced by Edman analysis. Usually, Edman analysis does not provide easy and unambiguous access to peptide hydroxylation data.

Recently, we have published the first (to our knowledge) almost complete tandem mass spectrometric (MS/MS) analysis of the hydroxylation profile of a native (rat) collagen I molecule [Xiong, et al., 2009]. Our present (improved since [Xiong, et al., 2009]) coverage for the α_1 and α_2 chains is 99.2% and 89.8%, respectively (Fig. 1a, for methods see [Xiong, et al., 2009]). Given the high precision of our data, we thought it might be interesting to compare it to the hydroxylation assignments of the *B. canadiensis* Type I collagen polypeptides. A BLASTP analysis of the *B. canadiensis* collagen Type I-derived polypeptides against those of the full-length rat α_1 and α_2 polypeptides yielded a single unambiguous homology in all cases (Fig. 4b). For five of the six *B. canadiensis* α_1-derived polypeptides the hydroxylation assignment is essentially identical to that found for rat tail-derived α_1 polypeptide, as determined by MS/MS (Fig. 4b). For the sixth polypeptide the hydroxylated proline residue in the rat sequence has been replaced by an isoleucine in the *B. canadiensis* sequence (Fig. 4b). Interestingly, and in stark contrast to the α_1 polypeptides, one of the two α_2-derived polypeptides from *B. canadiensis* does not show the same hydroxylation pattern as in the rat α_2 sequence, since the hydroxylable proline in the rat sequence has been replaced by alanine in the *B. canadiensis* sequence (Fig. 4b, lower). Nevertheless, for this *B. canadiensis* α_2 sequence, the distal hydroxylation sites neighbour those found for the rat α_2 sequence. For the second α_2 sequence, our partial sequence shows a hydroxylation difference at the third position. Although the rat distal sequences were not detectable, the hydroxylable proline in *B. canadiensis* is replaced by isoleucine in rat α_2. In summary, the hydroxylation assignments of the *B. canadiensis* are consistent with their experimental observation in a modern-day species (rat), but also show significant differences consistent with a long evolutionary divergence.

Mass spectrometry is now becoming a standard method in many biological and medical institutions. The results presented here, showed that the MS/MS based characterization of collagen, including mass determination and identification, can be easily applied as a very accurate procedure for quality control in the production and application of collagens. The method is of high accuracy and yields results in a very short time frame if compared to conventional methods. Moreover, for study of the collagens, in particular, for the same type of collagen in different tissues and different aging status, the MS/MS technique shows a unique advantage in being able to distinguish the above mentioned PTMs unambiguously.

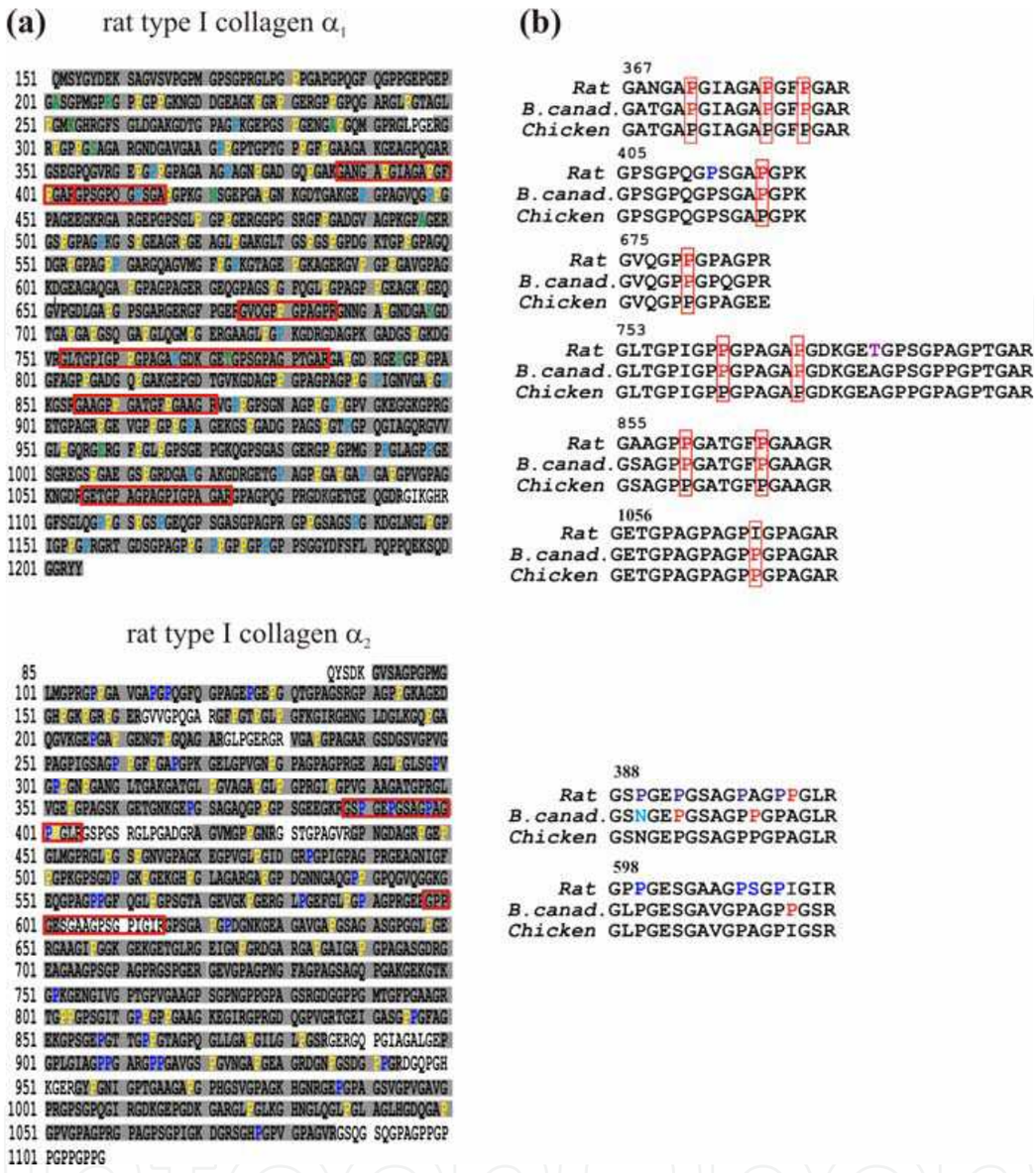


Fig. 4. MS/MS Sequence coverage with emphasis on the positional hydroxylation of proline as identifying feature for mammalian Type I collagen, as compared with published data from [Schweitzer, M.H., et al., 2007 & 2009]

(a) The amino acid sequences of the full-length mature Type I collagen α_1 (top) and α_2 (bottom) polypeptides (SP: P02454 (α_1) and P02466 (α_2), respectively. The gray shading indicates the sequences derived by MS/MS (see (3) for methods), and the positions of the homologous polypeptides from *B. canadiensis* are highlighted as red boxes. Persistently hydroxylated and partially modified proline residues shown by MS/MS are highlighted in yellow and blue, respectively. Residues differing from the SwissProt sequences were highlighted in green. (b) Detailed comparison of the homologous sequences from rat, *B. canadiensis* and chicken α_1 (top) and α_2 (bottom) polypeptides. The chicken sequence was chosen as this was considered extensively in (2) and because birds are thought to be evolutionarily closer related to dinosaurs than mammals. The red boxes indicate persistently hydroxylated proline residues

3.4 Applications of UC as biomatrix in tissue engineering

Type I collagen is one of the most widely-used biomaterials as a cell culture matrix and as a biomaterials in the clinic. However, as mentioned above, these applications have been hampered by many drawbacks, most of which are due to the production procedure. We demonstrated in our previous work that the UC-cultured 3T3 fibroblasts showed 3D-like cell morphology and all cell assays of gene regulation employed showed that the UC shows a significant benefit to the cultured cells if compared to plastic surface and acetic acid-extracted collagen. Moreover, we introduced the sol-gel concept to define our UC as a sol-gel like materials in high concentration in aqueous solution at neutral pH (water or culture media with pH 7.4). This product makes the long-term storage of collagen as a powder format possible and simplifies the delivery and application, as the powder can be easily redissolved on demand, even at high concentrations, without any detectable precipitation and or other change of properties. A very important property of the sol-gel UC is the avoidance of contraction of matrix during the cell culture time frame, thereby eliminating an important negative effect of the conventional collagen matrix. In the latter case, the collagen must be used in relative low concentrations and pre-gelled to give a solid gel-like structure. (for detailed results see [Xiong, et al., 2009])

In our study we also used the SEM technique to compare possible fibril formation of UC to that of the native structure of RTT. The SEM images showed random formation of large fibrils during/soon after the dialysis in water with a length more than 10 μm and an average diameter of approximately 2.4 μm. These dimensions are much larger than those of natural collagen (<100 nm). However, the arrangement of the large fibrils, examined using AFM, consists of many small fibrils and showed a visual similarity to those of native tissue (Fig. 3). The difference in fibril diameter is possibly attributable to the lack of additional components (bound carbohydrate, glycoproteins or lipid) present in the starting material and subsequently removed by gel filtration [Brodsky, & Eikenberry, 1982; Brodsky, & Persikov, 2005].

4. Outlook

Although collagen has been studied extensively during last decades, the understanding of its structure and biological functions are still limited, in particular due to the low quality of available collagen for highly accurate analysis. Based upon our newly-established urea-extraction procedure, we have not only demonstrated the high potential of UC as a biomatrix for tissue engineering but also showed indicated some new insights into understanding the structure, assembly, and function as well. We first showed a reversible assembly of UC from urea-solubilized α_1 and α_2 polypeptides in urea and water [Xiong, et al., 2009]. We then turned to the interesting question of whether this preparation can be used to design a new stable collagen-like/collagen containing biomatrix for tissue engineering. Throughout this review, we have tried to present some new ideas for the characterization of collagen, using modern highly accurate methods such as mass spectrometry and variety of newly established UV-spectroscopic methods for collagen quality control. We believe that there are still a great deal of information to be obtained concerning the post-translational modification of collagen, and that this is relevant for many commercial applications.

5. Acknowledgment

This work was supported by a fellowship of the Peter und Traudl Engelhorn Foundation to X.X.

6. References

Bailey, J.L., et al., Collagen oligomers modulate physical and biological properties of three-dimensional self-assembled matrices. *Biopolymers*, 2011. 95: 77-93.

Bann, J.G., Bachinger, H.P. and Peyton, D.H., Role of carbohydrate in stabilizing the triple-helix in a model for a deep-sea hydrothermal vent worm collagen. *Biochemistry*, 2003. 42: 4042-4048.

Becker, U. and Timpl, R., Cyanogen bromide peptides of the rabbit collagen a1-chain. *FEBS Lett*, 1972. 27: 85-88.

Berg, R.A. and Prockop, D.J., The thermal transition of a non-hydroxylated form of collagen. Evidence for a role for hydroxyproline in stabilizing the triple-helix of collagen. *Biochem Biophys Res Commun*, 1973. 52: 115-120.

Bornstein, P., The NH(2)-terminal propeptides of fibrillar collagens: highly conserved domains with poorly understood functions. *Matrix Biol*, 2002. 21: 217-226.

Bradford, M.M., A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal Biochem*, 1976. 72: 248-254.

Brodsky, B. and Eikenberry, E.F., Characterization of fibrous forms of collagen. In *Meth Enzymol*, Academic Press, Paris 1982. 82: 127-174.

Brodsky, B. and Persikov, A.V., Molecular structure of the collagen triple helix. *Adv Protein Chem*, 2005. 70: 301-339.

Chandrakasan, G., Torchia, D.A. and Piez, K.A., Preparation of intact monomeric collagen from rat tail tendon and skin and the structure of the nonhelical ends in solution. *J Biol Chem*, 1976. 251: 6062-6067.

Exposito, J.Y., et al., Evolution of collagens. *Anat Rec*, 2002. 268: 302-316.

Eyre, D.R., et al., A novel 3-hydroxyproline (3HYP)-rich motif marks the triple-helical C-terminus of tendon Type I collagen. *J Biol Chem.*, 2011. 286: 7732-7736.

Gelse, K., E. Poschl, and T. Aigner, Collagens--structure, function, and biosynthesis. *Adv Drug Deliv Rev*, 2003. 55: 1531-1546.

Iozzo, R.V., Matrix proteoglycans: from molecular design to cellular function. *Ann Rev Biochem*, 1998. 67: 605-652.

Jamall, I.S., Finelli, V.N. and Que Hee, S.S., A simple method to determine nanogram levels of 4-hydroxyproline in biological tissues. *Anal Biochem*, 1981. 112: 70-75.

Jenkins, C.L., et al., Effect of 3-hydroxyproline residues on collagen stability. *J Am Chem Soc*, 2003. 125: 6422-6427.

Kadler, K.E., Hojima, Y. and Prockop, D.J., Assembly of Type I collagen fibrils de novo. Between 37 and 41 degrees C the process is limited by micro-unfolding of monomers. *J Biol Chem*, 1988. 263: 10517-10523.

Kadler, K.E., et al., Collagen fibril formation. *Biochem J*, 1996. 316: 1-11.

Kadler, K.E., et al., Collagens at a glance. *J Cell Sci*, 2007. 120: 1955-1958.

Khoshnoodi, J., et al., Molecular recognition in the assembly of collagens: Terminal noncollagenous domains are key recognition modules in the formation of triple helical protomers. *J Biol Chem*, 2006. 281: 38117-38121.

Koivu, J., Identification of disulfide bonds in carboxy-terminal propeptides of human type I procollagen. *FEBS Lett*, 1987. 212: 229-232.

Kotch, F.W. and Raines, R.T., Self-assembly of synthetic collagen triple helices. *Proc Natl Acad Sci USA*, 2006. 103: 3028-3033.

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

Leikina, E., et al., Type I collagen is thermally unstable at body temperature. *Proc Natl Acad Sci USA*, 2002. 99: 1314-1318.

Lowry, O.H., et al., Protein measurement with the folin phenol reagent. *J Biol Chem*, 1951. 193: 265-275.

Malone, J.P., Alvares, K. and Veis, A., Structure and assembly of the heterotrimeric and homotrimeric C-propeptides of Type I collagen: Significance of the 2(I) chain. *Biochemistry*, 2005. 44: 15269-15279.

Miller EJ, Gay, S & Leon, WC, The collagens: An overview and update. In *Meth Enzymol*, Academic Press, Paris 1982. 82: 3-32.

Miller, E.J. and Rhodes, R.K., Preparation and characterization of the different types of collagen. In *Meth Enzymol*, Academic Press, Paris 1982. 82: 33-64.

Miller, E.J. and Gay, S., The collagens: an overview and update. In *Meth Enzymol*, Academic Press, Paris 1987. 144: 3-41.

Mizuno, K., et al., Hydroxylation-induced stabilization of the collagen triple helix. Acetyl-(glycyl-4(R)-hydroxyprolyl-4(R)-hydroxyprolyl)(10)-NH(2) forms a highly stable triple helix. *J Biol Chem*, 2004. 279: 38072-38078.

Mohs, A., et al., Mechanism of stabilization of a bacterial collagen triple helix in the absence of hydroxyproline. *J Biol Chem*, 2007. 282: 29757-29765.

Orgel, J.P., et al., The in situ supermolecular structure of Type I collagen. *Structure* (Camb), 2001. 9: 1061-1069.

Paramonov, S.E., Gauba, V. and Hartgerink, J.D., Synthesis of collagen-like peptide polymers by native chemical ligation. *Macromolecules*, 2005. 38: 7555-7561.

Peterson, G.L., A simplification of the protein assay method of Lowry et al. which is more generally applicable. *Anal Biochem*, 1977. 83: 346-356.

Privalov, P. L., Stability of proteins. *Adv Prot Chem*, 1982. 35, 1-104.

Prockop, D.J. and Kivirikko, K.I., Collagens: molecular biology, diseases, and potentials for therapy. *Ann Rev Biochem*, 1995. 64: 403-434.

Rajan, N., et al., Preparation of ready-to-use, storable and reconstituted Type I collagen from rat tail tendon for tissue engineering applications. *Nat Protocols*, 2007. 1: 2753-2758.

Ramachandran et al., A hypothesis on the role of hydroxyproline in stabilizing collagen structure. *Biochim Biophys Acta*, 1973 322: 166-171.

Schweitzer, M.H., et al., Analyses of soft tissue from Tyrannosaurus rex suggest the presence of protein. *Science*, 2007. 316: 277-280.

Schweitzer, M.H., et al., Biomolecular characterization and protein sequences of the Campanian hadrosaur *B. canadensis*. *Science*, 2009. 324: 626-631.

Shormanov, V.K. and Bulatnikov, G.G., Photometric determination of collagen. *J Anal Chem*, 2006. 61: 320-324.

Tsuchiya, S., et al., Collagen type I matrix affects molecular and cellular behavior of purified porcine dental follicle cells. *Cell Tissue Res*, 2008. 331: 447-459.

Weis, M.A., et al., Location of 3-hydroxyproline residues in collagen types I, II, III, and V/XI implies a role in fibril supramolecular assembly. *J Biol Chem*, 2010. 285: 2580-2590.

Wick, G., et al., The diagnostic application of specific antiprocollagen sera. II. Analysis of liver biopsies. *Int Arch Allergy Appl Immunol*, 1978. 56: 316-324.

Xiong, X., et al., A new procedure for rapid, high yield purification of Type I collagen for tissue engineering. *Process Biochem*, 2009. 44: 1200-1212.



Regenerative Medicine and Tissue Engineering - Cells and Biomaterials

Edited by Prof. Daniel Eberli

ISBN 978-953-307-663-8
Hard cover, 588 pages
Publisher InTech
Published online 29, August, 2011
Published in print edition August, 2011

Tissue Engineering may offer new treatment alternatives for organ replacement or repair deteriorated organs. Among the clinical applications of Tissue Engineering are the production of artificial skin for burn patients, tissue engineered trachea, cartilage for knee-replacement procedures, urinary bladder replacement, urethra substitutes and cellular therapies for the treatment of urinary incontinence. The Tissue Engineering approach has major advantages over traditional organ transplantation and circumvents the problem of organ shortage. Tissues reconstructed from readily available biopsy material induce only minimal or no immunogenicity when reimplanted in the patient. This book is aimed at anyone interested in the application of Tissue Engineering in different organ systems. It offers insights into a wide variety of strategies applying the principles of Tissue Engineering to tissue and organ regeneration.

How to reference

In order to correctly reference this scholarly work, feel free to copy and paste the following:

Xin Xiong, Herwig Brunner and Robin Ghosh (2011). Preparation and Characterization Urea-Solubilized Sol-Gel Type I Collagen and Its Possible Use in Applications, Regenerative Medicine and Tissue Engineering - Cells and Biomaterials, Prof. Daniel Eberli (Ed.), ISBN: 978-953-307-663-8, InTech, Available from: http://www.intechopen.com/books/regenerative-medicine-and-tissue-engineering-cells-and-biomaterials/preparation-and-characterization-urea-solubilized-sol-gel-type-i-collagen-and-its-possible-use-in-ap

INTECH
open science | open minds

InTech Europe
University Campus STeP Ri
Slavka Krautzeka 83/A
51000 Rijeka, Croatia
Phone: +385 (51) 770 447
Fax: +385 (51) 686 166
www.intechopen.com

InTech China
Unit 405, Office Block, Hotel Equatorial Shanghai
No.65, Yan An Road (West), Shanghai, 200040, China
中国上海市延安西路65号上海国际贵都大饭店办公楼405单元
Phone: +86-21-62489820
Fax: +86-21-62489821

© 2011 The Author(s). Licensee IntechOpen. This chapter is distributed under the terms of the Creative Commons Attribution-NonCommercial-ShareAlike-3.0 License, which permits use, distribution and reproduction for non-commercial purposes, provided the original is properly cited and derivative works building on this content are distributed under the same license.

IntechOpen

IntechOpen