Harris JR, Lewis RJ.

**The collagen type I segment long spacing (SLS) and fibrillar forms: Formation
by ATP and sulphonated diazo dyes.**

Micron 2016, 86, 36-47

Copyright:

© 2016. This manuscript version is made available under the [CC-BY-NC-ND 4.0 license](#)

DOI link to article:

<http://dx.doi.org/10.1016/j.micron.2016.04.008>

Date deposited:

13/07/2016

Embargo release date:

01 July 2017

This work is licensed under a
[Creative Commons Attribution-NonCommercial-NoDerivatives 4.0 International licence](#)

Manuscript Number: JMIC-D-16-00056R1

Title: The collagen type I segment long spacing (SLS) and fibrillar forms: Formation by ATP and sulphonated diazo dyes

Article Type: Full Length Article

Keywords: Collagen, segment long spacing (SLS) crystallite, fibril, D-banding, diazo dye, sodium tri(poly)phosphate, negative staining

Corresponding Author: Prof. James Robinson Harris,

Corresponding Author's Institution: University of Mainz

First Author: James Robinson Harris

Order of Authors: James Robinson Harris

Abstract: The collagen type I segment long spacing (SLS) crystallite is a well-ordered rod-like molecular aggregate, ~300 nm in length, which is produced in vitro under mildly acidic conditions (pH 2.5 to 3.5) in the presence of 1mM ATP. The formation of the SLS crystallite amplifies the inherent linear structural features of individual collagen heterotrimers, due to the punctate linear distribution and summation of the bulkier amino acid side chains along the length of individual collagen heterotrimers. This can be correlated structurally with the 67 nm D-banded collagen fibril that is found in vivo, and formed in vitro. Although first described many years ago, the range of conditions required for ATP-induced SLS crystallite formation from acid-soluble collagen have not been explored extensively. Consequently, we have addressed biochemical parameters such as the ATP concentration, pH, speed of formation and stability so as to provide a more complete structural understanding of the SLS crystallite. Treatment of collagen type I with 1mM ATP at neutral and higher pH (6.0 to 9.0) also induced the formation of D-banded fibrils. Contrary to previous studies, we have shown that the polysulphonated diazo dyes Direct red (Sirius red) and Evans blue, but not Congo red and Methyl blue, can also induce the formation of SLS-like aggregates of collagen, but under markedly different ionic conditions to those employed in the presence of ATP. Specifically, pre-formed D-banded collagen fibrils, prepared in a higher than the usual physiological NaCl concentration (eg 500 mM NaCl, 20mM Tris-HCl pH7.4 or x3 PBS), readily form SLS aggregates when treated with 0.1mM Direct red and Evans blue, but this did not occur at lower NaCl concentrations. These new data are discussed in relation to the anion (Cl⁻) and polyanion (phosphate and sulphonate) binding by the collagen heterotrimer and their likely role in collagen fibrillogenesis and SLS formation.

11 HackwoodPark
Hexham
Northumberland
NE46 1AX, UK

Ms Ref. No. JMIC-D-16-00058

Professor Filip Braet
Receiving Editor, Micron

Dear Professor Braet,

Professor Richard Lewis and I have revised our collagen manuscript for Micron, taking fully into account the comments of the two reviewers. We would like to thank the reviewers for their constructive comments.

Accordingly, we have made changes and hope that you may now find our manuscript to be acceptable for inclusion in Micron.

Best regards,

Robin Harris

Prof. J R. Harris

rharris@uni-mainz.de

UK Tel 0044 (0)1434 606981

Our Response

Reviewer#1:

1) We have included an opening paragraph to the Introduction, in which we cover the relevant background on collagen. To address all the points raised in a more thorough manner we consider beyond the scope of the present manuscript. It should also be borne in mind that the original version of this work (Ms. Ref. No.: JMIC-D-13-00097) did include a considerable amount of data relating to collagen fibrils, which was removed at the request of a Reviewer in order to concentrate upon the more significant SLS data. In view of the

widespread understanding of the D-banded collagen fibril and multi-fibril fibre, a schematic fibril diagram has not been included (one was removed from the previous ms). Indeed, the present ms is not directed towards the non-expert, similar to most research papers.

2) Figure 1d. We have included arrows to indicate the fibril D-banding misalignment within the collagen tectites.

Reviewer #2:

1) Arrows have been inserted as appropriate in Figures 1-4.

2) A scale bar has been inserted in Figure 10.

3) A statement has been included in the Discussion relating to the likely value of a detailed quantitative densitometric analysis, which for technical and other reasons cannot currently be performed, and would be best performed on currently unavailable unstained cryo-EM data,

4) SLS Dimensions: The figure of ~300 nm for the SLS length is widely established. Figures given for the varying SLS width during assembly/dissociation under the different conditions presented in Figs. 5, 7 and 8 are given as approximate numbers, to indicate the lack of a statistical average. Additions have been included within the legends to cover this point. In all figures scale bars are present from which SLS variation can be appreciated. It was not the intention of this study to provide quantitative information as to the SLS width variation, rather to address the formation conditions.

The collagen type I segment long spacing (SLS) and fibrillar forms: Formation by ATP and sulphonated diazo dyes

J. Robin Harris^{a*}, Richard J. Lewis^b

^aInstitute of Zoology, University of Mainz, 55099 Mainz, Germany

^bInstitute for Cell and Molecular Biosciences, University of Newcastle, Newcastle-upon-Tyne, NE2 4HH, UK.

*Corresponding author. Tel.: +44 (0)1434 606981

Email address: rharris@uni-mainz.de (J.R. Harris)

Keywords:

Collagen, segment long spacing (SLS) crystallite, fibril, D-banding, diazo dye, sodium tri(poly)phosphate, negative staining

Highlights

Under varying biochemical conditions collagen type I will form Segment Long Spacing (SLS) crystallites and other fibrillar states in the presence of ATP and sulphonated diazo dyes. Negatively stained TEM images of the various assembly products are presented.

Response to reviewers

We would like to thank the reviewers for their constructive comments.

Our Response

Reviewer#1:

1) We have included an opening paragraph to the Introduction, in which we cover the relevant background on collagen. To address all the points raised in a more thorough manner we consider beyond the scope of the present manuscript. It should also be borne in mind that the original version of this work (Ms. Ref. No.: JMIC-D-13-00097) did include a considerable amount of data relating to collagen fibrils, which was removed at the request of a Reviewer in order to concentrate upon the more significant SLS data. In view of the widespread understanding of the D-banded collagen fibril and multi-fibril fibre, a schematic fibril diagram has not been included (one was removed from the previous ms). Indeed, the present ms is not directed towards the non-expert, similar to most research papers.

2) Figure 1d. We have included arrows to indicate the fibril D-banding misalignment within the collagen tectites.

Reviewer #2:

1) Arrows have been inserted as appropriate in Figures 1-4.

2) A scale bar has been inserted in Figure 10.

3) A statement has been included in the Discussion relating to the likely value of a detailed quantitative densitometric analysis, which for technical and other reasons cannot currently be performed, and would be best performed on currently unavailable unstained cryo-EM data,

4) SLS Dimensions: The figure of ~300 nm for the SLS length is widely established. Figures given for the varying SLS width during assembly/dissociation under the different conditions presented in Figs. 5, 7 and 8 are given as approximate numbers, to indicate the lack of a statistical average. Additions have been included within the legends to cover this point. In all figures scale bars are present from which SLS variation can be appreciated. It is not the intention of this study to provide quantitative information as to the SLS width variation, rather to address the formation conditions.

The collagen type I segment long spacing (SLS) and fibrillar forms: Formation by ATP and sulphonated diazo dyes

J. Robin Harris^{a*}, Richard J. Lewis^b

^aInstitute of Zoology, University of Mainz, 55099 Mainz, Germany

^bInstitute for Cell and Molecular Biosciences, University of Newcastle, Newcastle-upon-Tyne,
NE2 4HH, UK.

*Corresponding author. Tel.: +44 (0)1434 606981

Email address: rharris@uni-mainz.de (J.R. Harris)

Keywords:

Collagen, segment long spacing (SLS) crystallite, fibril, D-banding, diazo dye, sodium tri(poly)phosphate, negative staining

Abstract

The collagen type I segment long spacing (SLS) crystallite is a well-ordered rod-like molecular aggregate, ~300 nm in length, which is produced *in vitro* under mildly acidic conditions (pH 2.5 to 3.5) in the presence of 1mM ATP. The formation of the SLS crystallite amplifies the inherent linear structural features of individual collagen heterotrimers, due to the punctate linear distribution and summation of the bulkier amino acid side chains along the length of individual collagen heterotrimers. This can be correlated structurally with the 67 nm D-banded collagen fibril that is found *in vivo*, and formed *in vitro*. Although first described many years ago, the range of conditions required for ATP-induced SLS crystallite formation from acid-soluble collagen have not been explored extensively. Consequently, we have addressed biochemical parameters such as the ATP concentration, pH, speed of formation and stability so as to provide a more complete structural understanding of the SLS crystallite. Treatment of collagen type I with 1mM ATP at neutral and higher pH (6.0 to 9.0) also induced the formation of D-banded fibrils. Contrary to previous studies, we have shown that the polysulphonated diazo dyes Direct red (Sirius red) and Evans blue, but not Congo red and Methyl blue, can also induce the formation of SLS-like aggregates of collagen, but under markedly different ionic conditions to those employed in the presence of ATP. Specifically, pre-formed D-banded collagen fibrils, prepared in a higher than the usual physiological NaCl concentration (*eg* 500 mM NaCl, 20mM Tris-HCl pH7.4 or x3 PBS), readily form SLS aggregates when treated with 0.1mM Direct red and Evans blue, but this did not occur at lower NaCl concentrations. These new data are discussed in relation to the anion (Cl⁻) and polyanion (phosphate and sulphonate) binding by the collagen heterotrimer and their likely role in collagen fibrillogenesis and SLS formation.

1. Introduction

Fibrous collagen is the prominent protein in connective tissues of all animals, present even in the sponges. Collagen fibres have been studied extensively by transmission electron microscopy since the 1940s, with the linear D-banding structural pattern emerging rapidly from unstained collagen samples as well as from metal shadowing and thin sectioning data. The periodic cross-striations of collagen fibres were indicative of a repeating linear molecular *quasi* crystalline organization. At the molecular level, type I collagen fibrils are constructed from the collagen heterotrimer, containing three left-handed proline-rich polypeptide chains twisted together as a right-handed trimer helix. The *in vitro* assembly of acid-solubilized collagen heterotrimers into fibres under defined biochemical conditions was established by Schmitt et al. (1942) and others, along with another non-fibrillar collagen assembly termed the segment long spacing (SLS) crystallite (Schmitt et al., 1953). Our present study places further biochemical and transmission electron microscopical emphasis upon this collagen SLS crystallite, its structure and formation.

The collagen type I SLS crystallite is a structurally-defined, experimentally-induced macromolecular assembly of collagen heterotrimers, with a length of ~300 nm. SLS crystallites, induced *in vitro* under mildly acidic conditions (pH ~3.0) by the addition of ATP, have been observed for collagen type I from several species and from different types of collagen since their first observation in the 1950s (*eg* Schmitt et al., 1953; Gross et al., 1954; Silver and Trelstad, 1980; Tillet-Barret et al., 1992). SLS crystallites are a rod-like bundle or aggregate of perfectly aligned collagen heterotrimers (all with the same N-/C-terminus polarity) thus possessing a markedly different molecular packing to that found in native and *in vitro*-produced D-banded collagen fibrils, and their thicker multi-fibril fibres (Harris and Reiber, 2007; Harris et al., 2013; Starborg et al., 2008). Though SLS crystallites do tend to aggregate, there is no evidence that they are able to form larger structures with truly crystalline order and consequently the use of the term *crystallite* is somewhat controversial. The wealth of early SLS data was reviewed by Serafini-Fracassini (1982) and Kühn (1982), who considered SLS crystallites to be a “powerful tool in collagen research” and emphasized the fact that they possess information on the length of the collagen heterotrimer and longitudinal features of its primary and secondary structure.

Most early electron microscopical studies on collagen SLS crystallites were performed using metal shadowing (Gross et al., 1954; Schmitt et al., 1953) and positive staining with uranyl acetate and phosphotungstic acid (Hodge and Schmitt, 1960; Bruns and Gross, 1970). The available SLS negative stain data, from both cationic uranyl acetate and anionic ammonium molybdate staining, is in general agreement with that from positive staining. These specimen preparation techniques have been applied to both the ~67 nm D-banded collagen fibril and the multiple-banded single ~300 nm SLS crystallite, establishing the presence of a lengthwise asymmetric pattern of protein bands, generally attributed to the punctate linear distribution and mass summation within the thickness of the crystallite, generated by the bulkier amino acid side chains along the length of individual collagen heterotrimers (Bender et al., 1982; Bruns and

Gross, 1973; Kobayashi et al., 1992). However, the additional mass contribution from bound ATP (a polyphosphate) was not considered, an aspect that could be addressed in the future by cryo-electron microscopy of unstained collagen specimens. In recent years atomic force microscopy (AFM) has been used (Fujita et al., 1997) to provide a model for the oligomeric substructure of the SLS crystallite (Paige and Goh, 2001). Of continuing significance is the relevance of the SLS crystallite to present day molecular models for the ~67 nm D-banded collagen fibril, where a well-defined linear collagen heterotrimer partial overlap (0.4D) and spacing (0.6D) is thought to be responsible for the organized/paracrystalline structure of the *in vivo* native and *in vitro* experimentally-formed collagen fibril.

Published data on ATP-induced collagen SLS crystallites is limited with respect to the concentration of ATP required for their creation, the speed of formation, the pH range over which this occurs and the influence of temperature. Furthermore, other nucleoside triphosphates, inorganic sodium tri(poly)phosphate, fructose 1,6-diphosphate and perdisulphuric acid can also produce SLS crystallites (Bowden et al., 1968), but the detailed influence of these reagents on SLS formation has yet to be studied in detail. Other polysulphates such as heparin, glycosaminoglycan and pentosan also remain to be studied. Thus, a number of unresolved questions remain, particularly in relation to SLS formation under varying biochemical conditions in the presence of ATP, their aggregation and dissociation, aspects that are addressed in the present work.

The influence of several sulphonated diazo dyes, known to be histochemical stains for collagen, on the formation of SLS-like structures from soluble collagen type I has also been investigated in the present study. Although Paige and Goh (2001) did not observe SLS-like structures following the interaction of the polyanionic dyes Cibacron blue and Amaranth with soluble collagen (under mildly acidic conditions), we have found that Evans blue and Direct red 80 (Sirius red) (containing four and six sulphonic acid groups, respectively) create thin sheet-like SLS bundles from pre-formed D-banded collagen fibrils, and directly from acid-soluble collagen heterotrimers, but only at neutral pH and in the presence of higher than usual NaCl concentration. These dye-induced SLS structures contain thin sheet-like aggregates of narrow collagen SLS rods, compared to the thicker discrete rod-like SLS crystallites induced by ATP. On the other hand, disulphonated Congo red and trisulphonated Methyl blue, known to interact with the crossed beta sheets present in amyloid fibres and with collagen fibres, do not induce collagen SLS formation. Therefore, the chemical structure and varying level of sulphonation of these synthetic diazo dyes is likely to be of significance for their interaction with collagen, as are the pH and ionic conditions employed. As for collagen fibril formation in saline solutions, the polyphosphate and polysulphonate interaction is thought to be mediated ionically through collagen amine side chains, predominantly from lysine (Tenni et al., 2006).

The biochemical approaches presented here, supported structurally by TEM analysis, extends the collagen interaction database and is of likely future use in the increasingly broad application of collagen within present day biotechnology.

2. Materials and Methods

2.1. Reagents

Purified acid-soluble rat tail collagen type I solution was purchased from SERVA (Collagen R Solution, Cat. No.47254), 2 mg/ml in 0.1% v/v acetic acid without pepsin digestion (this solution contains collagen heterotrimers with telopeptide N- and C-termini present). All other reagents of analytical and reagent grade were purchased from Sigma-Aldrich. Stock solutions of 100mM sodium ATP and sodium tri(poly)phosphate were stored as frozen aliquots, with subsequent dilution to the desired lower concentration for incubation with collagen type I heterotrimer. 100 mM stock solutions of the sulphonated diazo dyes (Congo red, Direct red 80/Sirius red, Evans blue and Methyl blue) were stored at room temperature, for direct dilution to the 0.1mM concentration employed during the collagen incubations. A 1.0 M stock solution of Tris-HCL (pH 7.4 and pH 8.0) and 100 mM Glycine-HCl (pH 2.5) was used to adjust the pH of the ATP, and 100 mM to 500 mM NaCl solutions. Narrow range pH paper (Whatman) was used to determine the pH of all collagen incubation solutions, by taking 5 μ l aliquots. A 1.0 M stock solution of NaCl was diluted as required in the buffered collagen solutions. x1 PBS and x3 PBS solutions were prepared from PBS tablets (Oxoid).

2.2. Treatment of collagen type I with ATP and sodium tri(poly)phosphate

All collagen incubations were performed at 22 °C for varying times, from 30 sec. to 24 h. as 1.0 ml volumes in polypropylene microcentrifuge tubes. Samples of collagen solution were mixed with sodium ATP and sodium tri(poly)phosphate (0.1 to 10 mM). The acetic acid-soluble collagen stock solution was diluted in the incubation solution to 0.02 or 0.04 mg/ml, followed by addition of selected volumes of ATP solution and thoroughly mixed by repeated inversion. Adjustment of the pH of collagen + ATP mixtures was made by rapid sequential addition of microlitre quantities of 1M Tris-HCl pH 8.0 or 100 mM Glycine-HCl pH 2.5, and checked using narrow range pH paper.

2.3 Treatment of collagen type I fibrils with sulphonated diazo dyes

For the diazo dye treatments, 1ml volumes of pre-formed D-banded collagen type I fibrils prepared in 0.2 M and 0.5 M NaCl, buffered with 20 mM Tris.HCl pH 7.5, or 20 mM sodium phosphate pH 7.5 (Harris et al., 2013) were treated with a 0.1 mM concentration of each dye for 24 h at 22°C, yielding SLS structures. Collagen fibrils prepared in the presence of x3 PBS were also utilized for the dye interactions, yielding essentially the same data as Tris-buffered 500 mM NaCl. Lower NaCl concentrations (100 and 200 mM) did not produce SLS structures.

2.4. Preparation of negatively stained EM specimens

20 µl aliquots of the different collagen solutions were taken following incubation and negatively stained specimens were prepared on glow-discharge treated continuous carbon support films, using the Parafilm droplet procedure (Harris, 1997). Collagen samples were adsorbed to the carbon film for 30 sec, washed rapidly up to four times with distilled water droplets (the number of wash steps depended upon the salt or buffer concentration in the sample), negatively stained with 2% w/v uranyl acetate solution (pH 4.5) or 5% w/v ammonium molybdate, 0.1% w/v trehalose (pH 7.0), and finally air-dried at room temperature.

2.5. Transmission electron microscopy

TEM studies were performed at ambient temperature using a Philips CM100, at 100kV, without using low electron dose. Digital images (8 bit) were recorded primarily at direct magnifications up to x 130,000, using an Optronics 1824x1824 pixel CCD camera with an AMT40 version 5.42 image capture engine, supplied by Deben UK. Image compilation for publication was performed in Adobe Photoshop.

3. Results

3.1. Formation of Collagen SLS Crystallites by ATP

3.1.1. pH dependency of SLS crystallite formation

From a survey of the available literature on collagen SLS crystallites it is clear that most previous studies used an ATP concentration in the range 0.2 % to 1.0 % w/v (equivalent to 0.36 to 1.8 mM) in a pH range of 2.5 to 3.5, usually in dilute acetic acid. We have extended the investigative pH range in the present study to pH 5.0 and pH 7.0, using 24 h, 22 °C incubation conditions with 0.02 mg/ml rat tail collagen type I (SERVA Collagen R). In the presence of 1 mM ATP characteristic clearly-banded SLS crystallites form (Fig. 1a,b), which have some tendency to cluster (see below). On the other hand, no SLS crystallites form at pH 5.0. Instead, diffusely D-banded ~25 nm diameter fibrils and multi-fibril fibres form (Fig. 1c), which bear a close resemblance to poorly formed collagen fibrils in sub-physiological concentrations of NaCl (Harris et al., 2013). At pH 7.0, in the presence of 1 mM ATP, D-banded fibril formation occurs with the production of broad spindle-shaped, tactoid multi-fibril collagen fibres that exhibit an oblique D-banding (Fig.1d). These observations emphasize the importance of maintaining mildly acidic conditions for the production of SLS crystallites, and links collagen-ATP interactions with the initial molecular aggregate, fibril and fibre formation by collagen in neutral pH saline and buffer solutions (Harris et al., 2013). Of considerable significance is that a low concentration of ATP (1 mM) at pH 7 is able to produce characteristic D-banded collagen fibrils, requiring a 100mM or greater concentration of neutral pH NaCl or Tris-HCl.

3.1.2. ATP concentration-dependency of SLS crystallite formation

At different concentrations the ATP-dependent association of collagen type I into SLS crystallites can be varied in a controlled manner when collagen is incubated for 24 h at 22°C

under mildly acidic conditions (pH 2.5). In 0.1 and 0.2 mM ATP, the collagen molecules start to associate as fine fibrils (Fig. 2a,b), with initial indication of linear punctation and banding (Fig. 2b). In 0.5 mM ATP this fibrillar banding becomes more pronounced (Fig. 2c) alongside the production of disordered SLS crystallites (Fig. 2d). In these low ATP concentrations not all the available collagen molecules are incorporated into the nascent SLS crystallites; by contrast, the clean background alongside the SLS crystallites in 1.0 mM ATP (Fig. 2e) indicates complete incorporation of all the available collagen. When the ATP concentration is further increased to 5.0 and 10.0 mM, it appears that production of thin SLS crystallites leads to their immediate association into disordered fibres (Fig. 2f). Because of lateral misalignment, there is no clear indication of the underlying SLS banding pattern or the creation of D-banding, yet the formation of collagen fibrils at this higher concentration of mildly acidic ATP has not been reported previously.

3.1.3. Speed of formation of ATP-induced SLS crystallites

Initially, all collagen SLS studies were routinely performed using a 24 h incubation time, after which time some SLS aggregation was often observed. However, the interaction of ATP with collagen is likely to take place over a much quicker timescale than 24 hours and, therefore, the kinetics of SLS crystallite formation was investigated. Figure 3a-d shows a limited time sequence of the interaction of 1.0 mM ATP with collagen type I (at pH 2.5), from 30 sec to 10 min. Narrow, diffusely-banded SLS form progressively throughout the shorter time periods (Fig. 3a-c) with pre-SLS aggregates present in the background. At the end of each time course (Fig. 3d), fully-formed ATP crystallites are present, with little indication of free collagen heterotrimers or pre-SLS aggregates on the background. It can, therefore, be concluded that collagen rapidly associates into SLS crystallites in the presence of 1.0 mM ATP. The strongly anionic triphosphate moiety of ATP is likely to produce rapid cross-linking between basic amino acid side chains of the collagen heterotrimer, creating the ordered and rigid alignment of the initially flexible collagen heterotrimers within the rod-like SLS crystallite.

3.1.4. SLS crystallite stability

The reversibility of the ATP-collagen interaction that stabilizes the heterotrimer within the SLS crystallites became apparent when preparing EM specimens, specifically when additional water washing was applied to the on-grid carbon-adsorbed SLS crystallites prior to negative staining. Cationic uranyl acetate (pH 4.5) has been shown to stabilize molecular structure (Zhao and Craig, 2003), but this is not the case with the anionic negative stain, ammonium molybdate (pH 6.9), that in principle could compete with ATP at the collagen cationic binding sites. Routinely, two on-grid water washes were applied to SLS preparations prior to negative staining with uranyl acetate, to remove excess ATP prior to the addition of the negative stain. When this was extended to four washes, it was found that swollen and unbanded SLS crystallites were present when negatively stained with uranyl acetate (Fig. 4a,b), indicating that the ATP can be leached from of the SLS crystallites, leading to instability. A similar, but more pronounced, situation was

encountered when ammonium molybdate was used at neutral pH for negative staining (Fig. 4c,d). Here, dispersal of the SLS crystallites during specimen preparation can be seen to release groups of collagen heterotrimers, with complete loss of banding and loss of rigidity. Prior stabilization of the SLS crystallites with 0.1% glutaraldehyde prevented this disruption (data not shown). This SLS instability indicates that considerable caution needs to be observed when water-washing some protein aggregates during the preparation of negatively stained specimens, but at the same time useful structural data of a biochemically informative nature may emerge.

3.1.5. SLS linkage and linear aggregation

When SLS crystallites aggregate as clusters, it is often the case that end-to-end overlapping of SLS rods also occurs. With increasing image magnification, it is clear that a ~27 nm overlap occurs at both ends of the SLS crystallites, highlighted by arrows in Fig. 5a,b,c, negatively stained with uranyl acetate. Thus, an ATP-induced interaction between the N- and C-terminal telopeptide regions of the SLS crystallite rods occurs, as well as a more generalized SLS aggregation. Although this 27 nm SLS overlap has been observed by others (*see* Discussion), this observation has not been fully accommodated within the currently accepted collagen heterotrimer overlap and spacing model for the D-banded fibril or the fibrous long spacing (FLS) fibrillar form of collagen. As with the single SLS crystallite, negative staining with ammonium molybdate exhibits dispersal of the heterotrimers within the linear SLS aggregates (Fig. 5d).

3.2. SLS Formation by other polyanionic reagents

3.2.1. Collagen treated with sodium tri(poly)phosphate

Nucleoside triphosphates other than ATP have been utilized previously for SLS creation, as has fructose-1,6-diphosphate (Bowden et al., 1968; Paige and Goh, 2001). Sodium tri(poly)phosphate (NaTP) has also been used, amongst other inorganic polyphosphates, under acidic conditions. As with our ATP study, we have investigated the varying conditions under which NaTP can generate collagen SLS crystallites. For practical reasons, this study has so far been performed in a more limited manner, with increasing concentrations of NaTP at pH 2.5. Increasing concentrations of NaTP (0.5 and 1.0 mM) led to the progressive formation of SLS crystallites from narrower, poorly banded intermediates (Fig. 6a,b); at 2.0 mM and 10 mM, NaTP led to clearer banding and linear SLS aggregation (Fig. 6c,d). It is not unreasonable to suggest that incubation time and pH will also influence SLS formation by NaTP, in a similar manner to ATP.

3.2.2. Collagen fibrils treated with polysulphonated diazo dyes

The histochemical staining of fibrillar collagen *in situ* by several polysulphonated diazo dyes has been known for many years. The specificity of staining varies; some of the dyes also stain the

range of crossed beta sheet-containing amyloid fibres. Consequently, fully formed D-banded collagen fibres prepared in 500 mM NaCl at pH 7.0 were treated in solution for 24 h with 0.1 mM Direct red 80/Sirius red, Evans blue, Congo red and Methyl blue, to investigate this interaction. The D-banded collagen multi-fibril fibres were disrupted in all cases. With Sirius red and Evans blue, thin, sheet-like 300 nm SLS structures, containing several precisely aligned ~25 nm diameter SLS rods were created (Figs 7 and 8), with a banding pattern closely resembling that of the rod-like SLS produced by ATP and NaTP. Similar data was obtained when collagen fibrils were prepared in x3 PBS and then treated with Direct red 80 and Evans blue. Also, the same SLS formation was observed when direct addition of acid-soluble collagen to 0.1 mM diazo dye in buffered 500 mM NaCl, but not lower NaCl concentrations (data not shown). D-banded collagen fibrils prepared in the presence of 100 or 200 mM NaCl or x1 PBS did not yield sheet-like SLS structures (Fig. 8b). With the diazo dyes Congo red and Methyl blue the situation is different, in that pre-formed D-banded collagen fibres prepared in 500mM NaCl, 20mM Tris-HCl pH 7.5 were dissociated into single fibrils, but were not transformed into SLS structures. The dispersed individual collagen fibrils remain intact after Congo red treatment (Fig. 9), but with clear indication that the dye is binding to the fibrils, as predicted from the histochemical properties of this dye (Fig. 9d). Corresponding results were obtained with Methyl blue (data not shown).

A significant difference between the diazo dye-induced collagen SLS structures, when compared to the ATP- and NaTP-induced rod-like SLS crystallites, is that a number of thinner ~25nm SLS banded rods align perfectly side-by-side to produce broader sheet-like SLS structures, whereas the ATP-induced SLS rod-like crystallites (300 nm in length) have a diameter of ~100nm; these latter do not aggregate laterally, although they do in a longitudinal manner (Fig. 5). The collagen-bound diazo dyes, as for the polyphosphates, contribute to the overall summated SLS mass at specific locations along the heterotrimer and therefore to the banding pattern within the negatively stained images, each contributing individual properties. A direct comparison of the banding pattern of SLS crystallite images, produced by ATP, NaTP and Direct red 80, is given in Fig. 10. In contrast to ATP and NaTP, direct addition of the diazo dyes to acid-soluble collagen solution at pH 2.5 produced a pigmented protein flocculate containing a disordered network of flexuous filaments, with no indication of SLS-like banding (Figs 7a, 8a and 9a), as shown by Paige and Goh (2001) for the diazo dyes Cibacron blue and Amaranth.

4. Discussion

Collagen SLS-like structures have only rarely been detected in normal and pathological tissues. Without ATP addition, procollagen SLS-like aggregates have been isolated from human skin fibroblasts (Goldberg 1974) and chicken embryo tendon fibroblasts (Bruns et al., 1979; Hulmes et al., 1983). Akiya et al. (1984) detected SLS aggregates in the developing rabbit vitreous humor, and Eyden and Tzaphlidou (2001) surveyed the range of diseases where SLS crystallites

have been detected. Pathological tissue inflammation, which could result in an increased extracellular acidity and ATP concentration, may have implications for collagen SLS formation *in vivo*.

Consequently, the study of experimentally-induced collagen SLS crystallites *in vitro* impacts directly on physiologically- and pathologically-relevant structural biology of collagen type I and the other fibre-forming members of the collagen superfamily. Despite the substantial amount of older literature on ATP-induced collagen SLS crystallites, the present work was undertaken to address a number of areas where limited standardized biochemical variation had been used in the previous studies on SLS formation. In particular, variation of pH and concentration of the ATP during SLS formation, the speed of formation, SLS stability and aggregation state had not been adequately addressed. In addition to our experiments with ATP, studies were also undertaken on the induction of SLS by sodium tri(poly)phosphate under varying conditions, yielding similar data. A number of sulphonated diazo dyes, known to bind to fibrillar collagen, were investigated as such dyes had previously been stated to be ineffective in relation to SLS formation (Paige and Goh, 2001).

Our data presented above shows clearly that when acid-soluble collagen type I is treated with 1 mM ATP at higher pHs than had been used previously for SLS formation (~pH 2.5 to 3.5), the product of collagen self-association is markedly different (Fig. 1c,d). Though chains of SLS crystallites were described at pH 5.0 (Hodge and Schmitt 1960), we have found that poorly formed fibrils, rather than SLS crystallites were created at this pH. Furthermore, the fibrils at pH 7.0 closely resemble the D-banded fibrillar tactites, shown by others at neutral pH in the presence chloride ions (Fig. 1d; *see also* Harris and Reiber, 2007). The data presented herein indicates that the interaction of collagen at neutral pH with 1 mM ATP is approximately equivalent to a 100 fold greater concentration of NaCl, implying that the polyphosphate group has a stronger interaction with and ability to cross-link basic amino acids in collagen than the chloride anion. At low pH, collagen (pI ~9) carries maximal net positive charge and will therefore be more reactive towards the polyphosphate group of ATP. This charge complementarity explains the ability of ATP to bind and efficiently crosslink individual collagen heterotrimers to create the precise heterotrimer orientation within the single SLS crystallite, which we have shown to occur rapidly (Fig. 3). At neutral pH the positive charge distribution on the collagen molecule will be reduced, thereby facilitating a different collagen cross-linking by ATP resulting in D-banded fibril production (Fig. 1c,d), not unlike higher concentrations of neutral pH NaCl.

The importance of utilizing an optimal ATP concentration for the creation of SLS crystallites has also been demonstrated in the present study. The progressive aggregation of collagen heterotrimers with increasing concentration of ATP from 0.1 mM to 1.0 mM (Fig. 2), with the gradual enhancement of the linear, punctated nature of the micro-fibril pre-SLS products, correlates well with the banding pattern of the SLS crystallite, which are more clearly defined in 0.5 mM ATP (Fig. 2d). It is also significant that the formation of SLS crystallites is

compromised at higher ATP concentrations (10 mM), with a fibril-like product predominating, apparently formed by the linear disordered aggregation of thin SLS crystallites (Fig. 2f).

That SLS crystallites exhibit instability, due to the leaching out/loss of bound ATP, was found when repeated on-grid water washing was applied prior to negative staining with uranyl acetate. Similar SLS disruption was shown by Schmitt et al. (1953), when SLS crystallites were washed with dilute acetic acid. SLS splaying also occurs readily when water-washed SLS crystallites are negatively stained with ammonium molybdate, which does not have the known protein stabilizing property of uranyl acetate (Zhao and Craig, 2003). The instability of SLS crystallites was also observed by Kobayashi et al. (1990) and Olsen (1967) using ammonium molybdate, which is known to have phosphate-binding properties, and low pH ammonium molybdate also dissociates keyhole limpet hemocyanin type 2 (Harris et al., 1995). The splaying out of collagen molecules from preformed SLS crystallites (Fig. 4), together with the data from increasing ATP concentrations, is in good agreement with an oligomeric collagen pre-SLS rod-like assembly or intermediate that aggregates further laterally to form the SLS crystallite, first proposed by Paige and Goh (2001) from AFM data.

That collagen SLS crystallites have a pronounced tendency to aggregate has been well-documented (Hirota et al., 2003; Kobayashi et al., 1985; Olsen, 1967; Schmitt et al., 1953; Silver and Trelstad, 1980). Whilst this aggregation does not create crystalline higher order structures, a reproducible feature has been the SLS terminal end-to-end overlap of ~27nm (Fig. 5). This tendency supports the widely accepted molecular overlap/gap model for the ~67 nm D-banded collagen fibril, within which the collagen heterotrimers associate in a linear manner with a ~27 nm overlap and a ~40 nm gap. D-banded collagen fibrils are thus created with periodic regions of greater net protein thickness (the overlap) and thinner gap regions within which negative stain can permeate. For the D-banded collagen fibril, the complex summated linear variation in overall amino acid heterotrimer bulkiness contributes to and creates the characteristic banding seen by TEM of negatively stained samples (Harris et al., 2013). With the SLS crystallite, the further mass contribution of bound polyphosphate or polysulphonated diazo dye has also to be taken into consideration when assessing the SLS banding pattern.

Apart from ATP, many other compounds can induce the formation collagen SLS crystallites, but in most cases detailed studies have not been performed. Sodium tri(poly)phosphate (NaTP) effectively formed SLS crystallites at pH ~3.5, but fructose 1,6-diphosphate and perdisulphuric acid were less effective (Bowden et al. 1968). Our data shows that NaTP produced slightly different SLS crystallites to those produced by ATP; the NaTP-induced crystallites were thinner than those formed by ATP and the banding pattern for the NaTP-induced crystallites was also less pronounced than that of their ATP counterparts. As with ATP, higher concentrations of NaTP produced fibrillar SLS aggregates (Fig. 6c,d). SLS formation has also been induced by a range of other substances, including nucleic acids, chondroitin sulphate and heparin (Schmitt et al., 1953). It is a characteristic feature for all these compounds is that they contain reactive anionic polyphosphate or polysulphate groups. Anion interactions with collagen have been

assessed by a number of groups (Komosa-Penkova et al., 1996; Mertz and Leikin, 2004; Weinstock et al., 1967), with the conclusion that sulphate and phosphate are the most reactive, in accordance with the Hofmeister series (Zhang and Cremer, 2006). The polyphosphates, polysulphates and polysulphonates are chemically more reactive than the single anions. There are no data that we are aware of on the interaction of soluble collagen with polysulphate compounds, such as pentosanpolysulphate, although some effort has been directed towards the interaction of fibrillar collagen with heparin sulfate and glycosaminoglycan polysulphate (Moraes et al., 2009).

Despite sharing a polysulphonate moiety, the dyes Amaranth and Cibacron blue do not interact with soluble collagen to create SLS crystallites at low pH, forming instead a fibrous collagen aggregate/precipitate (Paige and Goh 2001), an observation we have confirmed with four other diazo dyes. To analyze the diazo dyes further, we followed the interaction of Direct red 80/Sirius red and several other diazo dyes with preformed collagen fibrils at pH 7.0 in 500 mM NaCl solution and with acetic acid-soluble collagen type I added directly to 500 mM NaCl pH 7.0 solution. Direct red, with six sulphonate groups, has found widespread utility as a histochemical stain in the quantification of tissue collagen (Chen et al., 2013; Dapson et al., 2011; Lareu et al., 2010). Congo red carries two sulphonate groups, whereas Evans blue carries four; Amaranth and Cibacron blue both have three sulphonate groups. The negatively stained images of the dye-induced SLS structures, whilst closely resembling those of the ATP-induced SLS crystallites, are significantly different (Fig. 10). We propose that the addition of varying molecular mass to the collagen heterotrimer, imparted by basic amino acid residue-specific binding of polyanionic ATP, NaTP or sulphonated diazo dye, leads to this subtle image variation. A precise densitometric analysis of these structures would be desirable, ideally from unstained cryo-EM specimens, but was beyond the scope of the present study. With Congo red and Methyl blue, the D-banded collagen fibres were disrupted by the two dyes into single fibrils with particulate linear distribution of bound dye, but no SLS structures were formed. Sheet-like, ATP-induced collagen SLS structures, similar to those produced by Direct red 80 and Evans blue, have been observed previously following pepsin treatment (Schwartz and Veis, 1978) and in short chain cartilage collagen (Schmid et al., 1984), likely because of altered ATP binding resulting from removal of the N- and C-terminal telopeptides.

Because of the highly organized *quasi* crystalline packing of collagen heterotrimers within the ~67 nm D-banded collagen fibril (Ortolani et al., 2000; Holmes et al., 2001), interpretation of electron micrographs from negatively and positively stained images is inherently complex. The precisely overlapped and spaced heterotrimers along the length of the collagen fibril generates the D-banded image due to the varying vertical summation of protein thickness along the fibril length within the transmission electron image. Images of the single SLS crystallite are simpler to interpret because of the precisely orientated side-by-side alignment of collagen heterotrimers. The banding observed in the SLS negatively stained transmission electron micrographs is determined only by the summated thickness and location of superimposed “globular” protein sub-domains along the 300 nm length of perfectly aligned heterotrimers. This model is consistent

with the single collagen heterotrimer structure, where the mass of the larger, positively charged amino acid side chains of the three individual molecules summate as linear periodic bulkier “globular” sub-domains, spaced by thin rod-like helical domains within the heterotrimer (van der Rest and Garrone, 1990; Brown, 2012; Harris et al., 2013). This heterotrimer arrangement is repeated and summated within the SLS crystallite, resulting in the observed SLS banding (Bender et al., 1982; Silver et al., 2003; Kobayashi et al., 1988; Kobayashi et al., 1992; von der Mark et al., 1970) and *cf* Ortolani and Marchini (1993) for collagen fibrils.

The data presented here, together with the numerous previously documented attempts to understand the arrangement of collagen molecules within the ~300 nm SLS crystallite and correlate this with the molecular/heterotrimer arrangement within ~67 nm D-banded collagen fibrils, indicates the continuing relevance of these collagen SLS multimeric forms for the structural biology of this fibrous protein. The fact that Congo red and Thioflavin T (Morimoto et al., 2009) and other diazo dyes bind to collagen (Tuttolomondo et al., 2015) and to a broad range of amyloid fibrils, indicates that future TEM studies on dye binding in relation to amyloid protein oligomerization and fibrillogenesis are worthy of investigation. Moreover, in the future comparative cryo-electron microscopy of unstained D-banded collagen fibrils and SLS crystallites formed in the presence of different reagents could also yield informative data.

References

- Akiya, S., Uemura, Y., Fujiwara, T., 1984. Electron microscopic observation on the developing rabbit and human vitreous collagen fibrils by negative staining. *Ophthalmic Research* 16, 233-238.
- Bender, E., Silver, F.H., Hayashi, K., Trelstad, R.L., 1982. Type I collagen segment long spacing banding patterns. *Journal of Biological Chemistry* 257, 9653-9657.
- Borcuk, A.C., Niedt, G., Sablay, L.B., Kress, Y., Mannion, C.M., Factor, S.M., Tanaka, K.E., 1998. Fibrous long-spacing collagen in bacillary angiomatosis. *Ultrastructural Pathology* 22, 127-133.
- Bowden, J.K., Chapman, J.A., Wynn, C.H., 1968. Precipitation of collagen in the segment long-spacing form by various organic and inorganic compounds. *Biochimica et Biophysica Acta* 154, 190-195.
- Brown, E.M., 1012. Development and utilization of a bovine type I collagen microfibril model. *International Journal of Biological Macromolecules* 53, 20-25.

Bruns, R.R., Gross, J., 1973. Band pattern of the segment-long-spacing form of collagen. Its use in the analysis of primary structure. *Biochemistry* 12, 808-815.

Bruns, R.R., Hulmes, D.J.S., Therrien, S.F., Gross, J., 1979. Procollagen segment-long-spacing crystallites: Their role in collagen fibrillogenesis. *Proceedings of the National Academy of Sciences USA* 76, 313-317.

Chen, C., Yang, S., Zhang, M., Zhang, Z., Zhang, B., Han, D., Ma, J., Wang, X., Hong, J., Guo, Y., Okunieff, P., Zhang, L., 2013. In vitro Sirius red collagen assay measures the pattern shift from soluble to deposited collagen. *Advances in Experimental Biology and Medicine* 765, 47-53.

Croucher, L.J., Crawford, A., Hatton, P.V., Russell, R.G.G., Buttle, D.J., 2000. Extracellular ATP and UTP stimulate cartilage proteoglycan and collagen accumulation in bovine articular chondrocyte pellet cultures. *Biochimica et Biophysica Acta* 1503, 297-306.

Dapson, R.W., Fagan, C., Kiernan, J.A., Wickersham, T. W., 2011. Certification procedures for Sirius red F3B (C135780, Direct red 80). *Biotechnic and Histochemistry* 86, 133-139.

De Carlo, S., Harris, J. R. (2011) Negative staining and cryo-negative staining of macromolecules and viruses for TEM *Micron* 42, 117-131.

Doyle, B.B., Hukins, D.W.L., Hulmes, D.J.S., Miller, A., Woodhead-Galloway, J., 1975. Collagen polymorphism: Its origins and the amino acid sequence. *Journal of Molecular Biology* 91, 79-99.

Eyden, B., Tzaphlidou, M., 2001. Structural variation of collagen in normal and pathological tissues: role of electron microscopy. *Micron* 32, 287-300.

Fujita, Y., Kobayashi, K., Hoshino, T., 1997. Atomic force microscopy of collagen molecules, surface morphology of segment-long-spacing (SLS) crystallites of collagen. *Journal of Electron Microscopy* 46, 321-326.

Goldberg, B., 1974. Electron microscopic studies of procollagen from cultured human fibroblasts. *Cell* 1, 185-192.

Gross, J., Highberger, J.H., Schmitt, F.O., 1954. Collagen structures considered as states of aggregation of a kinetic unit. The tropocollagen particle. *Proceedings of the National Academy of Sciences USA* 40, 679-688.

Harris, J.R., 1997. Negative Staining and Cryoelectron Microscopy: the thin film techniques. *RMS Microscopy Handbook No. 35*, BIOS Scientific Publishers Ltd, Oxford.

Harris, J.R., Gebauer, W., Söhngen, S.M., Markl, J., 1995. Keyhole limpet hemocyanin (KLH): Purification of intact KLH1 through selective dissociation of KLH2. *Micron* 26, 201-202.

Harris, J.R., Reiber, A., 2007. Influence of saline and pH on collagen type I fibrillogenesis in vitro: Fibril polymorphism and colloidal gold labelling. *Micron* 38, 513-521.

Harris, J.R., Soliakov, A., Lewis, R.J., 2013. *In Vitro* fibrillogenesis of collagen type I in varying ionic and pH conditions. *Micron* 49, 60-68.

Hirota, A., Ebihara, T., Kusubata, M., Kobayashi, K., Kuwaba, K., Tanaka, K., Kiriya, T., Iric, S., Koyama, Y., 2003. Collagen of chronically inflamed skin is over-modified and upregulates secretion of matrix metalloproteinase 2 and matrix-degrading enzymes by endothelial cells and fibroblasts. *Journal of Investigative Dermatology* 121, 1317-1325.

Hodge, A.J., Schmitt, F.O., 1960. The charge profile of the tropocollagen macromolecules and the packing arrangement in native-type collagen fibrils. *Proceedings of the National Academy of Sciences USA* 46, 186-197.

Holmes, D.F., Graham, H.K., Trotter, J.A., Kadler, K.E., 2001. STEM/TEM studies of collagen fibril assembly. *Micron* 32, 273-285.

Hulmes, D.J.S., Bruns, R.R., Gross, J., 1983. On the state of aggregation of newly secreted procollagen. *Proceedings of the National Academy of Sciences USA* 80, 388-382.

Kobayashi, K., Hata, R., Nagai, S., Niwa, J., Hoshino, T., 1990. Direct visualization of affected collagen molecules synthesized by cultured fibroblasts from an osteogenesis imperfecta patient. *Biochemical and Biophysical Research Communications* 172, 217-222.

Kobayashi, K., Hashimoto, Y., Hayakawa, T., Hoshino, T., 1988. Further evidence for the correlation between the primary structure and the stain exclusion banding pattern of the segment-long-spacing crystallites of collagen. *Journal of Ultrastructure and Molecular Structure Research* 100, 255-262.

Kobayashi, K., Ito, T., Hoshino, T., 1985. Electron microscopic demonstration of acid-labile, 4D staggered intermolecular association of collagen formed *in vitro*. *Collagen and Related Research* 5, 253-260.

Kobayashi, K., Niwa, J., Hoshino, T., Nagatani, T., 1992. Electron microscopic visualization of collagen aggregates without chemical staining. *Journal of Electron Microscopy* 41, 235-241.

Komsa-Penkova, R., Koynova, R., Kostov, G., Tenchov, B.G., 1996. Thermal stability of calf skin collagen type I in salt solutions. *Biochimica et Biophysica Acta* 1297, 171-181.

Kühn, K., 1982. Segment-long-spacing crystallites, a powerful tool in collagen research. *Collagen and Related Research* 2, 61-80.

- Lareu, R.R., Zeugolis, D.I., Abu-Rub, M., Pandit, A., Raghunath, M., 2010. Essential modification of the sircol collagen assay for the accurate quantification of collagen content in complex protein solutions. *Acta Biomaterialia* 6, 3146-3151.
- von der Mark, K., Wendt, P., Rexrodt, F., Kühn, K., 1970. Direct evidence for a correlation between amino acid sequence and cross striation pattern of collagen. *FEBS Letters* 11, 105-108.
- Mertz, E.L., Leikin, S., 2004. Interactions of inorganic phosphate and sulfate anions with collagen. *Biochemistry* 43, 14901-14912.
- Morimoto, K., Kawabata, K., Kunil, S., Hamano, K., Saito, T., Tonomura, B., 20090. Characterization of type I collagen fibril formation using thioflavin T fluorescent dye. *Journal of Biochemistry* 145, 677-684.
- Moraes, J.R., Facco, G.G., Moraes, F.R., Engracia Filho, J.R., Miyazato, L.G., Beretta, D.C., 2009. Effects of glycosaminoglycan polysulphate on the organisation of collagen fibres in experimentally induced tendonitis in horses. *Veterinary Record* 165, 203-205.
- Olsen, B.R., 1967. Electron microscopical studies on collagen. V. The structure of segment-long-spacing aggregates consisting of molecules renatured from the isolated α_2 -fraction of rat tail collagen. *Journal of Ultrastructure Research* 19, 432-445.
- Ortolani, F., Giorfdano, M., Marchini, M., 2000. A model for type II collagen fibrils: Distinctive D-band patterns in native and reconstituted fibrils compared with sequence data for helix and telopeptide domains. *Biopolymers* 5r4, 448-463.
- Ortolani, F., Marchini, M., 1993. Correlation between amino acid composition and ultrastructural features of type I and type II native collagen fibrils. *Bollettino- Societa Italiana Biologia Sperimentale* 69, 99-106.
- Olsen, B.R., 1967. Electron microscope studies on collagen V. The structure of segment-long-spacing aggregates consisting of molecules renatured from the isolated α_1 -fraction of rat tail tendon collagen. *Journal of Ultrastructure Research* 19, 432-445.
- Paige, M.F., Goh, M.C., 2001. Ultrastructure and assembly of segmental long spacing collagen studied by atomic force microscopy. *Micron* 32, 355-361.
- Petruska, J.A., Hodge, A.J., 1964. A subunit model for the tropocollagen macromolecule. *Proceedings of the National Academy of Sciences USA* 51, 871-876.
- van der Rest, M., Garrone, R., 1990. Collagens as multidomain proteins. *Biochimie* 72, 473-484.
- Schmid, T.M., Mayne, R., Bruns, R.R., Linsenmayer, T.F., 1984. Molecular structure of short-chain (SC) collagen by electron microscopy. *Journal of Ultrastructure Research* 86, 186-191.

- Schmitt, F.O., Hall, C.E., Jakus, M.A., 1942. Electron microscope investigations of the structure of collagen. *Journal of Cellular and Comparative Physiology* 20, 11-33.
- Schmitt, F.O., Gross, J., Highberger, J.H., 1953. A new particle type in certain connective tissue extracts. *Proceedings of the National Academy of Sciences USA* 39, 459-470.
- Schwartz, D., Veis, A., 1978. Characterization of basement membrane collage of bovine anterior lens capsule via segment long spacing crystallites and the specific cleavage of the collagen by pepsin. *FEBS Letters* 85, 326-332.
- Serafini-Fracassini, A., 1982. The electron microscopy of fibrous protein of connective tissue. *In: Electron Microscopy of Proteins, Vol. 2. Ed. J. R. Harris, Academic Press, pp. 195-231.*
- Silver, F.H., Freeman, J.W., Seehra, G. P., 2003. Collagen self-assembly and the development of tendon mechanical properties. *Journal of Biomechanics* 36, 1529-1553.
- Silver, F.H., Trelstad, R.L., 1980. Type I collagen in solution. Structure and properties of fibril fragments. *Journal of Biological Chemistry* 255, 9427-9433.
- Starborg, T., Lu, Y., Kadler, K.E., Holmes, D.F., 2008. Electron microscopy of collagen fibril structure *in vitro* and *in vivo* including three-dimensional reconstruction. *Methods in Cell Biology* 88, 319-345.
- Tenni, R., Sonaggere, M., Viola, M., Bartolini, B., Enrica, M., Tira, A.R., Orsini, E., Ruggeri, A., Ottani, V., 2006. Self-aggregation of fibrillar collagens I and II involves lysine side chains. *Miicron* 37, 640-647.
- Tillet-Barret, E., Franc, J.-M., Franc, S., Garrone, R., 1992. Characterization of heterotrimeric collagen molecules in a sea-pen (Cnidaria, Octocorallia). *European Journal of Biochemistry* 203, 179-184.
- Trelstad, R.L., 1982. Multistep assembly of type I collagen fibrils. *Cell* 28, 197-198.
- Tuttolomondo, M.V., Galdópora, J.M., Trichet, L., Voisin, H., Coradin, T., Desimone, M.F. 2015. Dye-collagen interactions. Mechanism, knetic and thermodynamic analysis. *RSC Adv.* 5, 57395-57405.
- Usprech, J.F., 2010. A therapeutic dose of adenosine triphosphate (ATP) for cartilage tissue engineering. Master of Applied Science thesis, Queen's University, Kingston, Ontario, Canada.
- Weinstock, A., King, P.C., Wuthier, R.E., 1967. The ion-binding characteristics of reconstituted collagen. *Biochemical Journal* 102, 983-988.
- Zhang, Y., Cremer, P.S., 2006. Interactions between macromolecules and ions: the Hofmeister series. *Current Opinion in Chemical Biology* 10, 658-663.

Zhao, F.-Q., Craig, R., 2003. Capturing time-resolved changes in molecular structure by negative staining. *Journal of Structural Biology* 141, 43-52.

Figure Legends

Fig. 1. Collagen type I interaction with 1mM ATP at increasing pH, with incubation for 24h at 22°C. (a) At low pH (pH 2.5) characteristic 300 nm SLS crystallites readily form, with a tendency to link together as a network. (b) Higher magnification image of a single SLS crystallite, with the heterotrimer C- and N-terminal orientation indicated (following Kobayashi et al., 1985). Note the well-defined asymmetric banding pattern along the SLS crystallite, with respect to heterotrimer orientation. (c) At pH 5.0 the collagen molecules aggregate in a more fibrillar manner, with the creation of loosely associated pre-fibrils and fibrils showing an indication of D-banding. (d) At pH 7.0 the collagen molecules associate to form ~67 nm D-banded fibrils that aggregate as spindle-shaped fibre structures, sometimes termed tactoids. Note that within these tactoids, the banding of individual D-banded fibrils tends to be longitudinally misaligned with respect to one another (arrows) within the fibril bundle, unlike typical D-banded collagen fibres (*cf* Harris and Reiber, 2007, Fig. 5). All images are negatively stained with 2% w/v uranyl acetate. The scale bars indicate 100 nm.

Fig. 2. Collagen type I interaction with increasing concentrations of ATP (0.1 mM to 10 mM) at pH 2.5, with incubation for 24h at 22°C. (a and b) In 0.1 and 0.2 mM ATP the collagen molecules begin to associate as thin fibrillar aggregates, with initial production of linear punctuation/banding (arrows, b). (c, d) In 0.5 mM ATP this fibrillar punctuation is more marked (arrows), alongside the production of partly-ordered SLS crystallites (d). (e) In 1.0 mM ATP characteristic clearly banded SLS crystallites form; the absence of fine material on the background indicates maximal incorporation of collagen heterotrimers. (f) In 5.0 and 10.0 mM ATP thin SLS crystallites have aggregated to form disordered fibres, without any well-defined banding pattern. All images are negatively stained with 2% w/v uranyl acetate. The scale bars indicate 100 nm.

Fig. 3. Collagen type I interaction with 1 mM ATP at varying incubation times (0.5 min to 30 min) at 22°C. (a) After 0.5 min incubation, early faintly banded SLS crystallites (arrows, width upwards of 15 nm) are found within a background of thinner flexuous molecular aggregates. (b, c) After 1 and 2 minutes the formation of SLS crystallites proceeds, indicated by the progressive increase in the SLS width, but still with smaller molecular aggregates present. (d) After 10 min the formation of SLS crystallites has proceeded further, creating SLS widths in excess of 50 nm, with little unassociated collagen on the background. This data indicates that under these conditions, the speed of molecular association can be considered in terms of minutes rather than hours. All images are negatively stained with 2% w/v uranyl acetate. The scale bars indicate 100 nm.

Fig. 4. Collagen type I SLS crystallites can exhibit instability due to ATP leaching during EM specimen preparation. When additional slow water washing was applied to carbon-adsorbed SLS crystallites (formed in 1 mM ATP, pH 2.5 for 24h at 22°C), prior to negative staining with 2% uranyl acetate, they exhibit instability due to ATP removal, shown by disruption and flattening, with loss of the characteristic SLS banding pattern (arrows, a, b). An even more pronounced instability was shown when SLS crystallites are negatively stained with 2% ammonium molybdate (pH 7.0) after excess water washing (c, d). Here the individual collagen heterotrimers can be seen to separate (arrows, d). The scale bars indicate 100 nm.

Fig. 5. Collagen type I SLS crystallites have a tendency to aggregate, with a specific N/C-terminal overlap. Although SLS crystallites have been seen to aggregate randomly, there is also a pronounced tendency for end-to-end aggregation to occur, with a ~27 nm (average of 10 images) N/C-terminal overlap (arrows, a, b, c, with increasing magnification, uranyl acetate negative staining). The same feature was also shown with ammonium molybdate negative staining (d), even though some instability of the SLS crystallites is apparent (*cf* Fig. 4d). The scale bars indicate 100 nm.

Fig. 6. Collagen type I interaction with increasing concentrations of sodium tri(poly)phosphate at pH 2.5, for 24 h at 22°C. (a) 0.5 mM NaTP, (b) 1.0 mM NaTP, (c) 2.0 mM NaTP, (d) 10 mM NaTP. Note the progressive formation of compact ~ 30nm diameter SLS crystallites. These SLS crystallites show a tendency to aggregate in a linear manner, but little tendency to aggregate side by side. The banding pattern along the NaTP induced SLS crystallites is less pronounced than those produced by ATP (*cf* Fig. 1 and *see* Fig. 10). Some free collagen molecules are present on the background at 0.5 mM NaTP, with fewer at 1.0 mM and essentially none at 2mM and 10 mM NaTP. All images are negatively stained with 2% w/v uranyl acetate. The scale bars indicate 100 nm.

Fig. 7. Collagen type I interaction with the hexasulphonated diazo dye Direct red 80/Sirius red. (a) Addition of 0.1mM Direct red to collagen type I at pH 2.5 of 24 h, induced the formation of a disordered fibrillar pigmented flocculate, with no indication of ordered banding. (b) Pre-formed D-banded collagen fibrils in 500mM NaCl, 20mM Tris-HCl at pH 7.5 treated with 0.1 mM Sirius red for 15 min at 22°C. The early-stage of collagen aggregation into thin SLS-like bundles is shown. (c) Pre-formed D-banded collagen fibrils in 500mM NaCl, 20mM Tris-HCl at pH 7.5, treated with 0.1 mM Sirius red for 24h at 22°C. Broad, sheet-like SLS structures are present, that are formed from the side-by-side association of thinner ~ 17 nm SLS rods (average from within several sheets) (*Cf* Schwartz and Weis, 1980). (d) As for (c), but negatively stained with 5% w/v ammonium molybdate 0.1% trehalose (pH 7.0), showing fraying out of an SLS-like sheet in to

individual thinner SLS rods and heterotrimers, in accord with Fig. 4c,d. Images (a-c) were negatively stained with 2% w/v uranyl acetate. The scale bars indicate 100 nm.

Fig. 8. Collagen type I treated with the tetrasulfonated diazo dye Evans blue. (a) Acid-soluble collagen treated with 0.1 mM Evans blue at pH 2.5 for 24 h at 22°C. A disorganized protein flocculate was produced, with no indication of molecular order. (b) Collagen type I D-banded fibrils formed in the presence of 150 mM NaCl, 20mM Tris-HCl pH 7.5 (*see* Harris et al., 2013), followed by treatment with 0.1 mM Evans blue for 24h at 22°C. Note the disruption of the D-banded fibrils into a network of faintly banded dispersing fibril. (c, d) Collagen type I D-banded fibrils formed in the presence of 500 mM NaCl, pH 7.5, followed by treatment with 0.1 mM Evans blue for 24h at 22°C. Note the formation of thin sheets of from a number of aligned discretely banded (~17 nm) SLS fibrils (averaged from several sheets), that readily overlap and form fan-like structures (c). At higher magnifications (d), the dye binding by the parallel bundles of underlying collagen heterotrimers has generated a slightly different SLS image to that produced by ATP (*cf* Fig. 1, and *see* Fig. 10), but is essentially the same as that produced by the diazo dye Sirius red (Fig. 7). Negatively stained with 2% w/v uranyl acetate. The scale bars indicate 100 nm.

Fig. 9. Collagen type I treated with the disulfonated diazo dye, Congo red. (a) Acid-soluble collagen treated with 0.1 mM Congo red at pH 2.5 for 24 h at 22°C. A disorganized finely fibrillar pigmented collagen flocculate was produced, with no indication of underlying molecular order. (b) Preformed D-banded collagen fibrils formed in the presence of 500 mM NaCl 20mM Tris-HCl at pH 7.5, treated with 0.1 mM Congo red. Marked alteration of the characteristic collagen fibril D-banding is apparent, with fragmentation of the fibrils. (c, d) Preformed D-banded collagen fibrils formed in the presence of 1M NaCl at pH 7.5, treated with 0.1 mM Congo red; (c) slightly deeper negative stain than (d). Both (c) and (d) show detail of single fibrils with periodic binding of the diazo dye, without production of any SLS-like banding. Negatively stained with 2 % w/v uranyl acetate. The scale bars indicate 100 nm.

Fig. 10. A direct comparison of collagen type I SLS crystallites, formed (a) in the presence of ATP, (b) Sirius red and (c) sodium tri(poly) phosphate (NaTP). The variation in the SLS banding pattern is an indication of the binding by the collagen heterotrimers and differing molecular mass contribution of these three bound reagents within the SLS structures. All images negatively stained with 2% w/v uranyl acetate. The scale bar indicates 100 nm for a, b and c; the SLS lengths are ~300 nm.

Figure 1
[Click here to download high resolution image](#)

Figure 2
[Click here to download high resolution image](#)

Figure 3
[Click here to download high resolution image](#)

Figure 4
[Click here to download high resolution image](#)

Figure 5
[Click here to download high resolution image](#)

Figure 6
[Click here to download high resolution image](#)

Figure 7
[Click here to download high resolution image](#)

Figure 8
[Click here to download high resolution image](#)

Figure 9
[Click here to download high resolution image](#)

Figure 10
[Click here to download high resolution image](#)