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Collagen-Nano-Silver Complexes

The incorporation of ordered metallic nanoparticles into organopolymeric films endows the films with photo-optical properties. The case of silver incorporated into polyvinyl alcohol films is well established and has been used in polarization filters. Collagen fibers impregnated with silver or silver and gold show intense linear dichroism (LD) and abnormal dispersion of birefringence (ADB) based on spectral measurements of LD obtained by microspectrophotometry and ADB measurements obtained using compensators. Considering that silver is complexed with collagen fibers in the form of nanoparticles and that this event falls within the field of nanophotonics, the measurement of LD is highly desirable. Video image analysis combined with new and simpler methods of silver reaction with collagen can be used to some advantage to accurately detect and measure LD. The determination of nanodimensions for ordered binding of silver particles to collagen fibers of bovine tendon supports the hypothesis that the LD detected in silver-impregnated collagen is a type of textural LD. The aim of this work was to obtain detailed LD measurements by image analysis of collagen fibers bound silver nanoparticles.

INTRODUCTION

The photo-optical properties of liquid crystals related to molecular order when assembled with dichroic dyes have been accurately determined by measuring the spectral and dichroic characteristics of these complexes [1-4]. The introduction of ordered metallic nanoparticles into organopolymeric films endows the films with photo-optical properties [5]. The case of silver incorporated into polyvinyl alcohol films is well established and has been used in polarization filters [6]. Collagen fibers impregnated with silver or silver and gold show intense linear dichroism (LD) and abnormal dispersion of birefringence (ADB) based on spectral measurements of LD obtained by microspectrophotometry and ADB measurements obtained using compensators [7, 8]. Considering that silver is complexed with collagen fibers in the form of nanoparticles and that this event falls within the field of nanophotonics, the measurement of LD is highly desirable. Video image analysis combined with new and simpler methods of silver reaction with collagen can be used to some advantage to accurately detect and measure LD. The determination of nanodimensions for ordered binding of silver particles to collagen fibers of bovine tendon supports the hypothesis that the LD detected in silver-impregnated collagen is a type of textural LD. The aim of this work was to obtain detailed LD measurements by image analysis of collagen fibers bound silver nanoparticles.

MATERIALS AND METHODS

Achilles and flexor tendons of 5- and 21-day-old chicks were fixed in 4% paraformaldehyde in phosphate buffer at pH 7.2 during their removal and for a further 24 h. The tendons were kept at 10°C during the first 3 h. Sections 10 µm thick were used after deparaffinization and hydration. Hydrated sections with no prior treatment were incubated with ammoniacal silver solution until they became yellow or dark yellow, the precise color depending on the quantity and type of extracellular matrix, as well as the thickness of the section. After washing sections in bidistilled water, the sections were reduced with 10% formaldehyde solution for 5 min followed by extensive washing with distilled water and treatment with a 5% sodium thiosulfate solution [9]. Finally, the sections were washed, dehydrated, cleared and mounted in natural Canada balsam (n = 1.54).

Linear dichroism (LD) was analysed and measured using two microscopes: (1) a Zeiss Pol-Photomicroscope equipped with an image analysis system (IAS) + Image++97 and a DT 3155 framegrabber (Western Vision Software, LC-USA), a KODO 512KD CCD, an objective (Neofluar Pol 25x0.60 Optovar 1.25) and a condenser Pol 0.90; (2) a Zeiss Axioptot-2 photomicroscope equipped with Kontrol 400 2 IAS, a Sony Hyper HAD IRJS CCD, a 10x objective, and Optovar 2. A 100W halogen lamp was used as the light source in both microscopes. Monochromatic light was obtained using a λ = 546 nm interference filter, and images were analysed in gray values from 0 – 255 pixels. Gray values (GV) and gray average (GA) were transformed mathematically into absorbances (A). LD was studied by removing the analyzer and rotating the microscope stage so as to orient the long axis of the collagen fibers (or the tendon axis) successively parallel (A∥) and perpendicular (A⊥) to the azimuth of the polarized light (APL). To detect whether there was LD after non-polarized light traversed the sections, the analyzer was kept in position and the microscope stage was rotated.

The measurements were initially done using the two IAS and their respective microscopes. Line profile (LP) measurements were taken parallel and perpendicular to the longitudinal axis of the collagen bundles. For these two LP orientations the APL was A∥ and A⊥. Minitab 12 statistical software was used for all data manipulations and statistical analyses.

Various regions of objects (ROIs), ‘blobs’ or particles were obtained by segmentation after thresholding, with the collagen bundles under A∥ and A⊥ orientation. The area, GA, number of pixels per ROI image and entropy for particles were determined.

The dichroic ratio, DR = A∥/A⊥, was used to...
quantitatively express how many times A π > A l. Birefringence was used to detect wave-like structures (WLS) orienting the collagen bundles parallel to the crossed APL of the polarizers.

RESULTS AND DISCUSSION
Positive linear dichroism (A π > A l) was detected in all collagen bundles of the chick Achilles and digital flexor tendons examined after silver impregnation. To determine whether the silver-collagen fiber assembly (SCFA) could act as a polarizer, the SCFA was illuminated with non-polarized light which, after passing through the SCFA was tested by the analyzer for LD by rotating the microscope stage. Under these conditions, a strong LD of the same intensity as that normally detected using only a polarizer was observed. This is the first description of such an event and supports the conclusion that the SCFA has a polarizing effect on light.

The portion of the Achilles tendon that wraps around the tarsal–metatarsal articulation has a supra-organization designed to withstand compression and tension as also reported for other tendons [10]. Measurements in this region were obtained in the bundles involved in tension partitioning (Fig 1A–B).

LP measurements represent a track of successive co-occurring pixels of GV that correspond to transmittances at A π and A l. In this work, the range of the LP was 50 to 150 μm. All data reported here were obtained using HLImage++97 due to its facilities. LP obtained at positions A π and A l in this region showed a highly positive LD. Figure 2 is a graph of LP measurements in 5-day-old chick tendon sections. Measurements taken perpendicular to the tendon axis (with changes in APL) are shown in Fig 3. In this case, the peaks through the LP represent the fibers and their width. As expected for this orientation of LP, the standard deviations of the GV were higher than when the LP was parallel to the tendon axis. Table I shows the averages, medians and Mann-Whitney test for the two populations. Although the data were heteroscedastic so that ANOVA was not recommended, this analysis was nevertheless used here to allow comparison of the results one another. In 5-day-old chicks, in compressed tendons the collagen bundle birefringence gave birefringence images with no regular, discrete wave-like structures (WLS) when oriented parallel to one of the polarizers (Fig 4).

The LD along the tendon axis showed undulations depended on the fiber orientation and corresponded to the WLS; these undulations were more evident in older chicks (Fig 5 A, B). Discrete, regular WLS structures were observed in tendons subjected only to tension and in sections from 21-day-old chick tendons (Fig 6). LP measurements in the region of tension in 21-day-old chicks showed variations related to the WLS, and the LP reflected this situation, as shown by changes in the absorption peaks according to the fiber orientation (Fig 7). The statistics of these data were similar to the LP measurements obtained in sections from 5-day-old chicks (not shown).

The dichroic ratio (DR = A π and A l), calculated using the A π and A l. values from LP in regions of tension, showed variations that were related to the same causes reported above, i.e. changes in the fiber orientation in WLS. The high standard deviations indicated considerable variability in the measurements. Image analysis of segmented object regions, i.e. particles or blobs, was also used to cal-
The variability in the LP measurements and in the segmented areas was a characteristic of the tendon structure and demonstrated the precision of image analysis method for detecting and statistically representing this organization. Since collagen fibers result from self-recognition (SR) and self-assembly (SA) reactions to produce a highly ordered supramolecular structure that defines the tendon as a superstructure, the laws for SR and SA may be applicable [11, 12]. The laws of supra-molecular chemistry control the ordered binding of silver nanoparticles to collagen fibers [11]. Previous reports on binding of silver to collagen bundles [8, 9], together with the present paper, represent an attempt to establish a bridge between optical anisotropy and nanophotonics. This approach is a valuable means to studying molecular order as part of the feedback control between cells and collagen fibers [13].

CONCLUSIONS

As shown here, LD is an important method for quantifying and statistically describing molecular order, at the range of visible light since collagen is dichroic only at \( \lambda = 190-210 \) nm. Image analysis proved to be accurate and useful for obtaining peculiar line profiles. The software HUImage +97 was particularly more flexible and allowed one to obtain simultaneous LP in the same region and in the same points. IA was useful for obtaining large amount of data. LP and segmented ROI are therefore complementary means for surveying the distribution and variability of collagen fiber and macromolecular orientation.

REFERENCES


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