RETICULIN MORPHOMETRY AND COLLAGEN FIBERS OPTICAL ANISOTROPY AS ASSESSED BY IMAGE ANALYSIS AFTER SILVER IMPREGNATION

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ABSTRACT

A new method for the impregnation of tissue sections with ammoniacal silver carbonate in terms of single-step reaction, no gold toning, no oxidation step and silver impregnation after treatment with pancreatic α-amylase is described. This method yields silver nanocrystalloids with 18.40 nm in average diameter [16]. Natural carbohydrate radicals probably acting as natural reducing agents for silver are assumed to occur. Despite the fact that the method is not specific, reticulin fibers (type III collagen) were clearly distinguished from other collagen fibers types and easily and accurately segmented by image analysis (IA) systems, when using this procedure. Data from myocardial reticulin fibers are presented. Collagen bundles (type I collagen rich structures) exhibited intense linear dichroism (LD) in terms of gray averages measured by IA *(non-impregnated, natural, collagen fibers do not show LD)*. Anomalous dispersion of birefringence was also detected, highlighting typical morphologies, including crimp, which allow us to propose the method for molecular order studies and to assess collagen bundles as chiral objects, especially together with LD data.

Key words: Birefringence, collagen fibers-reticulin, image analysis, linear dichroism, silver carbonate impregnation

INTRODUCTION

Thin organopolymeric films have improved photo optical properties when ordered metalic nano-particles are incorporated into them. Highly oriented polyvinyl alcohol complexed with silver shows intense linear dichroism and its potential use as polarizer has been considered [5]. Silver crystalloids, measuring from 50 to 100 nm, introduce linear dichroism when bound to cellulose fibers [5]. Methods for impregnation with silver have been used for different biological materials since the early work by Golgi in 1898 [3]. For collagen bundles, particularly the identification of reticulin, many methodological variations have been described using different silver salt solutions. In all cases, an oxidative step precedes the treatment with the impregnating solution [3,4,6]. The various stages of impregnation with silver or gold introduce technical complications, including the time and the high cost involved, both of which being limiting factors to the more general use of the methods. Even so, the staining of cellular and extracellular structures by these procedures provides useful morphological [10,11], and even topochemical information since there is support that silver particles are bound to glycans [9-13].

Molecular order or crystallinity is being increasingly studied in non-biomedical fields in an attempt to correlate morphology with specific functional properties. The optical-anisotropic properties of collagen-based structures provide an excellent method for statistically determining molecular order in structure-function studies of the extracellular matrix.

Various accurate quantitative methods have been used to demonstrate that silver-impregnated collagen fibers show selective absorption of polarized light or linear dichroism (LD) and anomalous dispersion of birefringence (ADB), as well as interference color of birefringence, properties that non impregnated collagen fibers do not display. The ADB point of inflection which is close to the wavelength of maximal LD is related to the molecular order of the fibers which impose a helical, chiral arrangement on the silver crystals complexed to the collagen-chiral superstructure [7,16].

The introduction into cyto-histological routines of a technique involving only one reaction step with silver with non previous oxidation and no gold toning, that could provide topochemical and topophysical information, would be advantageous for morphometric studies and routine diagnoses.

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This report describes a procedure for the staining of extracellular matrix (ECM) constituents based on a reaction with a single solution of silver carbonate followed by reduction with formaldehyde. Under the conditions described, reticulin stains black and type I collagen fibers stain yellow and/or clear brown, showing measurable positive LD and abnormal colors of birefringence. Polarized light microscopy analysis allowed the detection and measurements of LD and of distinct interference colors resulting from the molecular arrangement and extent of aggregation of the impregnated substrate. The reaction with silver carbonate solution revealed useful for image analysis and, consequently, for different quantification levels.

MATERIAL AND METHODS

The tissues used were liver and heart samples from albino rats (*Rattus norvergicus albinus*), tarsal-metatarsal samples, including the region of the articulation, from 5-day-old chicken (*Gallus gallus*), and samples of the digital flexor tendon from bulls (*Bos taurus*). The tissue samples from chickens contained skin and all other anatomical structures normally found in the region sampled.

The materials were fixed in disodium phosphate buffer, pH 7.4 to which was added 25 g of paraformaldehyde that had been warmed at 60ºC to dissolve the crystals, the final concentration of paraformaldehyde was 2.5%. After cooling, the pH of the solution was adjusted to 7.4 and 1 ml of glutaraldehyde added. The chicken material, after fixation, was decalcified in 10% formic acid containing 4% formalin.

Following fixation, the tissues were embedded in paraplast (Paraplast, Oxford Labware, Division of Sherwood Medical, St. Louis, MO 63103, USA) and sections 7 µm thick were cut. The paraplast was subsequently removed from the slices by successive washings in xylene after which the sections were passed through ethanol and hydrated before metal impregnation.

Preparation of solutions and impregnation procedures

The ammoniacal silver carbonate solution was prepared by adding 10 ml of a 5% solution of sodium carbonate to 10 ml of a 10% solution of silver nitrate. This resulted in the immediate formation of a whitish-yellow precipitate which eventually turned a chestnut brown color. During the reaction, the precipitate was allowed to sediment without stirring and the supernatant drawn off with a pipet. The precipitate was then washed carefully three times and resuspended in the original volume of liquid (20 ml). This was followed by a slow, drop-by-drop addition of ammonia (20% in water) with mixing on a magnetic stirrer. Care was taken not to add excess ammonia. The final volume of the solution was then completed to 100 ml with glass bidistilled water. The use of high quality 90% ethanol instead of water is possible in this step and may prevent the sections from floating off the slides during processing. The ammoniacal silver carbonate impregnating solution is fresh prepared, completely used and immediately discharded after usage.

Other solutions required for the method included a reducing solution of 10% formaldehyde in bidistilled water, an aqueous solution of 5% sodium thiosulfate and a solution of pancreatic α-amylase (100-200 mg/ml in phosphate buffer, pH 7.0).

In some cases, before reacting with ammoniacal silver carbonate, the sections had to be digested with α-amylase, especially when the samples were from tissues rich in glycogen. Such digestion was usually done at 37ºC for 1 h in a humidified chamber, after which the sections were washed three times in bidistilled water before impregnation.

The sections were reacted with silver by submerging them in the ammoniacal silver carbonate solution until they became yellow or dark yellow. The precise impregnation color depended on the quantity and type of extracellular matrix and on the presence of glycogen and/or reducing groups. When sufficiently colored, the slides were washed in bidistilled water.

The next step involved a reducing reaction in which the sections were incubated in formol solution for 1-5 min followed by extensive washing with distilled water and soaking in 5% sodium thiosulfate before dehydration, clearing and mounting in Canada balsam with a refractive index of n = 1.54 necessary for image formation.

The sections were examined under polarized light to assess birefringence, interference colors caused by abnormal dispersion of birefringence (ADB) and linear dichroism (LD). The latter was measured by positioning the long structural axis (in this case, the long axis of the collagen fiber bundles) successively parallel and perpendicular to the azimuth of electrical vector of polarized light. Subsequent analyses and measurements were done with a polarized light microscope. Detection of ADB was improved using Sénarmont's method and a 1/4 λ compensator under polychromatic light. Image analysis was done using a Global Lab® Image system (Data Translation, Inc. USA) and a Zeiss-Kontron (Oberkochen/Munich) KS400-2 system. The parameters obtained by these analyses included absorbances, expressed on average gray values, i.e. gray average in pixels (GA), total area of the analyzed images (TA), the area with high reticulin density (HRD), low reticulin density (LRD) and areas without reticulin, i.e., clear areas (NR), and the ratio of covered areas (HRD and LRD) to total area, i.e. HRD/TA and LRD/TA. Further details of these measurements are provided in the Results section. Gray values (GV) were expressed in pixels and the areas in squared micrometers.

RESULTS

In general, the extent of silver impregnation depended on the structures examined. In sections not pretreated with amylase, the cytoplasm exhibited a strong black or grey color which made it difficult to see the reticulin. This was particularly the case in liver and skeletal and cardiac muscle. Following digestion with amylase, the reticulin fibers were seen more easily (Fig. 1A,B, 2A,B and 3).

Type I collagen-rich structures, including tendons, ligaments and bones, were stained a dark yellow or light brown and showed linear dichroism which varied with the structure examined. The detection of
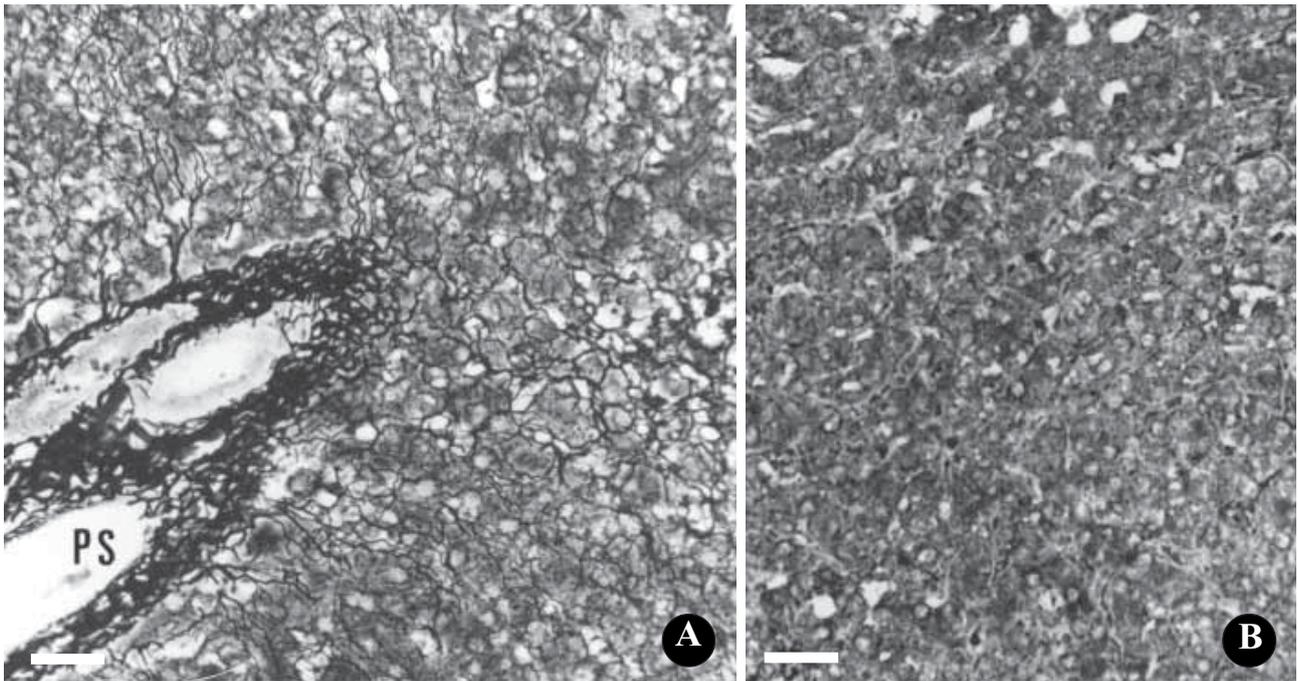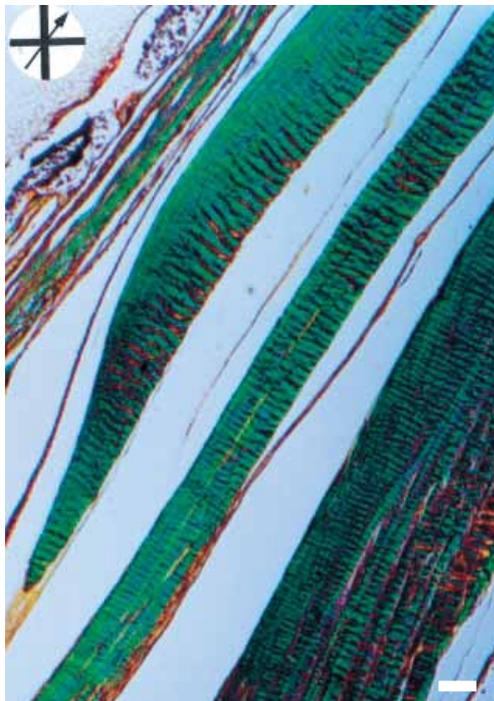
Figure 1. Silver-impregnated section of liver pretreated with pancreatic α-amylase, (**A**) and without pretreatment (**B**). Note the black color of the reticulin fibers in Figure 1A that form a reticulum covering the wall of the sinusoids. In the portal space (**PS**), there is a predominance of type I collagen fibers which, together with the reticular fibers, form the vascular walls. The appearance of the fibers in B is blurred. Bar = 50 μm.
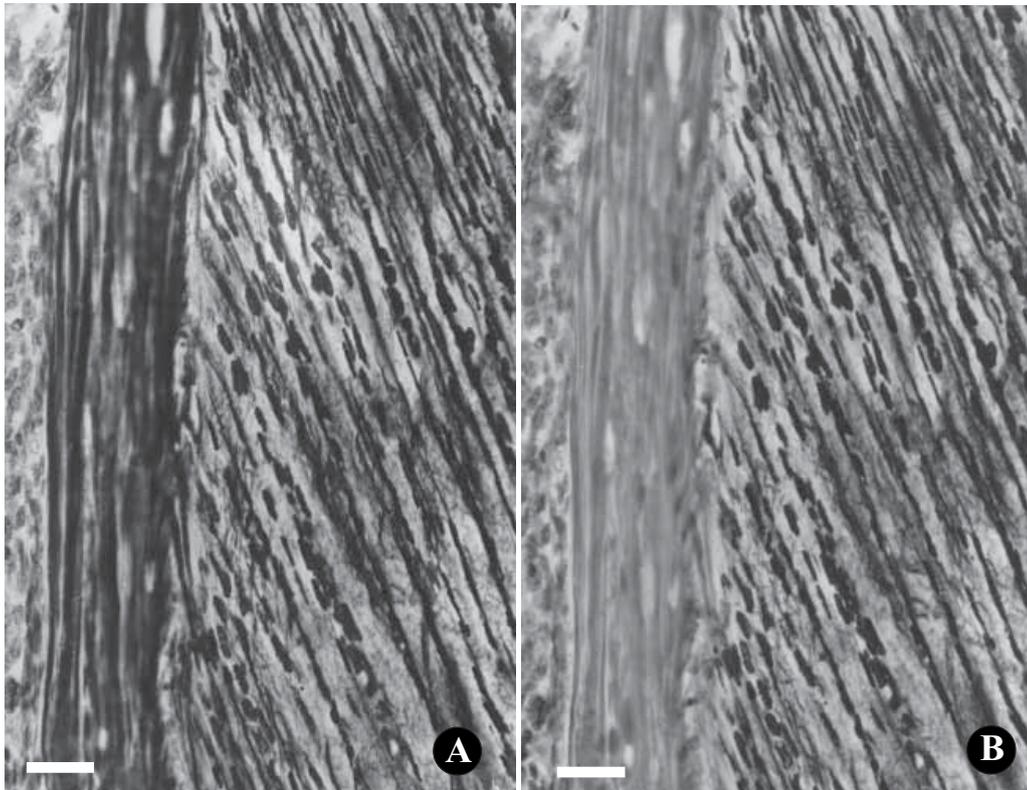
Figure 2. Silver impregnation after enzymatic digestion. A cortico-periosteal region with muscle insertions is shown. In both panels, the black reticulin fibers line and cover the myotubes. Linear dichroism is shown. In **A**, the long axis of collagen fibers of the cortico-periosteal structure is parallel to the EVPL and the fibers are darker (greater absorbance, lower GA values) than in **B** where they are perpendicular to the EVPL (positive linear dichroism). Bar = 50 μm.

interference colors indicated that there was anomalous dispersion of birefringence (Fig. 3 and 4). Undulating structures such as those found in tendons and ligaments were particularly clear (Fig. 3) and could be quantified (data not shown). Linear dichroism (LD) measurements could also be made on these structures in order to assess their molecular order. Image analysis provided accurate measurements of LD. GA were lowest when absorbances were greatest, as occurred when the collagen bundles were parallel to the electrical vector of polarized light (EVPL). Table I summarizes the measurements carried out in two different areas corresponding to structures depicted in Figure 2 A,B (the order of measurement of areas was 10^4 to 10^5 μm^2). LD in the same structure showed variability due to changes in molecular orientation in the supra-organization. Analysis of variance of the GA for fibers parallel and perpendicular to the EVPL yielded a highly significant F value of 25.38. Figure 5 displays the GA values under the conditions $A_{||}$ (parallel to the EVPL) and $A\perp$ (perpendicular to the EVPL). A double peak can be observed in the positive LD. This phenomenon was related to ADB [7,17] and the part played by the silver nanocrystalloid in the textural birefringence of collagen, the average diameter of the silver nanocrystalloids was 18.51 nm [16].

In rat cardiac muscle, the fibers were predominantly reticular and surrounded the muscle fibers, delimiting them in a manner similar to that in skeletal muscle. Using image analysis, it was possible to quan-
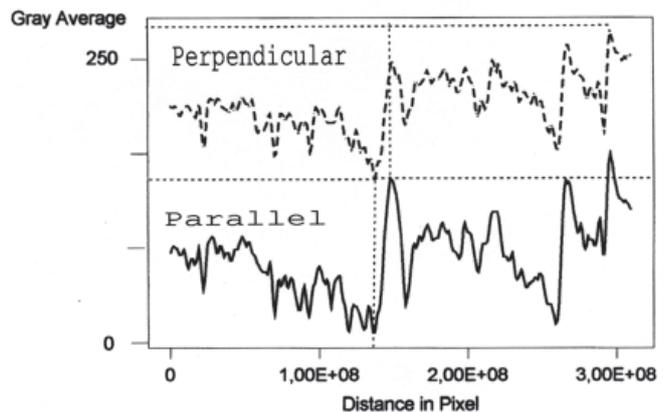

Figure 3. Anomalous dispersion of birefringence in a silver-impregnated section of bull tendon. The interference colors result from the different orientations of helically arranged fibers. Note the wave aspect of the fibers, even though these fibers are at an angle of 45º to the polarized light. The reticulin fibers are black stained. Bar = 50 μm.

Figure 4. Silver-impregnated section of ligaments from chicken tarsal-metatarsal articulation after digestion with α-amylase. The sections were examined under polarized light. Birefringence was compensated using a mica plate 1/4 λ compensator to detect interference colors by ADB. The vector indicates direction of the fiber´s axis as related to the polarizers. Bar = 100 μm.

Figure 5. Frequency histogram of gray average values (GA). Measurements done on sections of ligament of the tarsal-metatarsal articulation of chicken. Solid line stands for collagen fibers parallel ($A_{||}$) to the EVPL. Dotted-ashed line stands for collagen fibers perpendicular ($A\perp$) to the EVPL. Note the higher frequency of lower GA values (lower transmittance = higher absorbance) when the $A_{||} > A\perp$ condition prevails and positive LD is defined, here showing double peaks.

Table 1. Linear Dichroism Measurements Grey average (GA) in pixels, obtained by image analysis of the areas depicted in figures 2A,B. $A_{||}$, GA when the long axis of the structure is parallel to the EVPL; $A\perp$, when the same structure is perpendicular to the EVPL. The values represent two different areas to depict variability. GA and median of the gray values in the parallel relation to the EVPL are lower than in the perpendicular position. Total area is expressed as μm^2.

Relation to the EVPL	GA & Std	GV-Median	Total Area
$A_{||}$	188.16 - 35.95	187.00	216412
$A\perp$	215.71 - 27.83	217.00	216412
$A_{||}$	47.40 - 45.45	35.00	193842
$A\perp$	121.00 - 53.11	129.00	193842

Table 2 and **3**. Average gray values (GA) obtained by image analysis of rat heart sections impregnated with silver after treatment with pancreatic α-amylase. The results here shown are for areas of high reticulin densities (HRD, Fig. 5, Table II) and low reticulin densities (LRD, Fig. 6A, Table III). Abbreviations: NR stands for areas without reticular fibers. TA stands for total area = (NR + HRD) and/or (NR+LRD), in μm^2. - Ratio of areas: RA = (HRD)/TA or (LRD)/TA. %RF, percentage of area covered by reticulin fibers. Measurements were done using threshold gray-level (TGL) ranges of 0-75 pixels and 200-255 pixels. GA-NR corresponds to gray average for no reticulin areas; GA-HRD and GA-LRD stand for gray average of areas of high and low reticulin densities, respectively.

Table 2. HRD

TGL	200- 255 Pixels	TGL	0 - 75 Pixels
GA-NR	207.46 Std=69.9	GA-HRD	87.8- Std=22.4
NR	144 240.72	HRD	83 215.80
TA	231 155.00	TA	231 155.00
RA	0.624	RA	0.36
%RF NR	62.4	%RF HRD	36.0

Table 3. LRD

TGL	200-255 Pixels	TGL	0 - 75 Pixels
GA-NR	109 Std= 32.5	GA-LRD	22.25 Std=7.0
NR	46 203.20	LRD	1 247.52
TA	50 948.80	TA	50 948.80
RA	0.910	RA	0.025
%RF NR	91.0	%RF LRD	2.5

tify the area covered by reticulin fibers. Even after treatment with amylase and silver impregnation, the reticulin fibers showed little or no birefringence. Figures 6A,B show areas with high and low reticulin densities which accounted for 36% and 2.5% of the total area, respectively (Tables II and III).
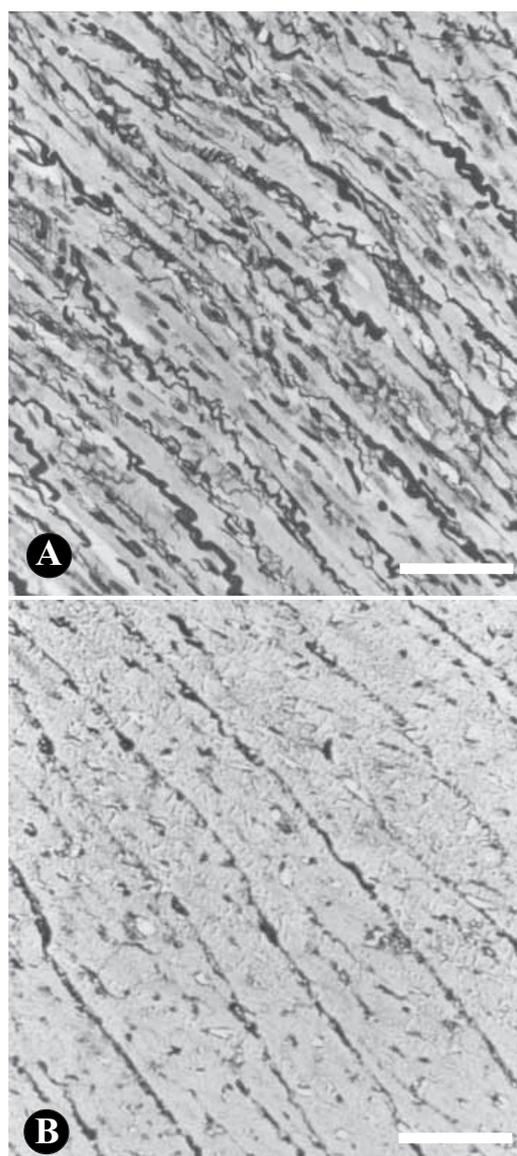
Figure 6. Silver-impregnated cardiac muscle after digestion with α-amylase. Areas of high (**A**) and low (**B**) reticulin densities are shown. See Tables II and III for morphometric data. Bar = 50 μm.

DISCUSSION

Natural reducing groups or groups derived from oxidations may be important in reducing silver to produce the classic reactions with this metal, in a manner similar to the periodic acid-Schiff reaction. Although the interactions of extracellular matrix components with silver may not be specific, they can nevertheless provide important insights into the mechanisms of the chemical reactions involved [10-12]. In particular, studies using enzymatic digestions and controlled oxidations have shown the importance of glycoconjugates in binding to silver [9,13]

In this study, no oxidative reactions that could have generated reducing groups were used, thus indicating that any such groups present occurred naturally. Carbohydrate groups or glycans are able to capture molecules of silver to give rise to different colors as shown above, even without prior oxidation. Similarly, the use of silver stains for electrophoresis gels also gives rise to different colors that reflect the arrangement of the silver grains on and around the proteins [8].

The silver impregnation of reticulin fibers resulted in differential staining that allowed them to be easily distinguished and quantified, regardless of the chemical reaction involved [13]. Immunohistochemistry is the specific method for collagen III, but in the present case, in order to have permanent slides and for quantifications, image analysis on silver-impregnated extracellular matrix fibers was more useful and recommended for experiments in that measurements of the areas and densities of reticulin or linear dichroism are required. The results described here are particularly relevant if previous reports indicating that reticulin fibers are made up of type III collagen fibers are considered [1,2,18]. Image analysis can also be of great value when studying blood vessel structure and angiogenesis, as well as in tissue bioengineering and biomimicking where there is an increasing need for knowledge of the structural interactions between muscle cells and the extracellular matrix, including reticulin.

The linear dichroism shown by silver-impregnated polymers is well known and is related to the orientation of the silver grains in polymers and on cellulose fibers [5]. In the case of collagen fibers, their molecular orientation and the object-chiral nature of the fibers and their bundles account for the ordered binding of silver, collagen is not dichroic in visible light. The interference colors produced by anomalous dispersion of birefringence allow certain physico-chemical inferences about the collagen-silver interaction, particularly with regard to the topology of these fibrous molecules within specific structures, as illustrated in Figs. 3 and 4. Physical and chemical inferences can be drawn because linear dichroism and anomalous dispersion of birefringence are caused by the molecular order of the structure with well-defined electron transitions, and because of the interaction between silver and collagen and between the silver nanocrystalloids themselves [16]. The wave forms (crimp) observed varied with the origin and region of the tissue samples examined and were also influenced by the age of the donor animal and the biomechanical properties of the structures analyzed. The numerous interference colors in the collagen fibers which make up the bundles reflected the helical arrangement of the former which produced a chiral object [14,15].

ACKNOWLEDGMENTS

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