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Structural comparison of carbohydrate and amyloid fibrils by polarization microscopy: a review

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ABSTRACT

Several simple carbohydrates and polysaccharide macromolecules are permanent and essential constituents of biological complex extracellular matrixes in human, animal, plant and bacterial cells. These sugar molecules have a variety of roles and have been implicated in many diseases and disorders. The study of carbohydrates or polysaccharides by polarizing optical microscopy involves the visualization of complex interactions among functional chemical groups, dye particles or molecules and extracellular matrix components of biological tissues. The primary aim of this review is to introduce the association of oligosaccharides and complex amyloid formations by polarization optical microscopy complemented by topo-optical staining reactions.

Keywords: *Carbohydrate/ Amyloid/ Birefringence/ Polarization microscopy/ Sugar histochemistry/ Topo-optical staining reactions/ Aldehyde-bisulphite toluidine blue reaction*

1. INTRODUCTION

Several simple saccharide units and polysaccharide macromolecules are permanent and essential constituents of complex extracellular matrixes across all taxa. Such ubiquitous sugar molecules are functionally diverse and have been implicated in many disorders. For example, monosaccharide glucoses are the energy sources for cells utilizing enzyme regulated glycogen metabolism, while defective enzyme activity can result in glycogenosis, a glycogen storage disease. While, deficiency of lectin-binding saccharides, glycoproteins or so called mucopolysaccharides cause lysosomal storage diseases. Changes in carbohydrate structures have also been linked to neurodegenerative diseases, such as Alzheimer's disease (AD). Therefore, a better understanding of how simple saccharide units and complex polysaccharide structures contribute to diseases and disorders is an important research focus. Characterization of glycosaminoglycan (GaGs) contribution to deposition and primary amyloidosis in human brain by topo-optical staining reactions and polarizing optical microscopy (POM) is an example of recent progress in this field. The study of carbohydrates or polysaccharides by POM involves the visualization of complex interactions between functional chemical groups, dye particles and other molecules with the extracellular matrix components of biological tissues. Together with histochemistry approaches, POM is a versatile and sensitive complementary tool for the visualization of local components and concentrations of selected light altering components [1]. Carbohydrates and biological tissues are structurally anisotropic formations with birefringence. Intrinsic (also known as crystalline) birefringence is the result of the anisotropic formation of chemical bonds and molecules and can arise from the polarizing properties of elongated structures with different refractive indices. Submicroscopic constituents in three distinct spatial orientations can alter the illuminated polarization light to produce two perpendicular refractive indices. As such, birefringent materials can be modified by selective attachment of various reagents.

The first part of this review introduces the structural differences of carbohydrates and polysaccharides in the context of POM, before focusing on complex carbohydrate-amyloid investigations. Complementary use of POM and histochemistry (also known as the topo-optical methodology) is a cutting-edge technology with potential application in molecular medicine, cell biology, pathology and clinical studies. If the geometry of the dye-binding structure is known, the ultrastructure of the biological specimen can be inferred from the dye orientation pattern. Histochemistry is often employed with POM to solve critical biosystematic problems, clinical diagnosis issues and investigate molecular/cellular events with the same reliability as protein sequencing. These histochemical methods are common practice, do not require special or complex equipment and can be performed using a single polarizing microscope. This review summarizes the contributions of many scientists who have used histochemical investigation together with POM in order to assess the utility of this technique in the study of carbohydrates and amyloid fibrils.

2. MONOSACCHARIDES

Carbohydrates represent important structural and functional components of cells and tissues including simple sugars, starches, animal and plant celluloses, chitin, galactoglucosamins and many other compounds. They consist of three basic building blocks; single or double bonded oxygen, carbon and hydrogen in various conformations. The most basic

carbohydrate units are monosaccharide, disaccharides and trisaccharides. Slightly higher polymeric degrees of carbohydrates are referred to as oligosaccharides, while ten of more linked monosaccharide units are classified as polysaccharides [2].

Monosaccharides can be further divided according to their carbon content as tetrose, pentose, hexose or heptose. At this point it should be mentioned that biopolymers are generally divided into three major groups; polysaccharides, nucleic acids (DNA and RNA) and proteins, however, the basic units can be similar. Lipids are another major group of biopolymers, with an important role in topo-optical histochemistry. For example, pentoses (5-carbon monosaccharides) are the components of ribonucleic acid (RNA) and deoxyribonucleic acid (DNA), with de-oxylation of ribose at the second carbon in the later. In nucleic acid formation, the hydroxyl group of the first positioned carbon atom is replaced with a nucleotide base. Hexoses are stereoisomers, having identical stereo epimers and form subunits of celluloses and most sugars in fruits and blood. D-glucose is a hexose repeating unit and the principal component of cellulose via the β-1-4 glycosidic linkage. In xylan and chitin, amylose (α-1-4-linked), xylose and N-acetyl-D-glucosamine (β-1-4-linked) are known as homopolysaccharides. β-1-4 linked polysaccharides are known as structural integrity in organisms. α-1-4-linked amylose is an energy-storage polysaccharide, denoted as a heteropolysaccharide since its repeating sugar unit consists of more than one type of sugar molecule.

There are seven distinct types of GaGs; hyaluronan (hyaluronic acid), chondroitin, chondroitin sulfate, heparin, heparan sulfate, dermatan sulfate and keratan sulfate [3]. The monosaccharide building blocks of charged glycosaminoglycans are hyaluronan (a D-glucuronic acid β-1-3-*N*-acetyl-D-glucosamine disaccharide unit linked via a β-1-4-glycosidic bond), chondroitin (a D-glucuronic acid β-1-3 *N*-acetyl-D-galactosamine disaccharide unit linked via a β-1-4 glycosidic bond) and heparin (a 2-O-sulfated iduronic acid unit linked via a β-1-4 glycosidic bond to 6-O-sulfated N-sulfated glucosamine) [4].

POM of monosaccharides. Polarized light is a light beam in which the planes of vibration of all light waves are parallel and the polarization states of all waves are uniform [5]. Polarization optical observation of mono-, di- and tri-saccharides has not been performed due to the structural simplicity, but their derivatives are widely studied [6-10]. For example, alkylated sugars (n-alkyl-1-O-β--D-glucopyranoside, n-alkyl-1-thio-α-D-mannopyranose), in which hydroxyl groups are responsible for smectic A phase separation, have weak birefringence in a natural state when induced polarized light interacts with their structures and show liquid crystal behavior [11].

The lysosome is a cellular organelle responsible for the degradation and recycling of several macromolecules, including homo- or hetero-polymers of monosaccharides, oligosaccharides, proteins and glycosaminoglycans. Lysosome dysfunction causes numerous types of lysosomal storage disorder, including glycogenosis, mucopolysaccharidosis or lipidosis. Because these disorders are associated with glycan derivatives, they can be investigated by topo-optical staining reactions and POM, with birefringent axes paralleling oligo or polymer axes and the detected light intensity changing according to spatial orientation.

M. Andersen syndrome is a rare autosomal recessive glycogenosis disease caused by a lack of amylo-1,4-1,6-transglucosidase enzyme. First investigated by Fischer (1978) [12] and subsequently by Makovitzky (2002) [13], altered transmission of polarized light by dye functionalized monosaccharide units as well as some extinction effects have been identified.

POM of polysaccharides. Starch (α-1-4-linked D-glucose) is an important energy-storage polysaccharide that has been extensively investigated by POM. In nature, starch exists as either a linear helix amylose structure or a highly branched amylopectin. Starch hydrolysis by acid or enzymes catalysts can convert amylopectin into dextrins, then syrups and maltodextrin. Plants starches have been investigated by POM and reported to have Maltese crosses with characteristic shapes (e.g., ellipsoidal, polyhedral and rounded) depending on their origin [14]. Polarized light is also used to visualize birefringence of Maltese cross in tissue biopsies, and intense periodic acid-Schiff (PAS) reactions are indicative of polyglycosan bodies with a high diversity of shapes [15]. Maltese cross patterning of light extinction under POM is a characteristic feature of spherulite structure and indicative of oriented molecular order or heterogeneous organization.

The unstained amylose (linear) and amylopectin (branched) show length-dependent linear negative birefringence [16-18], indicating that the ordinary and extraordinary refractive indexes are perpendiculars - a direct consequence of slow axis of transmission. Moreover, the large refractive index is perpendicular to the long axes of oriented OH groups during POM observation of starch.

Topo-optical reactions. This section introduces the most commonly used staining reactions and their usefulness when combined with POM. For clarity, we first describe some selected terms used in POM of biological structures. Anisometric submicroscopic particles influence the state of polarization light and distinguish three optical phenomena, called metachromasia, dichroism and birefringence (also known as double refraction) [19-21] and the theoretical basis of metachromasia and dichroism are discussed below. Metachromasia is a consequence of staining phenomenon, in which stained biological structures present colors that are different from the dye alone. Dichroism is an anisotropic absorption of polarized light by the molecules or crystals and represents an interaction between the dye and acidic radicals of biological specimens. This is related to the polarization plane and optic axis of dichroic structures.

One of the first selective staining techniques using the periodate reaction on mono-, di- and polysaccharides is the classical PAS reaction (reviewed in Romhányi 1978 [22]). This reaction produces an intense purple color, complementary to the linearly polarized green light, which is not transmitted by the structure. The specificity of the PAS reaction for the glycolytic OH groups is a key advantage, but the lack of birefringence (isotropic staining reaction) is a disadvantage [23]. Post-precipitation of the PAS-reaction with potassium iodide and potassium ferricyanide complex, it is possible to produce an anisotropic effect, however, this post-treatment remains unstable

[24]. The aldehyde-bisulphite toluidine blue (ABT)-based anisotropic PAS-reaction provides detailed information on changes in birefringence induced by dye molecules bound in a chemically ordered fashion or colorless molecules on micelle structures [12, 25], thus revealing otherwise hidden structural components. Depending on the oriented array of the dye molecules deposited on a structure, there are two possible types of topo-optical reaction: additive topo-optical reactions, where the vibrating plane of the oriented dye molecules or colorless chemical components parallel the micelle birefringence, thus increasing the original birefringence; and inverse topo-optical reactions where the vibrating planes are perpendicular, eliminating the original birefringence [26].

Induced topo-optical staining reactions on various conformations and using dye molecules lead to a better understanding of the molecular mechanisms underlying the reactions [19, 20]. There are three important assumptions used in topo-optical reactions: (1) When possessing a distinguished optical axis, planar dye molecules can induce topo-optical reactions; (2) Topo-optical reactions can shown on ordered micellar structures; (3) There should be high concentration of densely packed dye adsorbing places on the surface of the structure, which assist the coplanar association of flat dye molecules.

Romhányi was the first to investigate the polyanion converted staining reactions of tissue structure by a polarization optical-histochemical method [5, 27]: analyzing the Kramer and Windrum (1954) [28] sulphate method with toluidine blue reactions. In this reaction, the polyhydroxy component of the complex biological tissues can be characterized as post-sulphation reaction. In those biological structures where the OH groups are densely arranged, anisotropic reactions are introduced due to the coplanar association of dye molecules (e.g., polysaccharides in the hydroxyproline-rich connective tissue collagen) [20, 29]. Although there are several other OH containing biological structures, these generally lack the OH radical density required of coplanar association of dye molecules and therefore exhibit orthochromatic (disordered) behavior and show isotropy under polarized light. In the sulphation-type reactions, collagen (OH-proline and OH-lysine) and glucose components response simultaneously, with collagen showing linear dichroism (direction dependent absorption of polarized light) only in the ultra-violet region [30]. To dissociate these structures, Romhányi introduced the periodic acid sulphation reaction, in which the vicinal alcoholic-OH groups of polysaccharide components are oxidized and transformed to aldehyde prior to the sulphation, allowing selective staining of collagen [29].

The induction of aldehyde groups by prolonged oxidation with periodic acid and addition of bisulphite was named ABT reaction (Fig. 1) and results in the basophilic staining of polysaccharides by toluidine blue or thiazine dyes at pH 1. The ABT reaction of linear and spatially ordered glycol groups increases the sensitivity of the anisotropic staining.

By reaction with vicinal OH groups in polysaccharides, the ABT reaction can be used as an inverse topo-optical staining technique. The first step of this reaction is based on the selective oxidation of OH groups with 1% periodic acid (30 min at room temperature) and is equivalent to the first step in the PAS reaction, commonly used in polysaccharide histochemistry [23]. The oxidation mechanism is specific for the chemical groups of OH, NH_2, NH-R and double bond O. While the newly formed CHO groups react selectively with bisulphite, through which they are converted to sulfonic acid [12, 25]. Sulfonic acid has a strong negative charge and therefore the new aldehyde bisulphite-complex selectively reacts with basic dyes such as toluidine blue or various thiazine dyes (e.g., azure a, azure b, azure c, thionine, methylene blue) and 1.9-dimethyl methylene blue (1.9 dmmb) at pH 1. As described by [25], the ABT reaction is a useful method that produces a higher intensity reaction than PAS, and is selective for vicinal OH group identification by methachromatic basophilia and detection of the linear order of vicinal (glycolytic) OH groups in polysaccharide chains. Thus, polarization optical analysis makes it possible to determine the spatial arrangement of sugar chains at a molecular level, which is not possible with conventional or electron microscopic PAS reactions.

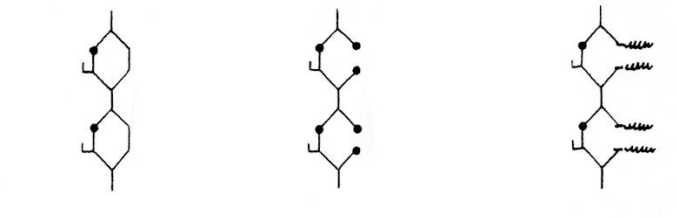

Figure 1. Scheme of the ABT reaction acc. to Romhányi 1975. In the first step the glycolitic OH groups are oxidized with periodic acid to form CHO groups. Then CHO group react with bisulphite to convert it sulphonic acid. The aldehyde bisulphite-complex can selectively react with toulidine blue at pH 1.

Selective reaction of O-acyl radicals of sugar molecules with a potassium hydroxide (KOH)-induced alkaline effect, followed by the PAS reaction is a well-established method [31-37]. Moreover, Fischer and Emödy (1976) [38] have selectively shown O-acyl radicals using the KOH-ABT reaction and a new periodic acid-borohydrate ABT reaction.

ABT polarization optical study of starches. After ABT reaction of starch, the signal is linear positive with dye molecules oriented parallel to the surface, indicating the radial arrangement of OH groups. The Maltese cross optical features of starches can only be shown for unmodified (non-hydrolized) starch granules (Fig. 2).

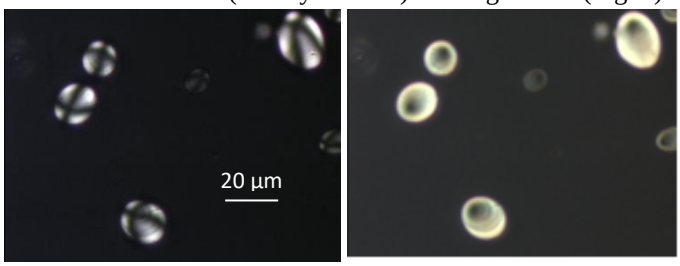

Figure 2. Starch granules. Maltese cross pattern of light extinction under polarizing microscope is a characteristic feature of starch granules. Unstained in linearly polarized light (upper) and in circular polarized light (down). According to Frey-Wyssling the sign of unstained starch granules is linear negative (radial positive spherit). The intensity of the birefringence indicates that the molecular orientation of amylose and amylopectin in a starch granule are identical.

In an artificially grown spherocrystal form of amylose, the amylose chain axis is aligned with the domain long axis, producing positive optical birefringence [39]. Steam pressure treatment can change the granular structure of acid modified starches and, in the case of maltodextrin, can result in complete disintegration (no birefringence) [40]. Similarly, Xue et al. (2008) [41] have shown that shear stress treatment of starch destroys the semi-crystalline structure, eliminating birefringence. Liu, Lelievre and Ayoung-Chee (1991) [42] studied starch gelatinization by OPM and found that a decrease in crystallinity can abolishes birefringence.

Topo-optical reaction of branched polysaccharide glycogen, dextran, galactan and inulin. Glycogen (sometimes referred to as animal starch) is a highly branched polysaccharide with a similar structure to amylopectin (α-1-4-D-glucose), but with a higher branching frequency, α-1-6 glycosidic linkages and shorter branch length. Glycogen is synthesized by the glycogen enzyme and the unstained form of glycogen is isotropic. Post-ABT staining reaction of glycogen, granular mosaic anisotropy with low intensity and an absence of dichroism has been shown [12]. ABT reaction of glycogen produces a red polarization color, as a non-optimal polarization color this is indicative of bundles of branched carbohydrate chains of various orientations. Toluidine blue dye also stains polyanionic structures in a metachromatic red color and, due to the ordered deposition of the dye molecules, enhances birefringence of stained structures. The metachromatic toluidine blue aggregate absorbs green light and is therefore strongly birefringent in green (its optimal polarization color). Longer (orange) and shorter (blue) wavelength light appears in the structure after additive compensation and subtractive compensation, respectively.

Dextran, also a branched polysaccharide formed of glucose molecules with α-1-6 D-glycosidic bonds and branching at α-1-3, is synthesized from sucrose. Galactan also has similar α-1-6 D-glycosidic bonds but between galactose molecules. Due to the non-specific orientation of the polysaccharide chains, both dextran and galactan are isotropic with ABT reaction. Inulins are another class of branched polysaccharide. Composed of β-2-1 glycosidic bonds between fructose units (typically with a terminal glucose unit), inulins have a starch-like structure and have weak anisotropy under polarized light [43].

POM analysis of cellulose. Cellulose is a natural biopolymer of linear β-1-4-linked glucose units, which (in contrast to starch) are oriented with a CH_2OH group at C5. Unstained cellulose has a low anisotropy in both water and gum Arabic, indicating that anisotropy is linear positive to chain length. Extending the POM analysis of pure cellulose, topo-optical staining techniques have been applied since 1945. In an additive topo-optical reaction, Wälchli (1945) [44] found that congo red dye molecules are bound parallel to the surface of plant cellulose and that the anisotropy signal is linear positive. Frey-Wyssling et al. (1948) [16] were the first to explore the plant cellulose fibrillar ultrastructure by electron microscopy. They found that essential and secondary cell walls exhibit a fibrillar texture. The ABT reaction is intensive for grape seed in paraffin sections with a green polarization color and linear negative anisotropy (also an inverse topo-optical staining feature) [12, 45]. Bacterial cellulose has also been investigated and a threaded structure observed [46, 47]. Congo red staining of bacterial cellulose results in a weak anisotropic effect and, similar to plant cellulose, can be observed after 50 min with 0.1% aqueous congo red. The bacterial cellulose signals are also linearly positive, with dye molecules bound in an optimal parallel orientation to the surface. Investigation of onion (*Allium cepa*) root cellulose by ABT staining reaction after pectinase digestion, has shown that the original birefringence signal is shifted from positive to negative [12]. This indicates that, in the case of plant cellulose, ABT follows an inverse topo-optical reaction. We have found that ABT and/or ABD reactions yield similar results with bacterial cellulose [48]. The polarization color (green) and metachromasia indicate optimal dye molecule binding to the surface in both reaction types. Bacterial cellulose is enriched with cellulose I_α-allomorph and differs from cellulose I_β in the conformation of the anhydroglycose units and in the β-1,4 linkages [49, 50], however the inverse staining signal is the same that produced with plant cellulose. The presence of ordered, arranged and thickly orchestrated aichromotropes on the surface of cellulose chains, results in selective metachromatic staining with the ABT reaction by the coplanar association of dye molecules. Negative dichroism and negative anisotropic absorption of polarized light show perpendicular introduction of color molecules regarding the cell wall.

A recent study of cellulose nanocrystal thin films demonstrated birefringence to be a result of intrinsic shape and optical anisotropy of rod-like cellulose nanocrystals, oriented by a spin-coating process. Birefringence varied with thickness and relative location to the spin axis, allowing preparation of solid samples ordered for maximilly anisotropy [51].

Using the crude cellulase enzyme from *Trichoderma viride*, positive- and negative-type spherulites can be formed by artificial polymerization of cellulose via cellobiosyl fluoride, as observed by optical polarization microscopy [52]. Like starch granules, spherules show optical birefringence, suggesting polymerization proceeds via two independent mechanisms. Under given polymerization conditions, the majority of the spherulites show a negative-type structure, indicating that cellulose chains within a spherulite have a predominantly tangential orientation [53].

The cellulose fibers delivered by the immediate disintegration of cellulose in a dissolvable have the non-exclusive name of lyocell. Pure lyocell fibers, resulting from direct chemical modification of cellulose, show a well-orientated structure, however, lyocell that has been surface modified with starch-based superabsorbent or hydrolyzed starch-grafted-polyacrylonitrile, show pure orientation under polarized optical microscopy. In this orientation, anisotropy is enhanced with increasing superabsorbent content. Swollen superabsorbent powders may intrude on the orientation of the lyocell fiber during wet spinning, but may diminish of the level of orientation, which appears to be useful to the infiltration of water diffusion into the fiber [54].

Polarization optical study of chitin and chitosan. Chitin is a glucose-based unbranched polysaccharide that differs from cellulose at the C2 carbon, where an acetamino residue replaces a hydroxyl group. Chitosan is as in part deacetylated polymer of acetyl glucosamine acquired through alkaline deacetylation of chitin. As a compound polymer of glucosamine and N-acetyl glucosamine, chitosan structure is very similar to that of cellulose, consisting of β-1-4-linked D-glucosamine residue with the 2-hydroxyl group substituted by an amino or acetylated amino group [55]. Due to its multiple functional groups, chitosan exhibits a high adsorption capacity towards many classes of dyes [56]. Unstained chitosan shows birefringence in water and gum Arabic medium with a linear positive signal. Fixed chitosan shows an intense anisotropic effect with 1.9-Dmmb (ABD), with purple birefringence indicating optimally oriented binding of 1.9-Dmmb to the chitosan surface. Metachromasia can be observed by light microscopy, which is negative in the linearly polarized light. Upon compensation with a λ4 compensator, anomalous colors appear. Anomalous color is produced by the orientation of light absorbing bonds and is detected by examining the specimen between crossed polaroids and varying the compensation by rotating the compensator plate [5]. Compensation is the neutralization of elliptically polarized light of one direction of rotation. When a dye has a single absorption peak, compensation produces elliptically polarized light in two opposite directions of rotation, which blend upon transmission to produce the observed color. In this way, neutralization allows the color produced by the opposite direction of rotation to appear. A horizontal orientated dye has directions of rotation at right angles to a vertically orientated dye. Compensation neutralizes one direction of rotation (horizontally) and the opposite direction (vertically), to produce two colors (rather than the blended color). In additive compensation, the horizontal and vertical sectors are green (absorption max.: λ=540 nm) and yellow, respectively. While in subtractive compensation, the horizontal sectors are yellow and vertical sectors are green. With the use of compensations, the permitted vibration direction of polarized light (slow and fast vibrating) can be ascertained by color changes. For example, narrower fibers exhibit a bluish-purple color, while thinner (<5 μm) parts are colored yellowish-blue. Thickness dependent changes in polarization colors at longer or shorter wavelengths can be observed in many biological samples. This is due to interference caused by retardation or the Cotton effect, which is a strong circular dichroism in the region of an absorption band [57].

Sulphated GaGs are not involved in PAS reactions because their constituents are not oxidized by periodic acid. During prolonged periodic acid oxidation, the hexuronic acid residues are oxidized yielding two aldehydes in one hexuronic acid. This allows bisulphite groups to bind the aldehydes, which can be detected with toluidine blue or other cationic dyes at low pH [5]. The chemically intensified version of this reaction is known as the basophilic reaction (CIBR) and with an adequate concentration of toluidine blue, can induce topo-optical reactions of chitosan to produce linear negative inverse birefringence (Fig. 3).

Congo red staining of chitosan reveals a weak congophilia and anisotropy by light microscopy. Subsequent potassium permanganate oxidation increases birefringence producing a linear positive signal. Weak congophilia demonstrates likewise a diminishedre measure of connections between protonated amino groups on the polymer chain and contrarily charged dye ions [58]. Rivanol stains absorbs to and induces fluorescence in the amino groups of chitosan to produce a peak fluorescence intensity at 488 nm argon laser light, while the birefringence signal remains positive.

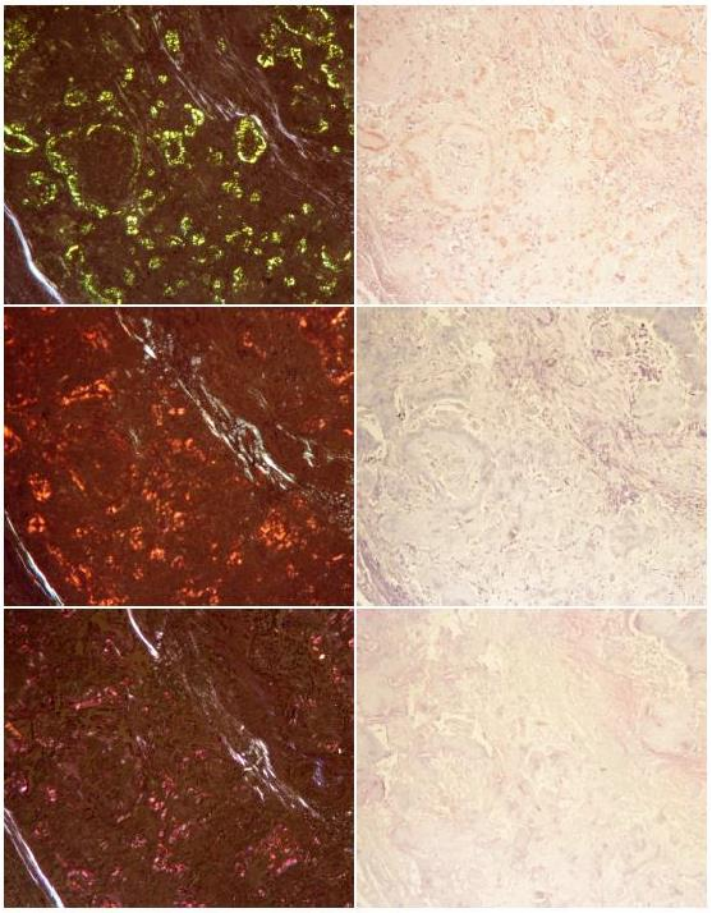

Figure 3. From top to bottom: congo red staining (a), ABT-reaction (b), Sialic acid specific topooptical reactions (c) of human amyloid (images are taken from the same place). From left to right: polarization and light optical images. Magnification: x80. On Fig.3b and 3c optimum dye adsorption can be seen.

It is known that natural unstained chitins have double refraction property [16, 59, 60]. Congo red and trypan blue staining show an intensive anisotropy of chitin, while birefringence properties are unchanged [59, 60].

The positive topo-optical staining reaction with 1.9-Dmmb indicates that chitosan *ab ovo* contains of glycosaminoglycan molecules. The ABD staining reaction of chitosan is an inverse topo-optical reaction, indicating that linearly ordered OH groups and dye molecules are perpendicularly oriented with the surface and that glucose chains are oriented parallel to the surface.

As chitin is an exceedingly insoluble molecule (chitosan is soluble) and a substrate for glycan-protein connections, chitin-like polysaccharides within the Alzheimer's disease (AD) brain

could give a framework to amyloid-β deposition. As such, glucosamine, which forms part of the chitosan and chitin polysaccharide structures, may facilitate the process of amyloidosis and/or give neuroprotection in the AD brain [61, 62]. In AD, high levels of glucose and glucosamine via hexosamine pathway activation caused by impaired glucose metabolism may lead to the production of glucose (starch) and glucosamine (chitin) polymers. In fact, the presence of glucose polymers (amylose) has been verified in the AD brain [63].

Due to its biocompatibility, biodegradability, non-toxicity, and adsorption properties, chitosan has potential as a functional material. Moreover, it has been reported that chitosan could regulate cellular processes such as differentiation, proliferation and cytokine production [64, 65]. Water-soluble chitosan can regulate inflammatory response and neurotoxicity in astrocytoma cells and may reduce and delay AD progression [66].

Polarization microscopic study of Amyloid. Amyloid is a pathological, abnormally folded protein deposit, with a highly organized fibrillar ultrastructure. POM together with distinctive topo-optical staining reactions can be useful for the submicroscopic analysis of amyloid deposits and tissue-isolated amyloid fibrils. Using POM analysis, Romhanyi (1963) [19] demonstrated that amyloids have a fibular structure, while Cohen and Calkins (1959) [67] found that amyloid deposits are highly linear ordered structures in double-stranded fibrils with a diameter of 70Å. Amyloid deposits are histochemically heterogeneous [68, 69] including at least five components: (1) the amyloid protein fibril and several non-fibril components; (2) insoluble polysaccharides (aggregated, aged glycogen); (3) sulphated glycosaminoglycans (GaGs); (4) a pentameric glycoprotein (amyloid P components) and (5) lipids [70-72]. The polysaccharide component includes sialic acid, glucosamine, glucose, galactose and mannose, the relative amounts of which vary across tissue types. The P component comprises up to 15% of the amyloid fibrils and consists of neutral hexoses and their derivates. Sialic acid, O-acyl-sialic acid and fucose are in the terminal position of the oligosaccharide chains. Toluidine blue and 1,9-dimethyl methylene blue topo-optical tinctorial reactions at pH 1 were used in the first efforts to investigate the role of GAGs in amyloid deposits and fibrils and demonstrated the existence of dichroic GAGs in amyloid deposits (Fig. 4).

Further discrimination of the components of GAGs was accomplished with the critical electrolyte concentration (CEC) method, modified for polarization microscopy by Módis (1991) [5]. Amyloid deposits in the presence of a range of $MgCl_2$ concentrations in a toluidine blue solution at pH 5.2 were also investigated, and the following components detected: hyaluronic acid at 0.1 mol, chondroitin sulphate at 0.5 mol, keratan sulphate at 1.0 mol and heparin sulphate at 1.8/1.9 mol. Between 0.1 and 1 mol $MgCl_2$, amyloid presented light optical metachromasy and intensive birefringence under polarized light. At $MgCl_2$ concentrations above 1.0 mol orthochromasy, anisotropy and positive staining with toluidine blue could be detected. Amyloid exhibits orthochromasy and intensive anisotropy, together with a positive staining reaction of toluidine blue below pH 3.5. Above pH 3.5, the optical polarization color was orange-red, exhibiting a typical red metachromasia. Increasing the pH above this threshold results the complete dissociation of the anionic binding sites.

As a result of the increased charge density, the anions of the chromotrope become sufficiently close to introduce an ordered coplanar methachromatic dye aggregate which shows birefringence in polarized light. At pH 3.5 to 4.0 amyloid displays methachromasia typical of an inverse topo-optical reaction. This is the results of an electrostatic interaction of toluidine blue molecules and the ionic groups (carboxyls of amino acids residues, sialic acids of amyloid proteins, sulfates and carboxyls of GaGs) [5].

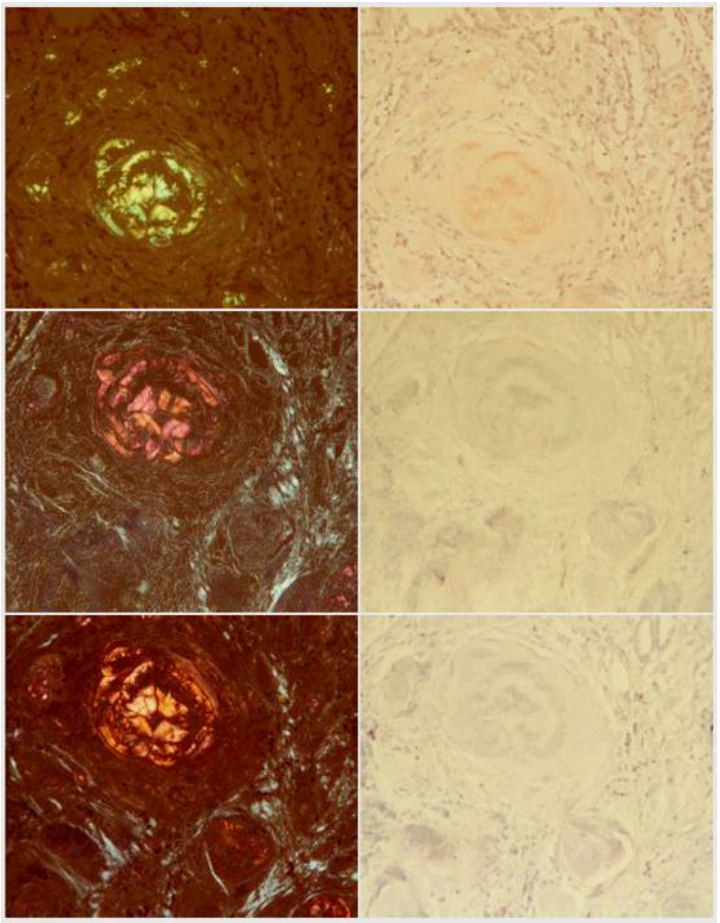

Figure 4. From top to bottom: congo red optimal staining (a), toluidine blue staining at pH 1 (b), CIBR-reaction for GAG-components (c) of human lung sections. From left to right: polarization and light optical images. Magnification: x80. Based on polarization optical analysis the hyaluronic acid components react in a linear negative (radial positive) birefringence, thus the polysaccharide chains are oriented parallel to the long axis, the linear oriented OH groups and the dye molecules are perpendicularly to the long axis of the amyloid fibril (Makovitzky 2003, Richter 2005).

The OH groups of glycoproteins in amyloid influence the orientation of dye molecule binding [5, 24, 72]. This reaction depends on a relatively large distance (5-7Å) from one substrate binding sites to the next. At pH >3.5 metachromasy and anisotropy can be observed with a linear negative signal (inverse-type reaction) and the polarization color is shifted to green. Fischer (1978) [12] and Módis (1991) [5] adapted the ABT-reaction for the submicroscopic analysis of glycosaminoglycans (one and/or two step method, the chemically intensified basophilic

reaction, the CIBR). The prolonged oxidation with periodic acid and bisulphite addition, and staining with toluidine blue or 1,9 dimethylmethylene/azure B at pH 1.0-1.6 results in an increased oriented dye binding of GAGs containing hexuronic acid residues [5, 18, 72, 73]. Based on POM analysis, the hyaluronic acid components react in linear negative (radial positive) birefringence, thus the polysaccharide chains are oriented parallel to the long axis of the amyloid fibril [24, 73].

Topo-optical investigation of P component and sialic acid. With the ABT/ABD-reaction it is possible to demonstrate P-glycoprotein components of amyloid. This component is linear negative to the length, thus the linear ordered OH-groups and the bound dye molecules are perpendicular to the axis and the sugar chains are oriented parallel to the surface. In sialic, and O-acyl sialic acid specific topo-optical staining reactions its amyloid P-glycoprotein components can be identified, with linearly ordered OH—groups and bound dye molecules perpendicularly oriented [24, 72].

For a comparison of the above methods congo red staining, ABT/ABD reaction, sialic and O-acyl-sialic specific topo-optical reactions, toluidine blue or l,9 dimethyl methylene blue staining reaction at pH 1.0, CEC and CIBR methods were performed on serial sections of tissues containing amyloid deposit. All sections were investigated for anisotropy and the staining was correlated. In general, a similar pattern of staining was observed with all of the topo-optical staining reactions under polarization microscopy. For an excellent review of congo red staining of amyloid and amyloid plaques, readers are directed to Howie and Brewer (2009) [74] and Kaminksy et al. (2006) [75], respectively.

Topo-optical investigation of ex vivo isolated human amyloid fibrils. Topo-optical staining reactions of *ex vivo* isolated amyloid fibrils compared with amyloid deposits in tissues show no differences under POM [68]. Likewise, the P component and GAGs can be identified (Fig. 5). Enzymatic digestion (hyaluronidase, chondroitinase and heparinase were performed to specify these reactions, increasing the birefringence effect after congo red staining. The lipid component of fibrils (similar to animal fibrils), could be demonstrated by Sudan b and fluoro-jade [76] staining after lipid extraction.

Using various topo-optical staining reactions the threedimensional amyloid model of Kisilevsky and Szarek, Inoue et al. (2002, 2002) [77-80] was confirmed. Amyloid has been shown to have a highly ordered structure in an oriented fashion: the center is formed by a glycoprotein core (AP), this is surrounded by helical structures of chondroitine and heparin sulphate, while a filament network of proteins (AA and AL and others) constitute the surface fibrils.

Makovitzky found that the helical structure of *ex vivo* isolated amyloid fibrils were reactive POM, regardless of whether stained or unstained with congo red, rivanol, toluidine blue and N,N'-dietyl-pseudiocyanin chloride (PSI) and pinacyanol stained preparations. These results demonstrate that unstained and stained tissue-isolated human and animal amyloid fibrils may have positive and/or negative birefringence with the axial isotropic band [24,81].

Polarization confocal laser scanning fluorescence microscopy studies have proven that structural similarity exists between the selected amyloid fibrils [82-85].

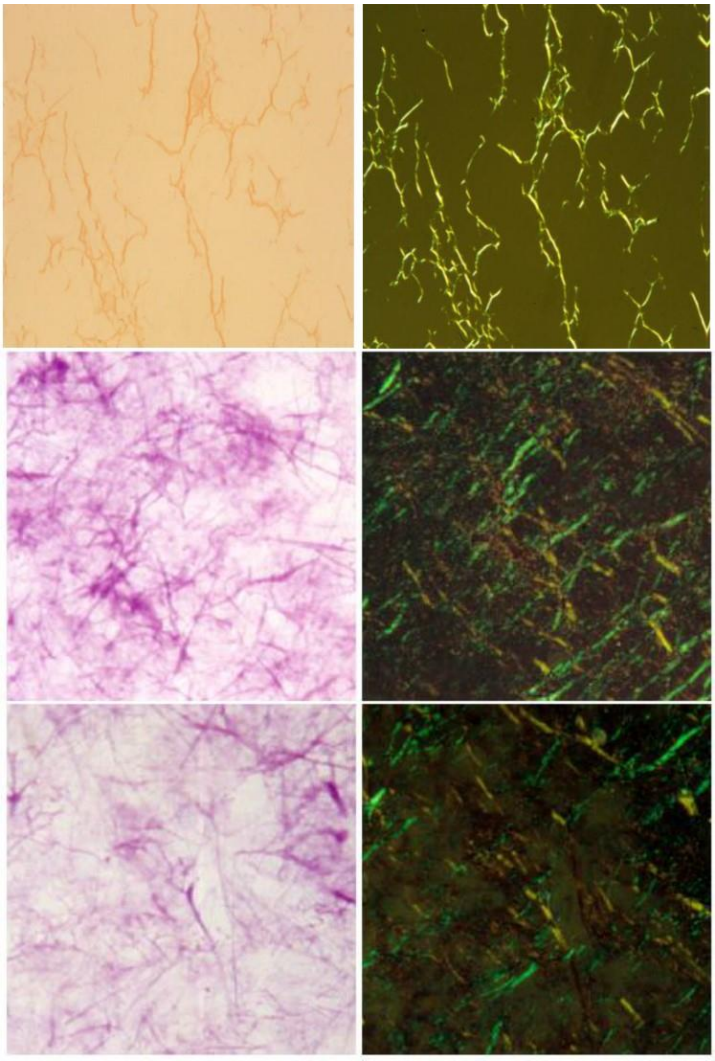

Figure 5. Topooptical staining reactions of human isolated amyloid fibrils from kidney. From top to bottom: congo red staining reaction (a), ABD-reaction (b), and reaction for GAG components (c) with 1,9 dimethyl methylene blue at pH 1.0. Magnification: x 40. The green color of amyloid on Fig. 5b and 5c indicate optimal dye adsorption and the black background is a consequence of crossed polars. From left to right: polarization and light optical images.

Steinbach and Makovitzky confirmed the polarization optical results on amyloid fibrils with differential polarization laser scanning microscopy. It was shown that congo red, rivanol, PSI, pinacyanol chloride have a positive reaction on *ex vivo* isolated human amyloid fibrils, with the dyes molecules of congo red, PSI, pinacyanolchloride positioned in a parallel oriented fashion and those of rivanol perpendiculary oriented (Steinbach and Makovitzky unpublished data).

2. CONCLUSION

Focused on the spatial structural comparison of polysaccharides and amyloid fibril depositions, this study introduces the natural birefringent state of GaGs sugar and sialic acid associated polysaccharides and amyloids. The birefringent state was determined using POM and topo-optical tinctorial reactions, which selectively stain the extracellular component of amyloid (GaGs and sialic acids) and simple oligosaccharides, thus proving a useful tool for determining the organization of highly oriented molecular structures.

3. REFERENCES

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