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Light scattering studies of proteoglycans

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Case Western Reserve University, 1991
LIGHT SCATTERING STUDIES OF
PROTEOGLYCANS

by

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Submitted in partial fulfillment of the requirements
for the Degree of Doctor of Philosophy

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GRADUATE STUDIES

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David Duane Zanoni
LIGHT SCATTERING STUDIES OF PROTEOGLYCANS

ABSTRACT

by

DAVID DUANE ZANGRANDO

Laser light scattering has been used to investigate the structure and hydrodynamic properties of proteoglycans. Two species of proteoglycans have been studied in this work. The first proteoglycan species is that isolated from bovine skin, and is called proteodermatan sulfate (PDS). The second proteoglycan species is isolated from bovine nasal septum cartilage, and exist in two forms: the subunit or monomeric form (PGS), and the aggregate form (PGA), in which PGS units are non-covalently attached to a linear strand of hyaluronic acid (HA).

Static light scattering was used to determine the weight-average molecular weight, Mw, z-average radius of gyration, Rgz, and the second virial coefficient, A2. Dynamic light scattering was used to obtain the z-average translational diffusion coefficient, Dtz, which at infinite dilution allows the frictional hydrodynamic radius, Rhf, to be determined using the Stoke's equation.

PDS was determined to have a Mw= 62,000 in 4M guanidine hydrochloride (GndHCl), which increased to 610,000 in 0.15M NaCl. The core protein was found to have a Mw= 39,000 in GndHCl, and this also significantly increased to 650,000 in 0.15M NaCl. In
contrast, the dermatan sulfate chain (DS) was determined to have a 
Mw= 24,000 in 0.15M NaCl, indicating minimal self-association. 
Comparison of the observed translational diffusion coefficient with 
various hydrodynamic models indicates that PDS has a "lollipop" 
conformation in dilute solution. The Rgz for PDS (53 nm) and core 
protein (34 nm) are significantly above expected values for their 
respective molecular weights. This discrepancy is consistent with a 
small degree (< 5%) of self-association of PDS and core protein in 4M 
GndHCl.

The dilute solution hydrodynamic behavior of PGS and PGA 
were found to deviate from conventional hydrodynamic theory. 
Initially, experimental error in the light scattering results was 
suspected. However, upon further review of proteoglycan literature, 
as well as literature on star- and comb-branched polymers, it was 
noticed that similar deviations from conventional theory also occurred. 
Following this observation, a review of more recent hydrodynamic 
theories was performed, which showed that branched molecular 
systems are not adequately explained by conventional theory.

Finally, the thermal stability of both the PGS and link-stabilized 
PGA was evaluated. This represents the first time the thermal stability 
of the subunit was investigated.
DEDICATION

To my family
ACKNOWLEDGEMENTS

The author wishes to express his appreciation to Dr. Alex Jamieson and John Blackwell for their patience and guidance in this study.

The author's special thanks to Dr. Paul Scott for providing the bovine skin proteodermatan sulfate used in this study, in addition to insightful discussions.

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CHAPTER 1
PROTEOGLYCANS

DEFINITION

Proteoglycans are biological macromolecules composed of a protein core to which at least one glycosaminoglycan is covalently attached [1]. They are found in almost all mammalian tissues, but are particularly abundant in the extracellular regions of some connective tissues, such as cartilage. This general definition encompasses a broad spectrum of proteoglycan structure in which the number of glycosaminoglycan (GAG) chains per core protein may range from one (e.g. in skin proteoglycan) [2], or up to 200 (e.g. in cartilage proteoglycan) [3,4].

CARTILAGE PROTEOGLYCANS

Structure

The current structural model of cartilage proteoglycans has remain basically intact with few modifications since it was first proposed by Shatton and Schubert in the early 1950's [5]. Prior to this time, it was believed chondroitin sulfate (CS) existed freely in a matrix of collagen molecules. The negatively charged sulfate ester groups of CS were believed to interact with the positive charges on collagen [6, 7]. Shatton and Schubert proposed that the anionic polydisaccharide CS chains were not freely dispersed throughout the cartilage matrix, but rather bound to a non-collagenous protein.
This new structure was termed a proteoglycan, and is schematically shown in Figure 1.1. As will be explained below, this structure is now referred to as proteoglycan subunit (PGS).

Cartilage proteoglycans are composed of 5-10% protein and 90-95% complex carbohydrate. Pendent to the protein core are numerous GAG chains, as well as smaller sized oligosaccharides. GAG's are polyanionic polysaccharides of variable chain length that have a unique disaccharide repeat unit containing a sulfate ester group and/or a carboxyl group [8]. The three major classes of mammalian glycosaminoglycans found in cartilage are shown in Figure 1.2. Hyaluronic acid (HA), chondritin sulfate, and keratan sulfate (KS) are all found in cartilage. The latter two are each occur as components of PGS covalently attached to the proteoglycan's core protein, but are arranged in a specific order.

CS is covalently attached to the serine residues of the protein core via a tetrasaccharide unit [8] as shown in Fig. 1.3. This tetrasaccharide linkage is followed by a repeating disaccharide sequence built from glucuronic acid and N-acetylgalactosamine linked B 1-3 and B 1-4 respectively. The hexosamine is sulfated at either the 4 or 6 position [9,10]. The total length of the disaccharide unit is about 0.96 nm [11]. The average molecular weight of the CS chain isolated from bovine nasal septum cartilage is approximately 20,000 [12], which corresponds to about 45 disaccharide units.
Fig. 1. *Schematic representation of cartilage proteoglycan and link protein structure*

The domain structures of cartilage proteoglycan and link protein are shown including: Ig fold: PTR; CS1 and CS2, chondroitin sulphate attachment region sequences. Disulphide bonds are marked with dashed lines. The polypeptide chains are drawn approximately to scale.

Figure 1.1. *Schematic of a proteoglycan subunit isolated from cartilage.* Ref. [27].
Figure 1.2. The three major glycosaminoglycans found in proteoglycans isolated from cartilage. Ref. [31].
**Figure 1.3.** Structures of the linkage region between glycosaminoglycan chains and the core protein of proteoglycan. Ref. [52].
The second GAG component of cartilage PGS, keratan sulfate, is attached to the core protein via a disaccharide linkage which is connected to a serine or threonine residue of the core protein, as displayed in Figure 1.3. The disaccharide repeat N-acetylglucosamine linked B 1-3 to galactose. The galactose is linked 1-4 to another disaccharide unit. Sulfation is usually at position 6 of the hexosamine although it can also occur at position 6 of the galactose moiety [13, 14]. The molecular weight of the KS chain ranges from 2,000 to 8,000 [15, 16], which is significantly lower than that of CS.

The other cartilage GAG is HA which does not interact covalently with the protein core, and therefore is not found as a component of proteoglycan subunit. Instead, it exists as a large single strand imbedded throughout the extracellular matrix [27]. The disaccharide repeat unit consist of B 1-4 linked D-glucuronic acid followed by B 1-3 linked N-acetyl-D-glucosamine which is linked B 1-4 to another disaccharide. It does however associate non-covalently with up to 75-90 % of PGS molecules in the cartilage matrix [18, 19]. The terminal regions of the core protein allows the formation of large structures called proteoglycan aggregates (PGA). The non-covalent association of PGS and HA chain is stabilized by a specific protein called link-protein, which enhances the binding between the HA and the "hook" region of PGS [20,21]. This protein has a molecular weight of 40,000-60,000. The structure of PGA is
shown schematically in Figure 1.4, and will be discussed in greater detail later in the chapter.

Cartilage proteoglycan core protein is one of the largest proteins produced by any cell [22], with a molecular weight of 200,000 - 350,000 [23, 24]. Amino acid analysis indicate a relatively large proportion of serine, glutamic acid, glycine, and proline [23]. Five distinct regions of the core protein can be defined: (1) the G1 globular hook region, (2) the G2 globular region, (3) KS-rich region, and (4) the CS-rich region and (5) the C-terminal globular G3 lectin binding region [27]. The G1 globular -shape hook region functions to bind the proteoglycan subunit to hyaluronic acid to create the aggregate species. The G2 domain does not bind collagen or link protein, and its function remains obscure. The G1 and G2 region of the protein core comprise a molecular weight of about 60,000 - 90,000, and is devoid of glycosaminoglycans. It does, however, contain oligosaccharides linked by N-glycolyamine bonds to asparagine unit in the core [25, 26]. Following this region is an area of about 30,000 molecular weight rich in keratan sulfate (100-120 KS chains) and O-glycosidically linked oligosaccharides [27]. In the CS-rich region [27], 80-100 CS chains are attached, usually in clusters of 4-10 chains [28], to a section of protein core with a molecular weight of about 110,000. This region also contains about half the KS chains in the subunit. In addition, a few O-linked
Figure 1.4. A schematic showing the structure and dimensions of the proteoglycan aggregate. Ref.[ 33].
oligosaccharides are present. The function of the C-terminal G3 region, although highly conserved, also remains unknown. The overall molecular weight of the subunit can range from 1-4 million [29, 30]. The methods of structural characterization will be discussed later in the chapter.

Function

Cartilage is first observed when the limbs begin to extend in the developing embryo [31]. A series of complex biochemical changes occur that commit undifferentiated mesenchymal cells into phenotypically distinct cartilage cells known as chondrocytes. It is the chondrocyte that synthesizes and extrudes the proteoglycans into the extracellular matrix. In addition, chondrocytes also synthesize and release collagen into the extracellular matrix [32]. The concentration of proteoglycans is high (about 50 mg/ml [33]) and the hyrodynamic interaction between these highly anionically charged molecules results in a compression of their volume to about 20% of their fully extended volumes [34]. The electrostatic interaction between the anionic sulfate groups on the glycosaminoglycans and the dipole moment of water creates hydration layers encompassing the charged groups. In addition, water is also trapped in the interstitial network of the proteoglycan aggregate. The net result of this hydration capability is that proteoglycan can create a volume 30-50 times greater than its dry mass volume [23, 25]. As limb development progresses, the
connective tissue matrix proximal to the direction of growth becomes mineralized with crystals of calcium hydroxyapatite [35, 36, 37]. After growth of the long bones is complete (termination of adolescence), the cartilage growth plate continues to exist at the articular surface where it functions to cushion and lubricate the joint surface.

The importance of proteoglycans in limb development is illustrated by two types of genetic disorders that affect the proteoglycan structure. A recessive gene in the cartilage-matrix-deficient mouse or nanomelic chicken results in the total absence of proteoglycans in the epiphyseal cartilage (cartilage at the growth plates of bones). As a result, severe skeletal deformities occur, in which the limbs are drastically shortened [38, 39]. The second example is the skeletal deformity that occurs in the brachymorphic mouse. Here proteoglycans are present, but structurally altered in such a way as to compromise their function [40, 41]. It was discovered that the chondroitin sulfate chains of the proteoglycan were undersulfated due to a mutant gene that impairs the chondrocytes ability to synthesize the sulfation factor phosphoadenosinephosphosulfate (PAPS) [41, 42]. This sulfation factor becomes the limiting reagent in tissues where proteoglycan synthesis is high, such as the growth plates of long bones, and the resultant proteoglycan can be up to 15% undersulfated. Since it is the anionic charges on the glycosaminoglycans that are responsible for structuring large amounts of water, undersulfation will naturally
lead to a deficiency in this capacity. As a result, the average hydrodynamic volume of the proteoglycan must decrease, and it is this smaller volume that perhaps results in the shorter limb structure.

The resiliency of cartilage or its ability to return to its original shape after removal of a compressive load is due in part to the structure of the proteoglycan molecule. As discussed previously, proteoglycans are highly charged due to the carboxyl and sulfate groups on the glycosaminoglycans. These charged groups interact with water molecules to form spherical hydration layers surrounding the charged groups. When pressure is placed on cartilage, the proteoglycan-water complex is compressed, which forces the water away from the proteoglycan. As the proteoglycan is compressed further, the anionic charges are brought into closer proximity, raising the electrostatic repulsive force which prevents further compression. When the compressive load is removed, water returns to the charge domains thereby restoring the cartilage matrix to its original form.

This is the way in which nourishment supplied to cartilage differs from that for tissues which possess vascular or lymphatic pathways. In the case of cartilage, nourishment and waste is transported by the constant flux of water into and out of the tissue by periodic compression and relaxation brought about by normal body movement. Long periods of joint immobilization will cause thinning and atrophy of the cartilage layer [92].

Physical Characterization of Cartilage Proteoglycans
The physical characterization of cartilage proteoglycans has been extensively pursued over the last 20 years, following the discovery and development of a simple two-step isolation procedure by Sajdera and Hascall [43]. Subsequent characterization of bovine nasal septum proteoglycan subunit by Hascall and Sajdera [23] using sedimentation-velocity and viscosity measurements determined a sedimentation coefficient of 15-35 S, with an average of 25 S. The intrinsic viscosity, \([n]\), was determined for PGS in 4M and 0.5M GdHCl and ranged from 131 ml/g to 141 ml/g respectively. Using these data in combination with the sedimentation coefficient, and assuming a spherical shape, they calculated a weight-average molecular weight of 2.5 \( \times 10^6 \) using the Mandelkern-Flory-Scheraga equation shown below.

Bovine articular cartilage proteoglycan has been characterized by sedimentation-velocity and viscosity methods by Rosenberg et al. [44]. They report a sedimentation coefficient of 56 S for an aggregate species, while the subunit proteoglycan has a value of 16 S in 3M GdHCl and an intrinsic viscosity of 184 ml/g. The calculated weight-average molecular weight was determined to be 1.6 \( \times 10^6 \). Both these studies had to assume a spherical shape of the proteoglycan in order to calculate the molecular weight using the Mandelkern-Flory-Scheraga equation [45], with a \( \beta \) value of 2.11\( \times 10^6 \).
\[ M[\eta] = \left( \frac{kT\beta}{D_t^0 \eta_0} \right)^3 \] (1.1)

The value of the parameter \( \beta \) is theoretically derived based on the particle's shape; it has a lower theoretical limit of \( 2.11 \times 10^6 \) for a hard-sphere morphology and increases for an ellipsoid depending upon the axial ratio. Therefore any molecular weight derived using this equation must be viewed with caution because of the assumption of the conformation of the proteoglycan molecule. This limitation does not apply to light scattering which requires no a priori knowledge of molecular shape, and gives an absolute weight-average molecular weight.

Pasternack et al. [46] studied the solvent-dependent changes in bovine nasal septum proteoglycan subunit using static light scattering and viscosity measurements. Using a range of ionic strengths of 0.05-4M GdHCl, they determined that the molecular weight was constant at \( 2.3 \times 10^6 \), while the radius of gyration varied from a high of 159 nm in 0.05M GdHCl to 57 nm in 4M GdHCl. Intrinsic viscosity measurements were relatively constant, ranging from 141-151 ml/g for 0.05M - 4M GdHCl respectively. These data suggest that PGS is a rather rigid structure in low ionic strength GdHCl, but at approximately 0.3M GdHCl the conformation changes to that of a more flexible coil.
Reihanian et al. [30] investigated the hydrodynamic properties for bovine nasal septum in 0.15M NaCl. Here the weight-average molecular weight of a A1D1D1 fraction was reported to be $3.97 \times 10^6$, and represents the upper part of the size distribution. The use of the terms A1(aggregate) and A1D1(subunit) refer to the method of proteoglycan isolation when the bottom fraction of an associative density gradient, A1, is recentrifuged in a dissociative density gradient and the bottom fraction, D1, is isolated [4]. The concentration dependence of the diffusion coefficient was found to be negative in 0.15M NaCl which was attributed to the formation of multimers of the subunit. However, when this solution was examined using the analytical ultracentrifuge with Schlieren optics, just the opposite concentration dependence was observed [47]. Harper et al. [47] concluded from this observation that two populations of proteoglycans exist in low ionic strength solution: a major fraction (>95%) consisting of free subunit, and a minor fraction consisting of an aggregate form of the subunit. The minor fraction would disproportionately influence the light scattering intensity and weigh the observations towards the aggregate population.

The morphology of cartilage PGA has been studied by Rosenberg et al. using electron microscopy [48]. For bovine articular cartilage, they observed a heterogeneous population of aggregates, in which the number of subunits per aggregate varied from 19-140. These were attached at fairly regular intervals (20-30
nm) to the hyaluronic acid backbone. The HA backbone of the aggregate varied in length from 400-4000 nm., and the length of the subunit protein cores ranged from 100-400 nm.

The method of preparing the proteoglycan aggregate is vitally important in determining its structure. Ohno et al. [49] utilizing dynamic light scattering and electron microscopy, showed the distribution of subunits per aggregate varied depending upon the protocol of isolation. Aggregates isolated under associative conditions (0.4M GdHCl) were found to have a weight-average number of 111 subunits per aggregate. When this structure is dissociated in 4M GdHCl and allowed to reassociate by dialysis to 0.4M GdHCl, the number of subunits per aggregate was reduced to 12. Isolation under dissociative conditions (4M GdHCl) and then reaggregation by dialysis in 0.4M GdHCl yields a proteoglycan structure with 32 subunits per aggregate.

In earlier work, Shogren et al. [50] compared the physical properties of the chick limb bud proteoglycan subunit with the aggregate structure using light scattering methods. The weight-average molecular weight of the subunit was determined to be 1.4 x10^6, while the aggregate was 45 x10^6. Based on hydrodynamic volumes obtained from dynamic light scattering, Shogren determined that the water structuring capability of the aggregate was not greatly different from that of the subunit. They concluded that the biological function of the aggregate structure is probably to
provide a more efficient anchorage of the proteoglycans in the tissue matrix.

In viscoelastic measurements of solutions of proteoglycan subunit and aggregate at physiologic concentrations, Mow et al. [33] observed differences in their complex shear moduli: \( |G^*| = [G' + G'' ] \). Here \( G' \) is the storage modulus, which defines the energy stored in the specimen due to an applied strain, and \( G'' \) is the loss modulus, which is a measure of the energy lost or dissipated by the specimen. The ratio \( G'/G'' \) is the phase shift between the stress and strain and is called \( \tan \delta \). \( \tan \delta \) was found to be higher for the subunit than for the aggregate, suggesting that the aggregate solution dissipates less energy relative to the energy stored. Thus, the biological significance of the aggregate may be two fold: (1) anchoring the proteoglycan subunits to prevent flow out of the tissue, and (2) providing more efficient energy absorption resulting in less tissue damage. In another study, Soby et al. [91] examined the viscoelastic properties of articular cartilage preparations of proteoglycan aggregate and subunit in 0.15M NaCl at concentrations 32 and 51 mg/cc. They observed that aggregate solutions exhibited predominantly an elastic response to an applied oscillating stress, whereas the subunit exhibited primarily viscous behavior. They concluded that the presence of the aggregate leads to the formation of a transient or weak-gel network. Since, in contrast to the subunit, aggregation leads to the formation of an
increase hydrodynamic volume (compared to that for the original subunit), which results in an increase in the rotational relaxation times, the role of aggregation is to shift the linear viscoelastic response from viscous flow behavior to more elastic relaxation behavior. They conclude that since the typical shear moduli of the proteoglycan solutions are significantly smaller than that of cartilage, the role of proteoglycan aggregate in the cartilage matrix is not to support mechanical loads, but to stabilize and organize the collagen fibers. It also appears proteoglycan may provide elastomeric support, thereby enabling cartilage to store, and dissipate abrupt energy inputs. Finally, the complex shear modulus of the proteoglycan aggregate solutions (10 Nm⁻²) is much smaller than for intact cartilage (10⁶ Nm⁻²). This underscores the composite nature of the proteoglycan-collagen interaction matrix in cartilage.

PROTEOGLYCANS ISOLATED FROM OTHER TISSUES

Structure

Proteoglycans from other connective tissues are basically similar to those for cartilage, but there are significant differences in structure. These are shown schematically in Figure 1.5. As can be seen, all the types of the proteoglycans shown are consistent with the generalized definition of having at least one glycosaminoglycan covalently attached to a core protein. However, it is the differences from the large aggregating cartilage proteoglycan just discussed that highlight the diversity of the proteoglycan molecule.
Figure 1.5. Diagrammatic representation drawn to scale of some of the main proteoglycans showing the different types and diversity of structure. Ref. [53].
Thus there appear to exist two broad families of proteoglycan in connective tissue which are distinct from the well-characterized cartilage proteoglycan described above; a large proteoglycan with molecular weight of $10^6$ or greater [51], and a second family which tend to be smaller, with molecular weights of $10^5$ or less [51, 53]. The suggested nomenclature for these two families of proteoglycans is given in Figure 1.6.

The core protein of the smaller proteoglycan has a molecular weight typically around 45,000, ranging from 17,000 [54] to 56,000 [55] depending on the source tissue. There is no hook region on the protein core, which probably accounts for the fact that the smaller proteoglycans do not interact with hyaluronic acid to form aggregates [53]. Amino acid analysis indicate a relatively large amount of aspartic acid, glutamic acid, proline, glycine, leucine, and lysine, with lower relative amounts of serine and threonine [51]. Although the core proteins from the small proteoglycans isolated from various tissues appear to be similar to each other in both size and amino acid composition, immunologic distinctions are evident that suggest genetic diversity [56].

The glycosaminoglycans of the small proteoglycans are similar in size (20,000 molecular weight) to the chondroitin sulfate chains of cartilage proteoglycans, but contain a varying proportion of iduronic acid [57, 58]. These iduronic acid containing glycosaminoglycans are known as dermatan sulfate (DS)
Figure 1.6. Suggested nomenclature for glycosaminoglycan-containing proteoglycans PG. Qualifying characteristics are indicated by the letter after the PG: L, large; Sm, small; A, aggregating, N, non-aggregating; CS, chondroitin sulfate; DS, dermatan sulfate.
glycosaminoglycans, in which some of the glucuronic acid units are epimerized to iduronic acid during synthesis. The linkage of iduronic acid or glucuronic acid is by an 1,3 linkage to N-Acetylgalactosamine, which is linked B 1,4 to either glucuronic acid or its epimer iduronic acid. In addition to having a different disaccharide composition, the number of DS chains attached per core protein is significantly lower, ranging from 1-5, compared to 80-100 CS chains of the cartilage subunit.

As might be expected from their structure, the biological function of the smaller proteoglycans is much different than the larger cartilage proteoglycan.

Function

Proteoglycans from fibrous connective tissues are far less abundant: than is the case for cartilage, in which they are minor components comprising less than 1% of the dry weight of some tissues [59]. The close association of the small dermatan sulfate proteoglycans with various types of collagen has suggested that they may have a significant role in the formation and functioning of connective tissues [60]. The preferential interaction of the dermatan sulfate proteoglycan with collagen fibrils has been demonstrated both biochemically [61, 62], and morphologically [60].

Vogel et al. [63] observed the specific inhibition of fibrillogenesis of types I and II collagen by the small dermatan sulfate proteoglycan of tendon. The kinetics of collagen
fibrillogenesis in solution was studied by following the development of turbidity due to the aggregating collagen fibrils. A limitation of this technique is that initial fibril formation or kinetics can not be observed since the collagen fibrils are still relatively soluble and thus invisible to turbidity detectors. However, this region of collagen fibril kinetics can be investigated using the method of dynamic light scattering. Mixtures of the small dermalan sulfate proteoglycan with either tendon type I collagen, or cartilage type II collagen displayed significantly slower fibrillogenesis rates in comparison to solutions of collagen devoid of proteoglycans. In addition, the final absorbance was lower for the collagen mixed with proteoglycan than for pure collagen. This indicated that the size of the final collagen fibril was smaller when proteoglycans were present. Treatment of the DS proteoglycan with alkali eliminated its ability to inhibit fibrillogenesis, in contrast to treatment with chondroitinase ABC, which had no such effect. This indicates that the function of the proteoglycan is based on the core protein. This is in contrast to the findings of Obrink [64], who concluded that chondroitin sulfate does form complexes with type II collagen. The functional significance of the core protein on type I collagen fibrillogenesis was also observed by Scott [65] using circular dichroism. Both the proteoglycan and core protein were effective in inhibiting collagen fibril formation. In contrast, addition of free DS chains to collagen actually enhanced the rate of fibril formation. The retarding effect of both the
proteoglycan and the core protein was destroyed when heated in the presence of the reducing agent 2-mercaptoethanol, which breaks S-S bonds by reducing them to -SH. Heating alone (i.e. without the reducing agent) did not destroy either the inhibiting effect of the proteoglycan or the core protein. The secondary structure was not regained when either species was cooled. It was therefore concluded by Scott that the secondary structure of the core protein was not functionally significant. Analysis of the core protein prior to reduction with 2-mercaptoethanol indicated the presence of three disulfide bridges. Scott attributes these internal loop structures of the core protein to be the functional locus of the collagen interaction.

Although fibril growth rates were reduced when dermatan sulfate chains were present in solution, the overall optical densities were the same, indicating that the final fibril morphology was unchanged. Electron micrographs showed periodic binding of the proteoglycan or the core protein along the collagen fibril. The core protein appeared to be adjacent to the fibril while the DS chain extended approximately perpendicular to the fibril axis. The observed binding periodicity was similar to the D-periodicity of the collagen fibril. Scott and Orford [66] observed this phenomenon with collagen isolated from rat tail. They localized the interaction to show it occurred specifically at the d-band of the D period. The structural significance of the d-band is its location in the gap zone of the fibril.
This is a gap of roughly 40 nm in length that exist between the individual collagen molecules (commonly referred to as tropocollagen) in the fibril assembly. The importance of this region is due to two important functions: first, it is the site of collagen fibril crosslinking and second, it is where calcification occurs by deposition of hydroxyapatite crystals. The first appearance of calcification in bone for example, occurs at the gap region [67]. Tendon calcifies in a similar manner [68]. Thus dermatan sulfate proteoglycans appear to play an important function in tissue composition, by influencing both the nature of the collagen fibril structure, as well as the degree of calcification of the collagen fibril.

Physical Characterization

The isolation and physical characterization of dermatan sulfate proteoglycans has been accomplished for a number of different tissues, such as bone [69, 70], corneal stroma [71], cervix [72], colon and colon carcinoma [73], human fetal membranes [74], ligament [75], aorta [76, 77], skin [54, 55, 78-86], tendon [59, 63], sclera [87, 88], and articular cartilage [89]. A summary of the physical characteristics of some of these proteoglycans in Table 1.1. The physical characteristics of skin dermatan sulfate proteoglycan are discussed in Chapter 3. Although dermatan sulfate proteoglycans are found in many different tissues, only those isolated from sclera and cartilage will be discussed in more detail.

Scleral Proteoglycans
Many of the values shown are approximate. Abbreviation: ND, not determined.

<table>
<thead>
<tr>
<th>$M_1$</th>
<th>Glycosaminoglycans</th>
<th>M, Chain number</th>
<th>M, Iduronic acid content (%)</th>
<th>Core protein</th>
<th>Binds to HA</th>
<th>Cell/tissue</th>
</tr>
</thead>
<tbody>
<tr>
<td>70000-100000</td>
<td>1-2</td>
<td>15000-24000</td>
<td>35-85</td>
<td>40000</td>
<td></td>
<td>Skin, Sclera, Cervix, Cartilage, Tendon, Placenta, Cornea, Fibroblast, Sclera, Glial, glioma cells, Cartilage (DS-PG1), Yolk sac tumour, Embryonic cartilage, Embryonic cartilage, Follicular fluid</td>
</tr>
<tr>
<td>100000-150000</td>
<td></td>
<td></td>
<td></td>
<td>40000</td>
<td></td>
<td></td>
</tr>
<tr>
<td>100000-150000</td>
<td>1</td>
<td>55000</td>
<td>10</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>100000-150000</td>
<td>ND</td>
<td>ND</td>
<td>25</td>
<td>40000</td>
<td>+</td>
<td></td>
</tr>
<tr>
<td>100000-150000</td>
<td>ND</td>
<td>ND</td>
<td>20</td>
<td>100000</td>
<td>+</td>
<td></td>
</tr>
<tr>
<td>100000-150000</td>
<td>2.5 × 10^4</td>
<td>50</td>
<td>8</td>
<td>200000</td>
<td>+</td>
<td></td>
</tr>
<tr>
<td>70000-100000</td>
<td>1-2</td>
<td>15000-24000</td>
<td>23-36</td>
<td>40000</td>
<td></td>
<td>Cartilage (DS-PG1), Yolk sac tumour, Embryonic cartilage, Embryonic cartilage, Follicular fluid</td>
</tr>
<tr>
<td>40000-800000</td>
<td></td>
<td></td>
<td></td>
<td>30000</td>
<td></td>
<td></td>
</tr>
<tr>
<td>500000</td>
<td>8</td>
<td>40000</td>
<td>10</td>
<td></td>
<td></td>
<td></td>
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<td>420000</td>
<td>1-2</td>
<td>40000</td>
<td>12</td>
<td>2 × 120000, 1 × 100000</td>
<td></td>
<td></td>
</tr>
<tr>
<td>(2-3) × 10^4</td>
<td>20</td>
<td>56000</td>
<td>9</td>
<td>500000</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Table 1.1. Molecular characteristics for DS proteoglycans isolated from various tissues. Ref.[53].
Costner et al. [87, 88] isolated two populations of dermatan sulfate proteoglycans from bovine sclera, which they termed DSPGI and DSPGII respectively. Sedimentation-velocity and sedimentation equilibrium gave weight-average molecular weights of 160,000-220,000 for DSPGI and 70,000-100,000 for DSPGII, both dissolved in 6M GdHCl. The diffusion coefficients normalized with respect to water at 20°C were 12.5 x 10^{-8} cm^2/s and 19.4 x 10^{-8} cm^2/s for DSPG-I and DSPG-II respectively. DSPG-I contained 45% protein (dry weight), whereas DSPG-II had a 60% protein content. The number of DS chains attached per core protein was determined to be 4-5 for DSPG-I and only 1-2 for DSPG-II. A self-association phenomenon was observed in 0.15M NaCl, after which the molecular weights increased to 500,000-800,000 for DSPG-I and to 90,000-110,000 for DSPG-II. Light scattering work on solutions in 0.15M NaCl gave extremely large molecular weights for DSPG-I (3.1 x 10^6) and DSPG-II (3.4 x 10^6). These values decreased in 6M GdHCl to 410,000 and 130,000 respectively. Light scattering studies of the cleaved DS side chains revealed a weight-average molecular weight of 100,000 in 0.15M NaCl, 75,000 in 0.15M KCl, and a "monomeric" molecular weight of 24,000 in 1.0M KCl. Addition of free DS chains to a 0.15M NaCl solution of scleral proteoglycans caused a 50-60 fold increase in molecular weight [90]. Fransson et al. therefore conclude that the interaction of proteoglycans involves the DS side chains, allowing the molecules to multimerize in 0.15M
NaCl. However, the aggregates of proteoglycans and DS chains did not dissociate under identical conditions, which may suggest interaction in the proteoglycan is not solely an effect of the side-chain interaction, but may involve protein-polysaccharide or protein-protein binding.

Articular Cartilage Dermatan Sulfate Proteoglycan

In addition to the large chondroitin sulfate proteoglycans abundant in cartilage, Rosenberg et al. [89] isolated and characterized a smaller dermatan sulfate proteoglycan. Repeated recycling on Sepharose CL-4B in 4M GdHCl gave a single unimodal peak showing no indication of separating into two or more components. Using SDS-PAGE electrophoresis at low ionic strength (0.10M phosphate) the dermatan sulfate proteoglycan again appeared as a single species with molecular weight ranging from 80,000-140,000. Treatment of the proteoglycan with chondroitinase AC or ABC in the presence of proteinase inhibitors gave the core protein which had a molecular weight of approximately 45,000, as estimated by SDS-PAGE electrophoresis. What appeared as a single species in 4M GdHCl and 0.10M phosphate was shown by Rosenberg to be two distinct proteoglycans. The separation of the two was accomplished using the fact that one population, termed DS-PGI, self-associates in high ionic strength (0.375M Tris), while the other population, termed DS-PGII, did not. The separation by gel electrophoresis in 0.375M Tris
gave an apparent molecular weight of 165,000-285,000 for DS-PGI, while the DS-PGII ranged from 87,000-120,000. The latter value indicates that DS-PGII does not aggregate at high ionic strengths. The core protein for DS-PGI had a molecular weight of 44,000 based on SDS-PAGE electrophoresis. The DS-PGII core protein showed two molecular weights of 44,000 and 47,000 respectively. The immunological identities of DS-PGI and DS-PGII were investigated by monoclonal and polyclonal antibodies which showed that they had different core proteins. At this point it appears that DS-PGI and DS-PGII represent two different species of dermatan sulfate proteoglycans. Light scattering characterization of these two populations of DSPG, as well as the DS chain was performed by Soby [93]. Results for DSPG-I in 4M GndHCl gave a Mw=77,889, and Rgz=13.5 (nm). Dynamic light scattering analysis indicated the presence of two population sizes: (1) Rh=4.98 nm, and (2) Rh=8.86 nm. For DSPG-II, the Mw=91,390 which reduced to 71,246 when corrected for polydispersity. Rg=19.1 nm. Again, dynamic light scattering analysis revealed the presence of two distinct population sizes, with (1) Rh=5.33 nm, and (2) Rh=16.6 nm.

Thus, although the physical characteristics of dermatan sulfate proteoglycans isolated from many different tissues vary (see Table 1.1), a general structure and biochemical picture emerges: The DSPG's have lower molecular weights as reflected by a smaller core protein, and fewer glycosaminoglycan side chains; vary with
the ability to associate with themselves, but not with hyaluronic acid; and they appear to have functional significance in influencing collagen fibrillogenesis and calcification of tissues by their close interaction with collagen.

The fact that up to 50-60% of these smaller proteoglycans is protein makes them difficult to characterize by methods such as size exclusion chromatography and gel electrophoresis. These methods need calibration with standard molecules of known molecular weight and shape. The hybrid structure of dermatan sulfate proteoglycans can also make molecular weight determination ambiguous, since the column or gels are either standardized with globular proteins, collagen, or glycosaminoglycans. The spatial interaction of these molecules with the three dimensional matrix of the separating medium (i.e. Sepharose 4B) will be different from each, as well as the proteoglycan in which two structurally different species (coil protein + stiff polysaccharide) are covalently adjoined.

Other manifestation of variability may be the solvent used for isolation and characterization. As shown by Coster et al. [88], scleral proteoglycans and DS chains self-associate in 0.15M NaCl but not in some other solvents. As mentioned previously, Rosenberg [89] showed the separation of two closely related dermatan sulfate proteoglycans by adjusting ionic strength. It is therefore important and necessary to be aware of these parameters when dealing with the isolation and characterization of proteoglycans.
In this work, we have used both static and dynamic laser light scattering to characterize both the intact proteodermatan sulfate, as well as its individual components of protein core and DS chain. The advantage of using light scattering is that it needs no calibration with standard molecular weight species, since it gives an absolute molecular weight value. In addition, unlike GPC measurements, we are able to further characterize these DS proteoglycans with respect to size and shape. These points are addressed further in Chapter 3 of this thesis.
REFERENCES

   Ginsberg, Wiley, N.Y..
   Proc., 4th Int. Symp. on Glycoconjugates, Ed. Gregory, I.,
   Jenaloz, R., Academic Press, N.Y.
   Frederiksen, D.W., Academic Press, N.Y.
    Connective and Skeletal Tissue, Ed. Engle, A., Larson, T.,
    Nordiska Bokhaneln, Stockholm.
    Soc. Trans., 18, 198.
    165, 427.
30. Reinhanian, H., Jamieson, A.M., Tang, L.H., Rosenberg, L.
    (1979) Biopolymers, 18, 1727.
    Alan, R. Liss, Inc., N.Y.
32. Tanzer, M.L. (1982) Collagen Biosynthesis and Degradation, 2,
    237.
    252, 3617.
    U.S.A., 69, 3385.
39. Rittenhouse, E., Dunn, L.C., Cookingham, J., Calo, C.,
Exp. Morph., 43, 71.


Ginsberg, U., Robinson, P., vol 1, Wiley, N.Y.


70. Fisher, L.W., Termine, J.D., Dejter, S.W. Jr., Whitson, S.W.,
    Yanagishita, M., Kimura, J.H., Hascall, V.C., Kleinman, H.K.,
72. Uldbjerg, N., Malmstrom, A., Ekman, G., Sheehan, J., Ulmsten,
74. Brennen, M.J., Oldberg, A., Pierschbacher, M.D., Rouslahti, E.
    Biol. Chem., 258, 5679.
77. Ehrlick, K.C., Radhakrishnamurthy, B., Berenson, G.S. (1975) J.
    Biochem. Biophys., 171, 361.
    257, 5523.
    132.
83. Pearson, C.H., Winterbottom, N., Fackre, D.S., Scott, P.G.,
CHAPTER 2
LIGHT SCATTERING THEORY

STATIC LIGHT SCATTERING [1, 2]

When a particle in space is subject to a polarized electromagnetic field, \( E \), its surrounding electrons are displaced in one direction, while the positively charged nucleus is displaced in the opposite direction. This separation of opposite but equal charge creates a dipole moment \( P \), which is oriented parallel to the direction of the incident electric field \( E \), provided that the particle is isotropic, i.e.- has no inherent dipole moment due to structural asymmetry.

The strength of the electric field, \( E_0 \), will influence the magnitude of the dipole moment:

\[
P = \alpha E
\]

(2.1)

where \( \alpha \) is known as the polarizability of the particle. The general equation for the electric field of a plane-polarized beam of light in an ideal gas is:

\[
E = E_0 \cos 2\pi (ut - x/\lambda)
\]

(2.2)
where $E_0$ is the maximum amplitude, $u$ and $l$ the frequency and wavelength of incident light respectively, $t$ the time, and $x$ the location along the line of propagation.

For small particles, much smaller than $l$, the electric field will be identical at all points in the particle. Using equations (2.1) and (2.2), $P$ can be expressed as an oscillating dipole moment:

$$P = \alpha E = \alpha E_0 \cos 2\pi(ut - x/l) \quad (2.3)$$

This oscillating dipole moment is itself a source of electromagnetic radiation, and hence is the origin of light scattered by the particle. Although this scattered light is radiated symmetrically in all directions from the scattering source, its field strength is not symmetric, but depends upon the orientation with regard to the dipole moment vector. This field strength is given as:

$$E_s = \frac{4\pi^2}{v^2} \frac{\alpha E_0 \sin \phi}{c^2} \cos 2\pi(ut - x/l) \quad (2.4)$$

where $\phi$ is the angle between the dipole axis and the line joining the point of observation to the dipole, $r$ is the distance from the dipole to the observer, and $c$ is the velocity of light used for dimensional consistency. From this equation, we observe that the
scattered incident field $E_s$ has the same frequency as the incident electric field (hence the term elastic light scattering). Note that the $E_s$ becomes a function of $\sin \phi$ and $r$.

Two important assumptions are made in this derivation: (1) that the medium containing the scattering particle does not absorb the incident radiation, and (2) that the particles do not absorb the incident radiation and reemitting it at a different frequency.

Experimentally what is measured is the intensity of energy in the scattered light wave. Here the ratio of the intensity of the electric field of the scattered light to that of the incident light is of interest and was first theoretically treated by Rayleigh [3], who derived the following expression from equation (2.4):

$$
\frac{i_s}{i_0} = \frac{16\pi \alpha^2 \sin \phi}{\lambda^4 r^2}
$$

(2.5)

The polarizability, $\alpha$, can be related to the dielectric constant $D$ by:

$$
D - 1 = 4\pi N \alpha
$$

(2.6)

where $N$ is the number of particles per cc.

Using Maxwell's relation [4] between the dielectric constant and refractive index $n$:
\[ D = \tilde{n}^2 \] (2.7)
	herefore
\[ \tilde{n}^2 - 1 = 4\pi N\alpha \] (2.8)

For dilute gases, the refractive index will be close to unity (i.e., \( n = 1 \) in a vacuum) so that expansion in a Taylor series gives:

\[ \tilde{n} = 1 + (\partial \tilde{n} / \partial c) c \] (2.9)

or
\[ \tilde{n}^2 = 1 + 2(\partial \tilde{n} / \partial c) c \text{ when the term } (\partial \tilde{n} / \partial c)^2 c^2 \text{ is } \ll 1. \]

Combining equations (2.8) and (2.10) gives:

\[ \alpha = \frac{c(\partial \tilde{n} / \partial c)}{2\pi N} = \frac{M(\partial \tilde{n} / \partial c)}{2\pi N_A} \] (2.11)

where \( M \) is the molecular weight of the particles, and \( N_A \) is Avogadro's number. This gives for a single scattering particle:

\[ i_s I_0 = \frac{4\pi^2 M^2 \sin^2 \phi (\partial \tilde{n} / \partial c)^2}{N_A^2 \lambda^4 r^2} \] (2.12)
where $\phi$ is the azimuthal angle, $M$ the molecular weight, and $(\partial \tilde{n}/\partial c)$ is the refractive index increment. To obtain the total scattering per unit volume we multiply both sides of equation (2.12) by $N_p$, the number of particles per cc.

In polymer solutions in which large molecules are suspended in a medium of much smaller molecules, the light scattered from the smaller molecules become negligible when compared to the larger particles. This follows because the scattering is due to the square of the molecular weight dependence of scattered intensity as seen in equation (2.12). In addition, since the scattering medium is no longer considered ideal as in the gas of a dilute gas, the refractive index can no longer be taken as unity. Hence equation (2.8) now becomes:

$$\tilde{n}^2 - \tilde{n}_0 = 2\tilde{n}_0(\partial \tilde{n}/\partial c)c \quad (2.13)$$

and equation (2.12) becomes:

$$\frac{\hat{i}_s}{I_0} = \frac{4\pi M^2 \sin^2 \phi(\partial \tilde{n}/\partial c) \tilde{n}^2}{N_A \lambda^4 \frac{4}{r^2}} \quad (2.14)$$
A more rigorous approach to the interpretation of scattering by a polymer solution was developed by Einstein [5] and Debye [6]. Here, they considered the polymer solution as composed of small volume elements, \( \psi \), the dimensions of which are much smaller than, \( \lambda \), the wavelength of incident light. Within these small volume elements may be many solvent molecules, and a few solute molecules. Since the polymer molecules are randomly distributed and diffusing throughout the various volume elements, the concentration, \( c \), within the microenvironment of the volume element will also fluctuate. This fluctuation will occur around a mean value \( c' \), by \( \delta c \). Here \( \delta c \) can be either positive or negative.

The fluctuations in concentration will give rise to fluctuations in the refractive index, and polarizability. Hence \( \alpha = \alpha' + \delta \alpha \) where the polarizability will fluctuate by \( \delta \alpha \) around its mean value \( \alpha' \), in time. Therefore according to Rayleigh's equation (5):

\[
\frac{i_s}{i_0} = \frac{16\pi}{\lambda^4} \frac{4(\alpha' + \delta \alpha)^2 \sin^2 \phi}{r^2}
\]

(2.15)

expansion of \((\alpha' + \delta \alpha)^2 = \alpha'^2 + 2\alpha' \delta \alpha + (\delta \alpha)^2 = \alpha'^2 + (\delta \alpha)^2 \) since \((\delta \alpha) = 0\). When we deal with the excess scattering, \( i_s = i_s(\text{solution}) - i_s(\text{solvent}) \), we may write:
\[ \Delta i_{g/l_0} = \frac{16\pi}{\lambda r^2} \frac{(\delta \alpha)^2 \sin^2 \phi}{r^2 \psi} \]  
\[ (2.16) \]

Since the temperature, pressure, and solute concentration are the independent variables affecting \( \alpha \) in the volume element, the fluctuation of \( \delta \alpha \) is related to the fluctuation of these parameters:

\[ \delta \alpha = (\frac{\partial \alpha}{\partial P})_{T,c} \delta P + (\frac{\partial \alpha}{\partial T})_P \delta T + (\frac{\partial \alpha}{\partial c})_{T,P} \delta c \]  
\[ (2.17) \]

Since the first two terms are considered the same for both the solvent and solution, they may be ignores. Because \( \alpha \) is related to \( n \) by:

\[ \frac{\partial \alpha}{\partial c} = \frac{\psi_n}{2\pi} \frac{\tilde{\partial} \tilde{n}}{\tilde{\partial} c} \]  
\[ (2.18) \]

we can express equation (2.16) as:

\[ \Delta i_{g/l_0} = \frac{4\pi \psi_n^2 (\tilde{\partial} \tilde{n}/\partial c)^2 \sin^2 \phi (\delta c)^2}{\lambda r^2} \]  
\[ (2.19) \]
The value \((\delta c)^2\) is a function of the free energy, \(F\), of the polymer solution, and varies with fluctuations in the solution concentration. It therefore means that the scattered intensity will be related to the colligative properties of the solution. Hence,

\[
(\delta c^2) = \frac{kT}{(\partial^2 F/\partial c^2)_{T,P}} \quad (2.20)
\]

To calculate \((\partial^2 F/\partial c^2)_{T,P}\) we let:

\[
(\partial F/\partial c)_{T,P} = (\mu_2 - \frac{\nu_2}{\nu_1} \mu_1) \frac{\psi}{M} \quad (2.21)
\]

where \(\nu_1\) and \(\nu_2\) are the partial molal volumes of solvent and solute respectively, \(\mu_1\) and \(\mu_2\) the chemical potentials and \(\psi = n_1\nu_1 + n_2\nu_2\) where \(n_1\) and \(n_2\) are the number of moles of solvent and solute respectively.

Therefore,

\[
(\frac{\partial^2 F}{\partial c^2})_{T,P} = -\frac{\psi}{\nu_1} (\frac{\partial \mu_1}{\partial c})_{T,P} \quad (2.22)
\]

For dilute solutions, \(\nu_1 = \nu^0_1\) and
\[ \frac{-1}{v_1 kT} \left( \frac{\partial \mu_1}{\partial c} \right)_{T,P} = N_A \frac{1}{M} + 2A_2 c + 3A_3 c^2 + ... \]

(2.23)

Equation (2.19) therefore becomes:

\[ \Delta i_{s} \mu_0 = \frac{2 \pi n \hat{n} \hat{c}^2 (1 + \cos^2 \phi) c}{N_A \lambda^4 \frac{2}{1} \frac{1}{M} + 2A_2 c + 3A_3 c^2 + ...} \]

(2.24)

Experimentally, equation (2.24) may be expressed in a more convenient form. To enable this, we set:

\[ R_\theta = \frac{i_{s} r^2}{i_0 (1 + \cos^2 \phi)} \]

(2.25)

which is called the Rayleigh ratio. The difference between the Rayleigh ratios of the solution and solvent is \[ \Delta R_\theta = R_\theta(\text{solution}) - R_\theta(\text{solvent}) \], and from equation (2.24) may be written:

\[ \Delta R_\theta = \frac{kc}{\left( \frac{1}{M} + 2A_2 c + 3A_3 c^2 + ... \right)} \]

(2.26)
where:
\[
K = 2\pi n^2 \left( \frac{\partial n}{\partial c} \right)^2 (1 + \cos^2 \phi) / N A^4 
\]
(2.27)

is the optical constant. Since \( \Delta R_{\theta} = \Sigma \Delta R_{\theta} = K \Sigma c_i M_i \), where \( c_i \) is the concentration of species with molecular weight \( M_i \), the average \( M \) is the weight-average molecular weight \( M_w = \Sigma c_i M_i / c \).

For small particles whose dimensions are small in comparison to \( \lambda \), there is no angular dependence to the scattered intensity and equation (2.26) may be used to determine the weight-average molecular weight, \( M_w \), and virial coefficients, \( A_2 \), and \( A_3 \). This is accomplished using a Debye plot of \( Kc/\Delta R_{\theta} \) vs. \( c \):

\[
\frac{Kc}{\Delta R_{\theta}} = \frac{1}{M_w} + 2A_2 c + 3A_3 c^2 + \ldots 
\]
(2.28)

whence the intercept equals \( 1/M_w \) and the limiting slope is related to the second virial coefficient.

Light scattering from particles whose dimension are greater than \( \lambda/20 \) will cause destructive interference in the scattered light as the scattering angle is increased. This angular dependence in the scattered light can give information about the size and shape of the
scattering particle, and is described by the particle scattering function $P(\theta)$:

\[
P(\theta) \quad \text{Scattered intensity for large particles} \quad \text{Scattered intensity for small particles}
\]

(2.29)

Here, $P(\theta)$ will vary from unity when $\theta=0$ to values $\ll 1$ at higher angles, and decreases more rapidly with increasing size of the scattering particle. $P(\theta)$ is related to the square of the z-average radius of gyration, $R^2_{gz}$, by:

\[
L_0 \, P(\theta)^{-1} = 1 + \frac{1}{3} q^2 R^2_{gz}
\]

(2.30)

where $q$ is the wave vector given by: $q = (4\pi n/\lambda) \sin \phi/2$.

Zimm [7] proposed combining the angular dependence and concentration dependence of the excess scattered light by the relation:

\[
\frac{k c}{\Delta R_\theta} = \frac{1}{M_w P(\theta)} + 2A_2 c + 3A_3 c^2 \ldots = \frac{1}{M_w (1 + \frac{q R^2_{gz}}{3} + 2A_2 c + 3A_3 c^2 \ldots)}
\]

(2.31)
A plot of \( Kc/\Delta R\theta \) vs. \( \sin^2\theta/2 + K'c \) (where \( K' \) is an arbitrary constant used to assist graphical display of the data points) then gives a common intercept of \( M_w \). The slopes of the limiting lines of \( \theta=0 \) and \( c=0 \) give \( A_2 \) and \( R_gz \) respectively.

**DYNAMIC LIGHT SCATTERING** [8, 9, 10]

Dynamic light scattering probes the dynamics or fluctuations of the intensity of light scattered by the particles. The scattering particle is in a constant state of flux due to Brownian translational motion, rotational motion, and internal motions if it is a flexible chain molecule. The interaction of the incident electric field with such a particle causes slight phase shifts that are dependent on the magnitude of these particle motions. The resultant scattered electric field, \( E_s \), is expressed as:

\[
E_s = \sum_j A_j(t)e^{i\vec{q} \cdot \vec{r}_j(t) - iw_0 t}
\]

(2.32)

Here, \( A_j(t) \) is the amplitude of the \( j \)th scattering element, \( r_j(t) \) is the position of the \( j \)th scattering particle at time \( t \), and \( \vec{q} \) is the scattering vector, defined (Figure 2.1) as the change of the wave vector \( K \) between the incident light and scattered light.
\[ |K_0| = \frac{2\pi n}{\lambda_0} \]

\[ |K_s| = \frac{2\pi n}{\lambda} \]

\[ |K| \approx 2|K_0| \sin \Theta / 2 = \frac{4\pi n}{\lambda_0} \sin \Theta / 2 \equiv |q| \]

since \[ |K_0| \equiv |K_s| \]

Figure 2.1. Quasielastic approximation where \(|q|\) is the change in the incident wave vector \(K_0\), and the scattered wave vector, \(K_s\).
The phase factor $e^{iq \cdot r(t)}$, depends on the translational diffusion motion of the scattering centers. This temporal motion of the scattering centers then causes $E_s$ to randomly fluctuate in time, which can be examined by using the temporal autocorrelation function of $E_s$. The autocorrelation function of the scattered electric field is given as:

$$g^{(1)}(\tau) = \langle E_s(t)E_s(t + \tau) \rangle = \lim_{T \to \infty} \frac{1}{T} \int_0^T E(t)E(t + \tau) d\tau$$

(2.33)

Graphically, it has the form shown in Figure 2.2.

Since most detectors produce a signal that is proportional to the intensity of the scattered light, it is the normalized intensity autocorrelation function which is measured:

$$g^{(2)}(\tau) = \frac{\langle I(t)I(t+\tau) \rangle}{\langle I^2 \rangle} = \frac{1}{T \langle I^2 \rangle} \int_0^T I(t)I(t+\tau) d\tau$$

(2.34)

$$= \frac{\langle |E_s(t)|^2|E_s(t + \tau)|^2 \rangle}{\langle E^4 \rangle}$$

For a Poisson's distribution of scattered photons, we have [11]:
Figure 2.2 Plot of the autocorrelation function of the scattered electric field.
\[ g^{(2)}(t) = \frac{|E_S(t)E_S(t)|^2 + |E_S(t)E_S(t+\tau)|^2}{\langle E^4 \rangle} \]  

(35)

Alternatively, we may write:

\[ g^{(2)}(\tau) = 1 + |g^{(1)}(\tau)|^2 \]  

(2.36)

where,

\[ g^{(1)}(\tau) = g^{*}(1)(\tau)|g^{(1)}(0)\]

\[ g^{(2)}(\tau) = g^{*}(2)(\tau)|g^{(1)}(0)|^2 \]

Substituting the expression for \( E_S \) into that for the autocorrelation function gives:

\[ \langle E_S(t)E_S(t+\tau) \rangle = \sum_{i}^{N} A_i \exp(i \overline{q}.\overline{r}(t)-\overline{w}_o(t)) \sum_{j}^{N} A_j \exp(i \overline{q}.\overline{r}(t+\tau)-\overline{w}_o(t)) \]  

(2.37)

The simplest theoretical case involves \( N \) numbers of identical spherical particles (or non-spherical, but optically isotropic) in the
scattering volume, each with a time-independent scattering amplitude $A_i = A_j = A$. Here we have,

$$<E_s(t)E_s(t + \tau)> = N|A|^2 \langle e^{-i\vec{q} \cdot \vec{r}(t)} e^{i\vec{q} \cdot \vec{r}(t + \tau)} \rangle e^{i\omega_0(t)}$$

(2.38)

Assuming the system to be ergodic, we can equate the time-average quantity $\langle e^{i\vec{q} \cdot \vec{r}(t)} e^{i\vec{q} \cdot \vec{r}(t + \tau)} \rangle$ to an ensemble-average:

$$< \int w_c(r_o, t | r_o + R, t + \tau) e^{i\vec{q} \cdot \vec{R}_d} d^3R > r_c$$

(2.39)

where $W^c$ is the conditional probability that a scatterer located at $r_o$ at time $t$ will be in a unit volume at $r_o + R$ at time $t + T$. The $\langle \rangle_{r_o}$ means that we are dealing with an ensemble average over $r_o$. Since the system is assumed to be isotropic, and stationary, $W^c$ cannot depend on $r_o$ or $t$, therefore:

$$\langle e^{-i\vec{q} \cdot \vec{r}(t)} e^{i\vec{q} \cdot \vec{r}(t + \tau)} \rangle = \int G_s(\vec{R}, \tau) e^{i\vec{q} \cdot \vec{R}_d} d^3R$$

(2.40)
The time evolution of $G_S(R,T)$ for a system undergoing translational diffusion is:

$$\frac{\partial G_S}{\partial t} = D_t V^2 G_S \text{ (Fick's second law)}$$

(2.41)

and has the known solution of:

$$G_S(\vec{R},\tau) = (4\pi D_t \tau)^{-3/2} e^{-R^2/4D_t \tau}$$

(2.42)

Therefore:

$$<e^{-i\vec{q}.\vec{r}(t)}_e i\vec{q}.\vec{r}(t+\tau)> = e^{-D_q \tau}$$

(2.43)

It follows that:

$$<E_S(t)E_S(t+\tau)> = N|A|^2 e^{-D_q \tau} e^{-i\omega \tau}$$

(2.44)
Equating \( \Gamma = D_1 q^2 \)

gives:

\[
\langle E_s(t)E_s(t+\tau)\rangle = |A|^2 e^{-\Gamma \tau} e^{-i\omega_0 \tau}
\]

(2.45)

and:

\[
\langle I_s(t)I_s(t+\tau)\rangle = N^2(A)^2 \beta^2 [1 + e^{-2D_1 q^2 \tau}] 
\]

(2.46)

Where \( \beta^2 = e^{-2i\omega_0 \tau} \)

**ANALYSIS OF POLYDISPERSITY**

For a monodisperse solution,

\[
|g^{(1)}(\tau)| = \exp(-\Gamma \tau) \quad (2.47)
\]

where \( \Gamma = D_1 q^2 \)

For a polydisperse solution, this equation must be generalized to a sum or distribution of exponentials:
\[ \left| g^{(1)}(\tau) \right| = \int_0^\infty G(\Gamma) \text{Exp}(-\Gamma \tau) d\Gamma \]

(2.48)

with \( G(\Gamma) d\Gamma = 1 \) and \( \Gamma = \int G(\Gamma) d\Gamma \)

The distribution function of the decay rates, \( G(\Gamma) \), can be narrow, broad, or multimodal. The goal of data analysis is to characterize this distribution \( G(\Gamma) \). There are a number of methods of achieving this, but only two will be discussed here. The first is the method of cumulants, and the second a modified Laplace transform of \( G(\Gamma) \).

The Method of Cumulants:

The exact formal correspondence between the form of the correlation function \( g'(\tau) \) and the moment-generating function \( M(-t, \Gamma) \) was first developed by Koppel [12]:

\[ M(-t, \Gamma) \ \text{Exp}\langle -\Gamma, \tau \rangle = \int_0^\infty G(\Gamma) \text{Exp}(-\Gamma \tau) d\Gamma \]

(2.49)
The cumulant-generating function is defined in terms of the logarithm of the moment-generating function:

\[ K(-\tau, \Gamma) = \ln M(-\tau, \Gamma) = \ln \left| g^{(1)}(\tau) \right| \]

(2.50)

where,

\[ K(-\tau, \Gamma) = \sum K_m(\Gamma) \left( \frac{-\tau}{m!} \right)^m \]

The mth cumulant \( K_m(\Gamma) \) is the coefficient of \((-\tau)^m/m!\) in the expansion of the cumulant-generating function, and is defined as:

\[ K_m(\Gamma) = \frac{d^m}{d(-\tau)^m} K(-\tau, \Gamma) \big|_{-\tau = 0} \]

(2.51)

The cumulants of the integral may be expressed as moments about the mean:

\[ K_1 = \bar{\Gamma} \]

(2.52)

\[ K_2 = \mu_2 \]

\[ K_3 = \mu_3 \]
where the mth central moment of \( G(\Gamma) \) is defined as:

\[
\mu_m = \int_0^\infty G(\Gamma)(\Gamma - \bar{\Gamma})^m d\Gamma
\]  
(2.53)

therefore,

\[
\ln |g^{(1)}(\tau)| = -\bar{\Gamma} \tau + \frac{1}{2!} \left( \frac{\mu_2}{\bar{\Gamma}^2} \right) (\Gamma_\tau)^2 - \frac{1}{3!} \left( \frac{\mu_3}{\bar{\Gamma}^3} \right) (\Gamma_\tau)^3 + ...
\]  
(2.54)

For a monodisperse solution, the above equation reduces to:

\[
\ln |g^{(1)}(\tau)| = -\bar{\Gamma} \tau
\]  
(2.55)

and a plot of \( \ln |g(\tau)| \) vs. \( \tau \) will be a straight line with slope \( \Gamma \) from which \( D_t \) may be determined via the relation \( \Gamma = D t q \). For a polydisperse solution, the higher terms of the solution become important. These coefficients contain information about the shape of \( G(\Gamma) \). For example, the normalized second moment, \( u_2/\Gamma^2 \), is related to the variance of the distribution, while the third, and forth normalized cumulants give information about the skew and kurtosis of the distribution. For most purposes, it is the normalized second
moment which is generally reported since this will contain information about the polydispersity of the sample.

Laplace Transform Inversion of $G(\Gamma)$.

A newer method involving the inversion of the Laplace transform of $g^{(1)}(\tau)$ [13, 14, 15] gives directly the distribution $G(\Gamma)$. The method is based on eigenfunctions $\psi_w(\Gamma)$ and eigenvalues $\Gamma_w$ of the Laplace transforms of $G(\Gamma)$, which must satisfy the eigenvalue relation:

$$\int_0^\infty \psi_w(\Gamma) \text{Exp}(-\Gamma \tau) d\Gamma = \lambda_w \psi_w(\Gamma)$$

(2.56)

The distribution function $G(\Gamma)$ may then be expanded in a continuous complete set of eigenfunctions:

$$G(\Gamma) = \int_{-\infty}^{\infty} a_w \psi_w(\Gamma) d\Gamma$$

(2.57)

where $a_w$ is a set of coefficients. Inserting this equation into equation (2.48) gives:
\[ g^{(1)}(\tau) = \int_{-\infty}^{\infty} \lambda_w a_w \psi_w(\Gamma) dw \]

(2.58)

Since \( g'(\tau) \) cannot be distinguished at large extremes of \( w \), it helps to "band-limit" \( G(\Gamma) \). There the distribution can be expressed as:

\[ \overline{G}(\Gamma) = \int_{w_{\text{min}}}^{w_{\text{max}}} a_w \psi_w(\Gamma) dw \]

(2.59)

Introducing the independent variable \( \log \Gamma \), the new function \( G(\log \Gamma) \) is defined as:

\[ G(\log \Gamma) d\log \Gamma = G(\Gamma) d\Gamma \]

(2.60)

Therefore,

\[ g^{(1)}(\tau) = \int_{\log \Gamma_{\text{min}}}^{\log \Gamma_{\text{max}}} \exp(-\Gamma \tau) G(\log \Gamma) d(\log \Gamma) \]

(2.61)

which by using an interpolation formula and inverting the sum and integral signs, can be rewritten as:
\[ g^{(1)}(\tau) = \sum_{n=1}^{N} G(\log \Gamma) \exp(-\Gamma_n \tau) \]  \hspace{1cm} (2.62)

here \( G(\log \Gamma) = \sum a_n \delta(\log \Gamma - \log \Gamma_n) \) where \( a_n = G(\log \Gamma) \)

This allows an easy fitting procedure to the correlation function. Using a least-squares fit of:

\[ \sum_{i=1}^{M} [g^{(1)}(\tau_i) - \sum_{n=1}^{N} a_n \exp(-\Gamma_n \tau_i)]^2 \]  \hspace{1cm} (2.63)

yields a set of \( a_n \) vs. \( \Gamma_i \). Therefore, the relaxation distribution \( G(\Gamma) \) can be obtained.

The usefulness of this method is that little a priori knowledge of the form of the distribution function is needed, and avoids sensitivity to the extremes of the distribution by using a logarithmic scale.

**EXPERIMENTAL APPARATUS**

A diagram of the light scattering apparatus is shown in Figure 2.3. The light source used is a 15mW Helium-Neon laser with a wavelength of 632.8 nm (Spectra Physics). The laser light beam is
Figure 2.3 Schematic representation of the light scattering apparatus.
focused using a lens, L, onto a point within a quartz vat which contains the sample tube. The vat is filled with an isorefractive index medium (toluene) to reduce the incidence of stray light scattered from interface of the sample tube. In addition, the toluene bath is housed in a thermally insulated jacket that allows the temperature to be adjusted (Neslab RTE-5DD) from 20 to 80°C and maintained to within +/- 0.1°C. The scattering sample and thermoregulated bath are mounted on a BI-240 goniometer supplied by Brookhaven Instrument Corp. A photomultiplier tube is attached to a focusing lense and mounted on an arm that can swing from 10° to 160°. The signal produced from the intensity of the scattered light is amplified using a voltage amplifier, and interfaced with a 264 channel Bi-2030AT real-time digital correlator. The correlator has four set of 64 channels that allows simultaneous sampling using different delay times t. The correlator is interfaced with an IBM-compatible desktop computer. Light scattering data is analyzed using software supplied by Brookhaven Instruments. The programs GEXPSAM which uses the method of Ostrowsky [13], and CUMULANT which uses the method described by Koppel [12] were used for all data interpretation.
REFERENCES


3. Lord Rayleigh (1871) Phil. Mag., 41, 447.


CHAPTER 3
BOVINE SKIN PROTEODERMATAN SULFATE

INTRODUCTION

Proteoglycans are a family of biopolymers which consist of a central protein chain to which glycosaminoglycans are attached as sidechains, generally at serine and threonine residues. The chondroitin sulfate (CS)-containing proteoglycan (CSPG) of cartilage has been studied extensively [1-5] and is distinguished by its large molecular weight ($M_w \approx 2.5 \times 10^6$), large core protein ($M_w \approx 2.0 \times 10^5$), and the presence of a large number of CS, keratin sulfate (KS) and oligosaccharide side-chains. There is also a binding site at one end of the protein core which participates in a specific non-covalent association with hyaluronic acid (HA) to form proteoglycan aggregate (PGA). Recently a second class of proteoglycans, which are much smaller in size, have been isolated from several tissues including skin [6-10], cultured skin fibroblasts [11-13], bone [14, 15], tendon [16-18], ligament [8], sclera [19-22], cartilage [23,24], aorta [25-28], cornea [29], and uterine cervix [30], human osteosarcoma cell [53], and human post-burn hypertrophic scar tissue [54]. Their reported molecular weights range from 70,000-250,000: the core protein has reported molecular weights from 30,000 to 57,000, and the glycoaminoglycan component is dermatan sulfate (DS). The protein core has 1-5 dermatan sulfate side-chains, with molecular weights in the range of 8,000-30,000, as well as a number of pendant
oligosaccharides. This class of proteoglycans are known as proteodermatan sulfates (PDS). In contrast to cartilage CSPG, PDS does not appear to form aggregates via binding to HA, although self-association in 0.15M NaCl solution has been observed [20, 21]. The core proteins of PDS from various sources are very similar to each other, in that they are rich in Asp, Glu, Pro, Gly, Leu, and Lys, and contain moderately high amounts of Ser and Thr. Recently Chopra et al [31] showed that the PDS from bovine skin has a single DS chain located at the 4-Ser position. Heinegard et al propose the existence of two distinct classes of PDS, based on studies of their immunological cross-relativity and peptide mapping [32].

This present chapter describes the use of laser light scattering to determine the molecular weight and structural dimensions of bovine skin PDS. With the exception of one sedimentation equilibrium study [8], all of the earlier studies on skin PDS have characterized this macromolecule, and its component core protein and DS chain, by empirical techniques such as SDS-electrophoresis or size exclusion chromatography. These methods require calibration with standards of known molecular weight and shape, and hence fail to provide absolute data. This is problematical in the case of the intact PDS, which is a block copolymer in which the protein and DS components have different hydrodynamic properties.
EXPERIMENTAL

**Specimen Preparation**

PDS was isolated from mature bovine skin as described previously [33]. Core protein was prepared by digestion of the PDS with chondroitinase ABC (Miles Laboratories) as described in ref. 33, but without the use of proteinase inhibitors. DS was prepared from the amino-terminal cyanogen bromide peptide of proteodermatan sulfate. Preparation of the latter peptide, which contains the only dermatan sulfate chain in the molecule [31], is described elsewhere [34]. This material (9 mg) was dissolved in 0.1M Na acetate/0.005M EDTA/0.005M cysteine (10 ml), adjusted to pH with acetic acid and digested with papain (12.5 mg; Sigma Chemical Co.) at 65°C for 4 h. The digest was cooled on ice, and cold trichloracetic acid was added to a final concentration of 6% (w/v). The white precipitate that formed on standing for 30 min. was removed by centrifugation and washed once with cold 6% trichloracetic acid. The combined supernatants were dialyzed exhaustively against water at 4°C, clarified by centrifugation, and freeze-dried. This product was then dissolved in 7M urea, 1.5M NaCl, 0.05M Tris, adjusted to pH 7.2 with HCl, and fractionated on a 1x110 cm column of Sephacyl-300 (Pharmacia Ltd.). Fractions from this column were monitored for uronic acid [8], pooled, dialyzed exhaustively against distilled water, and lyophilized. All preparations were characterized by amino acid analysis for which
samples were hydrolyzed in 6N HCl at 100°C for 24 h and run in duplicate on a Beckman 121MB automatic amino acid analyzer. The DS chains were terminated by Asp-Glu-Ala-Ser (60%) or Asp-Glu-Ala-Ser-Gly (40%) peptides.

**Light Scattering**

PDS and core protein were dissolved separately in 4M guanidine hydrochloride (GdnHCl), 0.05M sodium acetate buffer, 0.02% sodium azide (pH 6.8), and filtered through 0.45 micron Millipore filters. The solution was centrifuged at 5,000 rpm for 30 minutes prior to light scattering analysis. Solutions of PDS, core protein, and DS in 0.15M NaCl, 0.01M TES buffer, 0.02% sodium azide (pH 7.0) were prepared in the same way. Static and dynamic light scattering experiments were carried out at 25°C using a Brookhaven BI-240 goniometer, and BI-2030AT real-time correlator with 264 channels (Brookhaven Instruments, Ronkonkoma, N.Y.). Refractive index increments were determined at 633nm using a differential refractometer RF-600 (C.N. Wood Co.): the refractive index increments for PDS, core protein, and DS in 4M GdnHCl were found to be 0.15, 0.17, 0.12 respectively. The values in NaCl were similar to those in 4M GdnHCl.

Static or total intensity light scattering uses the procedure of Zimm [35] to obtain the weight average molecular weight, \( M_w \), the second virial coefficient, \( A_2 \), and the z-averaged radius of gyration, \( R_g \). Dynamic laser light scattering probes molecular relaxations
which arise from translational or internal motions due to Brownian forces in the solution [36,37]. The autocorrelation function of the intensity fluctuations in the scattered light is related to the apparent z-average translational diffusion coefficient, $D_t$, of the molecule. Extrapolation of $D_t$ to zero scattering angle and infinite dilution gives $D^o_t$, which can be used in the Stokes-Einstein equation to obtain the z-averaged reciprocal hydrodynamic radius, $<R_{\text{h}^{-1}>}_z$, whose inverse we will henceforth refer to as $R_{\text{h}}$.

Light scattering by block copolymers is complicated by the fact that the refractive indices of the component blocks will in general be different [38,39]. The refractive index increment of the copolymer is related to its composition by the expression:

$$\frac{dn}{dc} = W_A\left(\frac{dn}{dc}\right)_A + (1 - W_A)\left(\frac{dn}{dc}\right)_B$$  \hspace{1cm} (3.1)

increments of the homopolymer components A and B in the same solvent and at the same temperature, and $W_A$ is the weight fraction of component A. Treatment of the block copolymer as a homopolymer leads to determination of an apparent molecular weight, $M^*_w$, which needs to be corrected using the following equations [38,39] to obtain the true molecular weight.
\[ M_w^* = \frac{1}{2} \left( W_A M_w^A \nu_A^2 + (1 - W_A) M_w^B \nu_B^2 + 2 \nu_A \nu_B M_w^{AB} \right) \]

in which \( M_w^A \) and \( M_w^B \) are the weight-average molecular weights of components A and B of the AB block copolymer respectively; \( \nu_A, \nu_B \), and \( \nu \) are the refractive index increments of components A, B, and the block copolymer respectively. \( M_w^{AB} \) is given as:

\[ M_w^{AB} = \frac{1}{2} \left( M_w - W_A M_w^A - (1 - W_A) M_w^B \right) \]

in which \( M_w \) is the true weight average molecular weight of the block copolymer.

An expression by Leng and Bencit [40] allows the determination of the true radius of gyration for a polydisperse block copolymer:

\[ M_w^* R_g^*^2 = M_w \frac{\nu_A \nu_B}{\nu^2} R_g^2 + W_A M_w^A \frac{(\nu_A - \nu_B)^2}{\nu^2} \nu_A R_g^2 + (1 - W_A) M_w^B \frac{(\nu_B - \nu_A)^2}{\nu^2} \nu_B R_g^2 \]

\[ (3.4) \]

in which \( R_g^*^2 \) is the apparent mean-square radius of gyration obtained from the light scattering experiment, and \( R_g^2_A \) and \( R_g^2_B \) are the mean-square radii of gyration for the two components A and B of the block copolymer.
RESULTS

Table 3.1 lists the values determined for $M_w$, $R_g$, $A_2$ (the second virial coefficient), $D^o_t$, $R_h$, and $\mu_2/\Gamma^2$ (the second moment of the correlation function of dynamic light scattering) for the specimens of PDS, core protein, and DS dissolved in 4M GdnHCl or 0.15M NaCl. Zimm plots for PDS and core protein in 4M GdnHCl are shown in Figures 3.1 and 3.2. These data yield $M_w = 65,000$ and $R_g = 51\text{nm}$ for PDS and $M_w = 39,000$ and $R_g = 34\text{nm}$ for the core protein in this solvent. The second virial coefficient, $A_2$, is small and negative for each species. The equivalent Zimm plots for PDS and core protein dissolved in 0.15M NaCl are shown in Figures 3.3 and 3.4. These data yield appreciably larger molecular parameters: $M_w = 610,000$ and $R_g = 87\text{nm}$ for PDS, and $M_w = 650,000$ and $R_g = 88\text{nm}$ for the core protein. For this solvent, the $A_2$ values are small but positive for both specimens. A Debye plot for the specimen of DS dissolved in 0.15M NaCl is shown in Figure 3.5 and yields $M_w = 24,000$. Insufficient DS sample was available to determine $D^o_t$.

The translational diffusion coefficients $D_t$ were derived from the dynamic light scattering data for solutions of PDS and core protein in 4M GdnHCl. $D_t$ extrapolated to zero concentration in Figures 3.6 and 3.7, yield $D^o_t = 15.0 \times 10^{-8} \text{cm}^2/\text{s}$ for PDS and $20.5 \times 10^{-8} \text{cm}^2/\text{s}$ for core protein, respectively. Using the Stokes-Einstein equation, $R_h$ is calculated to be 13nm for PDS, whereas the protein core has $R_h =$
Table 3.1 Molecular parameters for bovine skin proteoglycan, core protein, and dermatan sulfate.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Solvent</th>
<th>$M_w \times 10^4$</th>
<th>$R_g$ (nm)</th>
<th>$A_2 \times 10^4$</th>
<th>$D \times 10^8$ cm$^2$/s</th>
<th>$R_h$ (nm)</th>
<th>$\frac{\mu^2}{I^-}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>PDS</td>
<td>4M GdnHCl</td>
<td>6.5 (6.2)*</td>
<td>51 (53)*</td>
<td>-2.5</td>
<td>15.0</td>
<td>13.0</td>
<td>0.31</td>
</tr>
<tr>
<td>Core protein</td>
<td>4M GdnHCl</td>
<td>3.9</td>
<td>34</td>
<td>-1.3</td>
<td>20.5</td>
<td>9.8</td>
<td>0.25</td>
</tr>
<tr>
<td>PDS</td>
<td>0.15M NaCl</td>
<td>61.0</td>
<td>87</td>
<td>1.0</td>
<td>7.4</td>
<td>32.0</td>
<td>0.35</td>
</tr>
<tr>
<td>Dermatan Sulfate</td>
<td>0.15M NaCl</td>
<td>2.4</td>
<td>-</td>
<td>2.7</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Core protein</td>
<td>0.15M NaCl</td>
<td>65.0</td>
<td>88</td>
<td>0.63</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

*with Copolymer Correction
Figure 3.1
Zimm plot of intensity measurements for bovine skin PDS in 4M GdHCl. Temp. 25 C. Mw= 65,000, Rgz= 51 nm, and A2=-2.5x10^-4 mole-cm^3/gm.
Figure 3.2

Zimm plot of intensity measurements for bovine skin PDS core protein in 4M GdCl. Temp. 25°C. Mw = 39,000, Rg = 34 nm, A2 = -1.3 x 10^-4 mole-cm^3/gm.
Figure 3.3  
Zimm plot of intensity measurements for bovine skin PDS in 0.15M. Temp. 25 C. Mw= 610,000, Rgz= 87 nm, A2 =1.0 x 10^-4 mole-cm^3/gm.
Figure 3.4  Zimm plot of intensity measurements for bovine skin PDS core protein in 0.15M NaCl. Temp. 25 C. 
Mw= 650,000, Rgz= 88 nm, A2=0.63 x10^{-4} mole-cm^{3}/gm.
Figure 3.5  Debye plot of intensity measurements for bovine skin DS in 0.15M NaCl. Temp. 25°C. Mw=24,000 and A2=2.7 x 10^-4 mole-cm^3/gm.
Figure 3.6. Plot of the translational diffusion coefficient as a function of concentration for bovine skin PDS in 4M GdHCl. Temp. 25 C. Extrapolation to zero concentration yields $D_t^0 = 15 \times 10^{-8} \text{ cm}^2/\text{sec}$, and $R_h = 13 \text{ nm}$. 
Figure 3.7. Plot of the translational diffusion coefficient as a function of concentration for bovine skin PDS core protein in 4M GdCl. Temp. 25 C. Extrapolation to zero concentration yields $D_{t0} = 20.5 \times 10^{-8}$ cm$^2$/sec, and $R_h = 9.8$ nm.
9.8 nm. Both plots have negative slopes, consistent with the negative second virial coefficients determined from their respective Zimm plots. The equivalent data for PDS in 0.15 M NaCl (Figure 3.8) yield $D^c_t = 7.4 \times 10^{-8}$ cm$^2$/s and $R_h = 32$ nm; the concentration dependence of $D^c_t$ is positive, in accord with its positive $A_2$ from total intensity light scattering. Insufficient specimens were available for measurement of $D^c_t$ for the protein core in 0.15 M NaCl solution.

**DISCUSSION**

The static and dynamic light scattering results are consistent in that they indicate PDS and its core protein self-associate in 0.15 M NaCl. In addition, the raw $M_w$ values for PDS and its core protein 4 M GdnHCl suggest that we may be dealing with a monomeric preparation in this solvent. The difference of 26,000 between the molecular weights of PDS and core protein in 4 M GdnHCl is approximately the same as the value of $M_w = 24,000$ for the DS component in 0.15 M NaCl. This is consistent with the proposed structure for PDS consisting of a core protein and a single DS chain [31]. The result for the DS component also indicates that DS does not self-associate in 0.15 M NaCl, in contrast to PDS and the core protein, which implies that the self-association of PDS is due to the protein component.

$M_w$ and $R_g$ for PDS in 4 M GdnHCl are raw values in that we need to take account of the block copolymer structure in which the
Figure 3.8
Plot of the translational diffusion coefficient as a function of concentration for bovine skin PDS in 0.15M NaCl. Temp. 25°C. Extrapolation to zero concentration yields D \text{Dz} = 7.4 \times 10^{-8} \text{ cm}^2/\text{sec}, and Rh = 32 nm.
protein and DS chains have different optical properties [38,39]. Taking 0.61 and 0.39 as the weight fractions of protein and DS, 65,000 for the raw $M_w$ of PDS, and $M_w = 39,000$ and $M_w = 24,000$ for the protein core and DS components, respectively, we obtain (via equations 3.2 and 3.3) a value of 62,000 for the corrected $M_w$ for PDS. The correction is small, i.e. the block copolymer structure distorts the measurement of molecular weight by less than 5%, which is comparable to the experimental uncertainty.

The corrected $R_g$ for PDS in 4M GdnHCl was calculated using equation 3.4. It was impossible to determine $R_g$ for DS by light scattering methods and therefore a calculated value was obtained using the Kratky-Porod [41] model for stiff worm-like chains:

$$R_g^2 = \frac{2q^2}{y^2} \left( \frac{y^3}{6} + \frac{y^2}{2} + y - 1 + \exp(-y) \right)$$

(3.5)

The persistence length $q$ is estimated to be 4nm [42], and the contour length $L = Yq = 55.7$nm is computed from $Y$, the number of disaccharide units (53), and $l$, the length of one disaccharide unit (1.05nm). Substituting these values into Eq. 3.5, we obtain a calculated mean-square $R_g^2$ of 65 nm$^2$ for the DS. This value can then be inserted into Eq. 3.4 to obtain the corrected root mean-square $R_g$ of 53nm for PDS, which is within experimental error of the apparent value of 51nm. Thus no significant anomalies occur in the
static light scattering properties of PDS due to its block copolymer character.

In previous work on the same bovine skin PDS, Pearson and Gibson [8] determined $K_{av} = 0.89$ on a Sepharose 2B Column. This corresponds to $M_w = 62,000$ using the molecular weight - $K_{av}$ calibration equation derived by Ohno et al [4] for cartilage proteoglycans on Sepharose 2B, which is in excellent agreement with our corrected molecular weight for PDS. It is also interesting to compare our measured molecular weights with those reported previously, based mainly on empirical measurements. Damle et al studying pig skin PDS [9] determined a molecular weight of 70,000 using sedimentation equilibrium. The protein core comprised 60% of the molecule, with a molecular weight of 44,000 determined by SDS-gel electrophoresis, which dropped to 41,000 following treatment with HF to remove all oligosaccharides. Silbert [43] recently isolated a PDS from human skin fibroblast in which the core protein has $Mr = 45,000$ before and 40,000 after removal of the oligosaccharides. Glossl [12] determined $M_w = 38,000$ for the core protein of human skin fibroblast proteodermatan sulfate. Less consistent are the results of Fujii and Nagai [6] who obtained molecular weights of 115,000 for calf skin PDS, 56,000 for the core protein and 17,000 for the DS, using SDS-gel electrophoresis. They proposed a model for the PDS in which 3-4 DS chains are attached to the core protein. Rat skin PDS has been characterized by Miyamoto
and Nagase [7] using gel chromatography, who reported $M_w = 36,000$ for PDS and 23,000 for the DS.

Light scattering methods have been applied previously by Fransson et al [10] to determine $M_w$ for pig skin DS. They report $M_w = 30,000$ and 25,000 for two specimens of pig skin DS containing 90 and 75% iduronic acid respectively. These figures are in good agreement with the value of 24,000 obtained in this study. Analysis of the PDS used in this investigation (P.G. Scott, unpublished data) showed the presence of 39 moles of galactosamine per mole of protein. Given that there is only one DS chain in the molecule [31], this would correspond to $M_n \sim 18,000$.

It is interesting that the molecular weights reported by Fransson et al were for DS in 0.15M KCl. When the same two specimens were dissolved in 0.15M NaCl, large aggregates were formed with $M_w = 224,000$ and 322,000 respectively. Using the technique of affinity chromatography to study DS:DS interaction [44], this phenomenon was found to be most pronounced for oligosaccharides containing approximately equal proportions of iduronosyl and glucuronosyl residues, especially when these two uronic acids alternated in the polymer sequence. Iduronic acid comprises 76% of total hexuroniate in the DS chain of mature bovine skin PDS [8], an almost identical content to that of the self-aggregating pig skin DS [10]. It is therefore perhaps surprising that the sample of DS studied here did not appear to self-aggregate in 0.15M NaCl. No definitive explanation can be
given. It is possible that our DS and that studied by Fransson and co-workers differ in arrangement of iduronosyl and glucuronosyl residues. We have no data on this. It may be relevant to note that the DS used here was obtained from a purified PDS (in fact a pure GAG-bearing peptide of this PDS) rather than from intact skin, so that a self-aggregating fraction, or a component promoting aggregation, of DS may have been lost during preparation.

In contrast to the behavior of DS in 0.15M NaCl, the high molecular weights of PDS (610,000) and the PDS core protein (650,000) reflect extensive self-association. Self-association in 0.15M NaCl has also been demonstrated for bovine scleral PDS [20]: molecular weights determined by light scattering for two proteodermatan sulfates, PDS-I and II, were 3.1 and 3.4 x 10^6 respectively, and a mixture of PDS-II and DS had M_w = 85 x 10^6. The apparent self-association of the small PDS from bovine sclera was also recently observed in the electron microscope [45].

The values of R_g and R_h for PDS and its core protein are in each case significantly larger than would be expected based on their measured molecular weights. In addition, the values of the ratio \( \rho = R_g/R_h \) are larger for PDS (\( \rho = 3.9 \)) and the PDS core protein (\( \rho = 3.5 \)) than seem reasonable. A similar anomaly is implicit in the earlier light scattering study of bovine scleral PDS by Coster et al [20]. If the protein is modeled as a gaussian random coil, the radius of gyration can be estimated from \( R_g^2 = C^\infty (M_w/M_0) 1^2/6 \), where \( C^\infty \) is the
characteristic ratio, which has a value of about 9 for a random coil protein containing 20% glycine residues [46]; \( l \) is the length of the peptide bond and is taken to be 3.8\( \AA \) [46]; \( M_0 \) is the average monomer molecular weight, taken as 115 (neglecting the oligosaccharides). Substitution of these values into the expression for \( R_g^2 \) leads to \( R_g = 9 \text{nm} \), which would correspond to \( R_h = 6 \text{nm} \) using the Kirkwood-Riseman equation \( (R_g = 1.51 \cdot R_h) \) for a flexible coil. These computed values are comparable to the experimental radii seen for protein random coils of similar molecular weight [47]. The observed \( R_g \) and \( R_h \) for the monomeric core protein in 4M GdnHCl, deduced from our studies (Table 3.1) are greater by a factor of 4 for \( R_g \) and 1.6 for \( R_h \). Thus, allowing for a degree of chain expansion due to excluded volume, the experimental value of \( R_h \) seems physically reasonable, but that of \( R_g \) is too large.

The likely source of this effect is the presence of trace amounts of PDS and core protein aggregates in 4M GdnHCl. To see this we have simulated the effect of such aggregates on the light scattering results using the equations

\[
M_w = w_1 M_1 + w_2 M_2
\]

\[
R_g^2 = M_w^{-1} \left( w_1 M_1 R_{g1}^2 + w_2 M_2 R_{g2}^2 \right)
\]  

(3.6)  

(3.7)
\[ R_h = \frac{M_w R_{h1} R_{h2}}{(w_1 M_1 R_{h2} + w_2 M_2 R_{h1})} \]  

\[ \frac{\mu_2}{\Gamma^{-2}} - 1 = \frac{(R_h)^2 (w_1 M_1 R_{h2} + w_2 M_2 R_{h1})}{M_w R_{h1} R_{h2}} \]

where \( w_1 \) and \( w_2 \) are the weight fractions of monomer and aggregate in the solution \( (w_1 + w_2 = 1) \). It is easy to show that if the contribution of the aggregates \( w_2 M_2 \) to the measured \( M_w \) is sufficiently small as to be within the experimental error \( (\pm 5\%) \), \( R_h \) deviates from the monomeric value \( (R_{h1}) \) by no more than 10\%, whereas \( R_g \) can deviate by a much larger amount \( (\sim 300\%) \). Values of \( R_g, R_h, M_w \) and \( \mu_2/\Gamma^{-2} \) for PDS and its core protein were estimated from equations (3.6)-(3.9) assuming: a) \( M_1 (\text{PDS}) = 59,000 \) and \( M_1 (\text{core}) = 35,000 \), values consistent with the known composition of PDS \( (60\% \text{ protein, } 40\% \text{ carbohydrate}) \), and the measured value of \( M_w = 24,000 \) for the DS chain; b) \( w_1 = 0.995 \) and \( w_2 = 0.005 \) for both PDS and its core protein; c) \( R_{h1} (\text{PDS}) = 12\text{nm} \) and \( R_{g1} (\text{PDS}) = 18\text{nm} \), and \( R_{h1} (\text{core}) = 9\text{nm} \) and \( R_{g1} (\text{core}) = 13\text{nm} \); d) \( M_2 (\text{PDS}) = 1.4 \times 10^6 \), \( R_{g2} (\text{PDS}) = 150\text{nm} \), and \( R_{h2} (\text{PDS}) = 100\text{nm} \); e) \( M_2 (\text{core}) = 840,000 \), \( R_{g2} (\text{core}) = 100\text{nm} \) and \( R_{h2} (\text{core}) = 65\text{nm} \). The ratio \( R_g/R_h \) for each species has been taken to be equal to 1.5, the value for flexible coil molecules. We determine for PDS, \( M_w = 65,700 \), \( R_g = 51.8\text{nm} \), \( R_h = 13.2\text{nm} \) and \( \mu_2/\Gamma^{-2} = 0.08 \) while, for the core protein, we obtain \( M_w \)
= 39,000, \( R_g = 35nm \), \( R_h = 9.9nm \) and \( \mu_2/\Gamma^2 = 0.08 \). Clearly these simulated values are in good agreement with the experimental results in Table 3.1. The measured second moments \( \mu_2/\Gamma^2 \), are slightly larger than the calculated values, which may be due to polydispersity of the PDS monomer. This calculation illustrates that the effect of trace amounts of aggregates on the measured \( R_g \) is large but is small for \( R_h \).

We note that it is also possible that the protein core exists in a relatively expanded conformation because of its unusual peptide composition, which contains a high percentage (\( \sim 30\% \)) of charged amino acids, and because of the presence of three oligosaccharide side chains (P.G. Scott, unpublished work). In studies of the conformation of a number of mucin glycoproteins by light scattering, Shogren et al [48] observed the same kind of behavior: specifically, log-log plots of \( R_g \) or \( R_h \) vs. fractional mucin protein molecular weight were linear, but were found to be translated to larger radii by a factor of \( \sim 2.7 \) when compared to similar plots of denatured random coil proteins. However, in these glycoproteins, the value \( R_g/R_h \) is close to the random coil value (\( R_g/R_h \sim 1.7 \)). Thus we feel the large \( R_g \) values for PDS are more likely an indication of residual aggregation. Recently, the small proteodermatan sulfates, DSPG-I and DSPG-II, from bovine articular cartilage were examined by light scattering methods [55]. The DSPG-I and DSPG-II had \( M_w \) of 77,889 and 91,390 in 4M GndHCl respectively. The latter value dropped to
71,246 when corrections for polydispersity were made. The \( R_g \) for DSPG-I was 13.5 nm, while for DSPG-II it was 19.1 nm. Interestingly, both species had a bimodal hydrodynamic size distribution, as determined from dynamic light scattering. Results for DSPG-I gave a \( R_h(1) \) of 4.98 nm, and \( R_h(2) \) of 8.89 nm. Conversely, DSPG-II gave a \( R_h(1) \) of 5.33 nm and \( R_h(2) \) of 16.6 nm. The DS chain had a \( M_w \), \( R_g \), and \( R_h \) in 4M GdnHCl of 33,160, 34.5 nm, and 10.6 nm respectively, while in 0.15M NaCl, the same structural parameter values were 43,770, 33.2 nm, and 11.5 nm respectively. Thus the DS chain did not self-associate in either solvent.

Finally, it is of interest to compare the light scattering data for PDS and its core protein in 4M GdnHCl with those predicted for structural models. Unfortunately, the effect of polydispersity precludes an interpretation of the \( R_g \) data. However, it is possible to compare the observed \( R_h \) values with those computed from theoretical models. One particular "lollipop" structure has been proposed for PDS based on electron microscopy observations [49]. \( R_h \) for such a model can be estimated using theoretical expressions for the translational frictional coefficient of rigid macromolecular complexes composed of \( N \) spherical subunits [50]. The lollipop conformation is modeled as a large sphere of hydrodynamic radius \( \sigma_1 \), to which a rod-like tail or stem is attached, composed of a string of \( N-1 \) spheres of hydrodynamic radius, \( \sigma_2 \). The frictional coefficient, \( f_0 = 6\pi\eta_0 R_h \) is given by
\[ f_0 = 6\pi \eta_0 \sigma_1 \left( 1 + \sum_{i=1}^{3} \sum_{j=1}^{3} b_{ij} \left( \frac{L}{\sigma_1} \right)^i \left( \frac{\sigma_1}{\sigma_2} \right)^{j-1} \right) \] (3.10)

in which \( L \) is the length of the tail (\( L = 2(N-1)\sigma_2 \)). The values of the \( b_{ij} \) coefficients are given in ref. 50 for the lollipop model.

The radius of the large sphere is taken, of course, to be \( \sigma_1 = 9.8 \text{nm} \), corresponding to the hydrodynamic radius obtained experimentally for the core protein monomer in 4M GdnHCl. Unfortunately, because of limited sample, we were unable to obtain a comparable value for the DS chain. However, reasonable estimates for the dimensions to be used for the DS chain can be inferred from our knowledge of the polysaccharide chain statistics. As noted above, the most likely conformation is that of a worm-like coil of contour length \( L \), and persistence length \( q \). Yamakawa and Fujii [51] have derived an expression for the hydrodynamic values of non-draining wormlike coils which may be expressed in simplified form

\[ R_h^{-1} = \left( \frac{2}{L} \right) \left[ A_1y + A_2 + A_3y^{-1/2} + A_4y^{-1} + A_5y^{-3/2} \right] \] (3.11)

where \( L = yq \), and the \( A_i \) are themselves functions of \( (dq^{-1}) \), where \( d \) is the thickness of the coil. Using values specified earlier in connection with eq. (3.5), \( L = 55.7 \text{nm} \), \( q = 4 \text{nm} \), and taking the thickness of the chain to equal the diameter of one saccharide unit,
\[ d = 0.525\text{nm} \text{ we estimate } R_h = 4.3\text{nm for a DS chain of } M_w = 24,000. \text{ This is to be compared with a } R_g = 8\text{nm determined form eq.}(3.5). \text{ It is also pertinent to note that previous light scattering studies of a DS chain of } M_w = 27,000 \text{ by Jamieson } et al \text{ [52] determined } D_t^o = 3.2\times10^{-7} \text{ cm}^2/\text{s in water at } 20^\circ\text{C} \text{ and hence would yield } R_h = 5.6\text{nm via the Stokes-Einstein equation. We may thus set } N = 2 \text{ and } \sigma_2 = 4.3\text{nm or } \sigma_2 = 5.6\text{nm in eq.} \,(3.10). \]

It is found that we can neglect all terms in eq.(3.10) beyond \(b_{11}\) and thus eq.(3.10) reduces to

\[
\left(\frac{R_h}{\sigma_1}\right) = 1 + 2b_{11}\left(\frac{\sigma_2}{\sigma_1}\right) \tag{3.12}
\]

with \(b_{11} = 0.1052\). Inserting \(\sigma_1 = 9.8\text{nm and } \sigma_2 = 4.3\text{nm or } \sigma_2 = 5.6\text{nm leads to } R_h = 10.7\text{nm or } R_h = 11.0\text{nm respectively, which is slightly smaller than the observed value } \left(\text{R}_h = 13\text{nm}\right). \text{ Thus it is possible that the dimensions of the DS and/or protein components linked together in the PDS molecule may be somewhat expanded over the free values, perhaps because of mutual repulsions of the chemically dissimilar components.}

**CONCLUSIONS**

Laser light scattering data leads to absolute molecular weights of 62,000 for PDS, and 39,000 and 24,000 for the component protein core and DS core protein, respectively. The ratio of the radius of
gyration to the hydrodynamic radius for both the proteodermatan sulfate and core protein under denaturing conditions are large relative to those for non-glycosylated random coil proteins of comparable molecular weights. These effects are most likely due to the polydispersity resulting from the presence of a small fraction of aggregates in 4M GdnHCl solution. In 0.15M NaCl both the proteodermatan sulfate and core protein form large self-associating aggregates of molecular weight 610,000 and 650,000 respectively, which is in contrast to the dermatan sulfate, which remains monomeric in this solvent (24,000). It is concluded that the self-association of PDS occurs via interaction of the protein core. Model calculations suggest that the hydrodynamic properties of PDS in 4M GdnHCl are consistent with a "lollipop" conformation consisting of an expanded globular protein head and a worm-like coil DS tail.
REFERENCES


CHAPTER 4
HYDRO_DYNAMIC PROPERTIES OF CARTILAGE
PROTEOGLYCANS

INTRODUCTION

Dynamic and static laser light scattering methods were employed in conjunction with viscosity measurements to determine the Mandelkern-Flory-Scheraga (MFS) parameter $\beta$, for bovine nasal septum (BNS) proteoglycan subunit (PGS) in 4M GndHCl and in 0.15M NaCl. By way of comparison, the MFS parameter was also determined for the proteoglycan aggregate (PGA) by similar methods in 0.15M NaCl solution.

The ratio of the hydrodynamic radii obtained from the intrinsic viscosity ($R_{\eta}$) and the translational diffusion coefficient ($R_{D}$), $\rho_2 = \frac{R_{\eta}}{R_{D}}$, was determined, as well as the radius ratio $\rho_1 = \frac{R_{g_z}}{R_{D}}$, where $R_{g_z}$ is the z-average radius of gyration obtained from static light scattering, and $R_{D}$ is the reciprocal z-average Stokes radius, $\frac{1}{R_{D}}$.

For branched-chain molecules, $\rho_1$ and $\rho_2$ are known to depend numerically on branch frequency, branch type, and molecular polydispersity. Even after correction for polydispersity, our experimental results for both PGA and PGS give $\beta$ and $\rho_2$ values significantly below the hard-sphere limit of conventional hydrodynamic theory.
A review of proteoglycan literature was performed in order to determine if such hydrodynamic behavior had been previously observed and if any explanation had been proposed. In addition, a review of the experimental hydrodynamic properties of synthetic star-branched polymers was carried out for further comparison with our results.

Lastly, recent theories developed to explain the effects of branching on hydrodynamic behavior are discussed as well as Monte Carlo simulations. Each approach appears to better approximate the spatial and relaxation characteristics of the synthetically produced or computer-generated branched structures than the older and often used Kirkwood-Riseman (KR) theory. These more recent theories have found values for \( \beta \) and \( \rho_1 \) and \( \rho_2 \) below the lower limits set by the KR model. This may indicated that our results are not inconsistent with recent hydrodynamic modeling.

EXPERIMENTAL

Specimen Preparation: Bovine nasal septum proteoglycan subunit was isolated and prepared as previously reported [2]., giving subunit labelled as A1D1-CL2B. This designation indicates a specimen which has been purified by density gradient centrifugation under associative , followed by dissociative conditions, and subsequently isolated as an excluded fraction following gel permeation chromatography on a cross-linked (CL) Sepharose 2B
analytical column. The aggregate sample was obtained as the void volume fraction on a Sepharose 2B column and thus is designated A1-2B. Lyophilized samples of each were weighed on a Perkin-Elmer Autobalance AD-2 to within a 10th of a milligram.

Light Scattering: The subunit A1D1-CL2B was dissolved in 4M guanidine hydrochloride (GdHCl), 0.05M sodium acetate (NaOAc), 0.02% sodium azide (pH 6.8). A second solution was prepared of the same specimen in 0.15M NaCl, 0.05M MES, 0.02% sodium azide (pH 7.0). To determine whether the method of sample preparation in 0.15M NaCl may influence the solubility, a solution of subunit was first dissolved in 4M GdHCl and following equilibration, dialized to 0.15M NaCl. All subunit solutions were filtered through a 0.22u Millipore filter. The aggregate species were dissolved in 0.15M NaCl and filtered through a 0.80u Millipore filter. The solutions were centrifuged at 4,000 rpm for 15 minutes immediately prior to light-scattering analysis. Samples whose fluctuations of total scattered light intensity were within 1% were used for analysis.

Refractive index measurements (dn/dc) were determined at 633 nm using a differential refractometer RF-600 (C.N. Wood Co.). Refractive index increments for subunit in 4M GdHCl was 0.145, while in 0.15M NaCl a value of 0.150 was obtained.

Static and dynamic light scattering experiments were carried out at 25 C using a Brookhaven BI-240 goniometer, and a BI-2020 correlator with 256 channels, capable of simultaneously computing
the correlation function in four groups at different sampling values. (Brookhaven Instruments, Ronkonkoma, N.Y.).

Static or total light scattering intensity was analyzed using the procedure of Zimm [3] to obtain the weight-average molecular weight, \( M_w \), z-average radius of gyration \( R_gz \), and second virial coefficient \( A_2 \):

\[
\frac{K_c}{\Delta R_\theta} = \frac{1}{M_w} \left( 1 + \frac{16\pi^2 \sin^2 \theta / 2R_g^2}{3\lambda^2} + 2A_2 \right)
\]

(4.1)

Where:

\[
K = 4\pi^2 n_0^2 (\partial n / \partial c)^2 / \lambda^4
\]

(4.2)

Dynamic light scattering probes molecular relaxations which arise from the translational and/or internal motions due to Brownian forces in the solution. The time autocorrelation function of the fluctuations in the total scattered electric field can be interpreted by the cumulant method of Koppel [4]:

\[
\ln g^{(1)}(\tau) = -Gt + \frac{\mu_2}{2\Gamma^2} (\tau \Gamma)^2 - \frac{\mu_3}{3\Gamma^3} (\tau \Gamma)^3 + ...
\]

\[
\frac{2\Gamma}{3\Gamma}
\]

(4.3)
where the first moment, \( \Gamma \), (the initial slope) is related to the \( z \)-average translational diffusion coefficient \( D_{tz} \) by:

\[
\Gamma = D_{tz}q^2 \quad \text{where} \quad q^2 = (4\pi n_0 \lambda_0)^2 \sin^2 \theta / 2
\]

(4.4)

**Viscosity Measurements:** Dilute solution viscosity measurements were performed on solutions of subunit in 4M GdHCl and in 0.15M NaCl. Viscosity measurements of aggregate were performed in 0.15M NaCl. All solutions were clarified using filtration methods described above for light scattering. Measurements were made using a #25 Cannon-Ubbelohde viscometer in a constant temperature (25 C) water bath. All times were in excess of 100 minutes, indicating insignificant shearing forces. Viscosity data was analyzed using the Maron-Reznik equation [5] which includes the second-order terms in the Huggins-Kramer equation:

\[
\frac{\Lambda}{c^2} = \frac{1}{2} [\eta]^2 + (k_1 - 1/3)[\eta]^3 c + ... 
\]

(4.5)

where

\[
\Lambda = \eta_{sp} - 1 \eta_r
\]

(4.6)

Viscosity measurements were extrapolated to the limiting concentration using linear least-squares analysis providing the intrinsic viscosity \([\eta]\).
Hydrodynamic Expressions: Following the suggestion of Flory and Fox [6, 7], the intrinsic viscosity is related to an average linear dimension of the polymer chain:

\[ [\eta] = k_T M^{1/2} \alpha^3 \] (4.7)

where the constant of proportionality \( K_T = \Phi \langle r_0^2 \rangle / M \) and \( \langle r_0^2 \rangle \) is the unperturbed mean-square radius of gyration. On the basis of experimental data, \( \Phi \) was suggested to represent a universal constant, being the same for all polymers with an experimental value of approximately \( 2.1 \times 10^{21} \). A similar argument for the frictional coefficient was developed by Mandelkern and Flory [8] giving the expression:

\[ f_0/\eta_0 = K_f M^{1/2} \alpha \] (4.8)

where \( K_f = \Phi \langle r_0^2 \rangle / M \), \( \alpha = \langle r^2 \rangle / \langle r_0^2 \rangle \), and \( \Phi \) should be a universal constant with a value of approximately \( 5.1 \). Combining these two equations yields:

\[ f_0/\eta_0 = \beta^{-1} (M[\eta])^{1/3} \] (4.9)

where \( \beta = \Phi^{-1} \alpha^{1/3} \). Each hydrodynamic parameter, \( f_0 \) and \( [\eta] \), as well as the structural parameter, \( M \), are determined independently from dynamic light scattering, dilute solution viscosity, and static light scattering experiments respectively. \( f_0 \) may be determined by combining the translational diffusion coefficient \( D^0 t \) via dynamic light scattering with Einstein's equation \( D^0 t = kT/f_0 \). Experimental results [8] indicate a \( \beta \) value of \( 2.5 \times 10^6 \) for Gaussian linear coils.
which is consistent with the KR values of $P=5.1$, and $\Phi = 2.1 \times 10^{21}$.

Stoke's equation relates the frictional coefficient of a sphere to the hydrodynamic radius of an equivalent sphere ($R_{h_f}$) in the non-draining limit:

$$f_0 = 6\pi \eta_0 R_{h_f} \quad (4.10)$$

Similarly, Einstein showed for an impermeable hydrodynamic sphere:

$$[\eta] = \left(\frac{10}{3}\right) \frac{\pi N_A}{M} R_{h_f}^3 \eta \quad (4.11)$$

Substituting expressions for $f_0$ and $[\eta]$ of the above two equations allows the $\beta$ parameter to be given in terms of the equivalent hydrodynamic radii $R_{h_f}$ and $R_{h_\eta}$:

$$\beta = \left[\left(\frac{N_A \pi}{30}\right)^{1/3} / 6\pi\right] (R_{h_\eta} / R_{h_f}) \quad (4.12)$$

The Kirkwood-Riseman theory predicts that $R_{h_\eta}/R_{h_f}$ in the non-draining limit equals 1.295 [9]. A review of classical theory of the hydrodynamic radii was performed by Yamakawa [10]. Results based on the use of the preaveraged Oseen hydrodynamic interaction tensor indicates for all these calculations:
\[ \rho_2 = \frac{R_{\eta}}{R_{\eta f}} > 1.21 \]

(4.13)

Note that an added degree of difficulty is introduced, when comparing experimental results, and theory when with polydisperse samples, since the diffusion \( (R_{\eta f}) \), and viscosity \( (R_{\eta}) \) measurements determine different moments of the molecular weight distribution [11]. This will be addressed in more detail in the discussion section.

The ratio of the radius of gyration \( R_g \) to the frictional radius \( R_{\eta f} \) is defined as \( \rho_1 \). The numerical value of this parameter (and that of \( \rho_2 \)) is strongly influenced by both the solvent quality of the solution, as well as the molecular structure of the molecule. It is also sensitive to the presence of chain branching. It is noted that, when dealing with macromolecules that can be modelled as impermeable hard-sphere particles, \( R_g = \frac{3}{5} R_h \), and \( R_{\eta f} = R_{\eta} \). In this case, \( \beta = 2.11 \times 10^6 \). However, it has recently been pointed out that experiment and theory suggest that for partially permeable spheres, \( \beta \) may decrease slightly below the hard sphere value [12]. For an oblate impermeable ellipsoid, \( \beta \) increases from 2.12 to 2.15 x 10^6 for an axial ratio increase from 1 to 200. Therefore, it is often assumed when dealing with compact macromolecules (e.g. globular proteins) that if \( \beta > 2.15 \times 10^6 \) they can be modelled as prolate.
ellipsoids [13]. After presenting the experimental data, I will discuss more recent theoretical developments.

RESULTS

Zimm plots of BNS subunit in 4M GdHCl and in 0.15M NaCl are shown in Figures (4.1) and (4.2) respectively. Results indicate a Mw of 1.93x10^6, Rgz of 608 Å, and A2 of 1.98x10^{-4} moles-cm^3/gm for the subunit in 4M GdHCl. In contrast, the subunit molecular weight Mw increases to 3.01x10^6 in 0.15M NaCl, while the Rgz increases to 866 Å, and the A2 value drops to 1.18x10^{-4} mole-cm^3/gm. Figure (4.3) shows the Zimm plot of the subunit sample initially dissolved in 4M GdHCl and then dialyzed to 0.15M NaCl. Results indicate a Mw of 2.46x10^6, Rgz of 817 Å, and A2 of 1.60x10^{-4} mole-cm^3/gm. Figure (4.4) gives the Zimm plot of the PGA in 0.15M NaCl indicating the Mw to be 67x10^6, and Rgz of 2576 Å.

Dynamic light scattering measurements of finite concentrations were extrapolated to zero angle and then to infinite dilution. This procedure yields D_{t2}^o from which we can obtain the Stoke's hydrodynamic radius R_h of the macromolecule using the Stoke's-Einstein relation:

\[ D_{t2}^o = kT/6\pi\eta o R_h \]  
(4.14)
Figure 4.1

Zimm plot of intensity measurements for bovine nasal septum PGS A1D1-2B in 4M GdHCl.
Temp. 25 C. Mw=1.93x10^6, Rgz= 608A, A2= 1.98x10^-4 moles-cm^3/gm.
Zimm plot of intensity measurements for bovine nasal septum PGS A1D1-2B in 0.15M NaCl.
Temp. 25 C. MW=3.01x10^6, Rg=866A, A2=1.18x10^-4 moles-cm^3/gm.
Figure 4.3  Zimm plot of intensity measurements for bovine nasal septum PGS A1D1-2B dissolved initially in 4M GdHCl, and then dialyzed to 0.15M NaCl. Temp. 25 C, Mw=2.46x10^6, Rgz= 817A, A2 = 160x10^-4 mole-cm^3/gm.
Figure 4.4 Zimm plot of intensity measurements for bovine nasal septum proteoglycan aggregate A1-2B in 0.15M NaCl. Temp 25 C. Mw=67 x10^6, Rgz=2567 A.
Figures (4.5) and (4.6) show the concentration dependence of the diffusion coefficient for the subunit in 4M GdHCl and in 0.15M NaCl, giving $D_0t$ of $3.95 \times 10^{-8}$ cm$^2$/sec and $D_0t$ of $3.70 \times 10^{-8}$ cm$^2$/sec respectively. Inserting these values into the above equation produces $R_{th}$ of 490 Å in 4M GdHCl and 633 Å in 0.15M NaCl. Figure (4.7) shows the concentration dependence of the diffusion coefficient of the subunit dissolved in 4M GdHCl and dialyzed to 0.15M NaCl. Here, the $D_0t$ is $3.94 \times 10^{-8}$ cm$^2$/sec which corresponds to an equivalent hydrodynamic radius of 594 Å. Figure (4.8) gives the concentration dependence of $D_t$ for PGA, with an extrapolated infinite dilution $D_0t$ of $1.26 \times 10^{-8}$ cm$^2$/sec and $R_{th}$ of 1951 Å.

Maron-Resnik plots for the subunit solution in 4M GdHCl and 0.15M NaCl are shown in Figures (4.9) and (4.10) respectively. Intercept values using least-squares analysis can be used to obtain the intrinsic viscosity values of 120 ml/g and 215 ml/g for 4M GdHCl and 0.15M NaCl solution respectively. Figure (4.11) gives the Maron-Resnik plot for PGA with an intrinsic viscosity value of 310 ml/g. A summary of light scattering and viscosity results are given in TABLE (4.1).

$R_g/R_{th}$ ($\rho 1$) values for PGS in 4M GdHCl and in 0.15M NaCl give 1.24 and 1.37 respectively, while for PGA in 0.15M NaCl the $\rho 1$ value is 1.32. $R_{h\eta}/R_{th}$ ($\rho 2$) values are 0.74 and 0.86 for subunit
Figure 4.5  Plot of the translational diffusion coefficient as a function of concentration for bovine nasal septum PGS A1D1-2B in 4M GdHCl. Extrapolation to zero concentration yields $D_{t^0} = 3.95 \times 10^{-8} \text{ cm}^2/\text{sec}$, and $Rh = 490 \text{ A}$. Temperature 25 C.
Figure 4.6  Plot of the translational diffusion coefficient as a function of concentration for bovine nasal septum PGS A1D1-2B in 0.15M NaCl. Extrapolation to zero concentration yields $D_t^0 = 3.70 \times 10^{-8} \text{ cm}^2/\text{sec}$, and $R_h = 633 \text{ A}$. Temperature 25 C.
Figure 4.7  Plot of the translational diffusion coefficient as a function of concentration for bovine nasal septum PGS A1D1-2B in 0.15M NaCl following dialysis from 4M GdHCl. Extrapolation to zero concentration yields $D(t=0) = 3.94 \times 10^{-8}$ cm$^2$/sec, and $R_h = 594$ A. Temperature 25 C.
Figure 4.8  Plot of the translational diffusion coefficient as a function of concentration for bovine nasal septum PGA A1-2B in 0.15M NaCl. Extrapolation to zero concentration yields $D_{t0} = 1.26 \times 10^{-8}$ cm$^2$/sec and $R_h = 1951$ A.
Figure 4.9  Maron-Resnik dilute solution viscosity plot of bovine nasal septum PGS A1D1-2B in 4M GdHCl. Extrapolation to zero concentration yields in conjunction with Equation (3) a [n] = 120 ml/gm.
Figure 4.10  Maron-Resnik dilute solution viscosity plot of bovine nasal septum PGS A1D1-2B in 0.15M NaCl. Extrapolation to zero concentration yields in conjunction with Equation (3), [n]= 215 ml/gm.
Figure 4.11  Maron-Resnik dilute solution viscosity plot of bovine nasal septum PGA A1-2B in 0.15M NaCl. Extrapolation to zero concentration yields in conjunction with Equation (3), \([n] = 310 \text{ ml/gm.}\)
Table 4.1 Molecular parameters for cartilage proteoglycan subunit and aggregate in 4M GdnHCl and 0.15M NaCl.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Solvent</th>
<th>$M_w \times 10^{-6}$</th>
<th>$R_g \AA$</th>
<th>$A_2$</th>
<th>$D_t^0$</th>
<th>$R_h [\eta]$</th>
<th>$\rho_1$</th>
<th>$\rho_2$</th>
<th>$\beta \times 10^6$</th>
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<tbody>
<tr>
<td>4NGd</td>
<td>HCl</td>
<td>1.93</td>
<td>608</td>
<td>1.98</td>
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<td>490</td>
<td>120</td>
<td>1.24</td>
<td>0.74</td>
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<td>0.15M</td>
<td>NaCl</td>
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<td>866</td>
<td>1.18</td>
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<td>633</td>
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<td>817</td>
<td>1.60</td>
<td>3.94</td>
<td>594</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>0.15M</td>
<td>NaCl</td>
<td>67.</td>
<td>2567</td>
<td>-</td>
<td>1.26</td>
<td>1951</td>
<td>310</td>
<td>1.32</td>
<td>0.85</td>
</tr>
</tbody>
</table>

BNS-A1-2B

| 0.15M   | NaCl    | 67.                 | 2567     | -    | 1.26    | 1951          | 310    | 1.32   | 0.85              |
in 4M GdHCl and 0.15M NaCl respectively. A summary of these results are shown in TABLE (4.I).

The MLF parameter (β) was determined utilizing absolute and independently produced values via static and dynamic laser light scattering techniques, as well as viscometry. As can be seen in TABLE (4.I), β, values of 1.51 x 10^6, and 1.65 x 10^6 for BNS subunit in 4M GdHCl and 0.15M NaCl respectively, and 1.60 x 10^6 for PGA are significantly below the theoretical lower limit based on a hard sphere conformation using the classical Kirkwood-Riseman theory. In addition, the ratio of the hydrodynamic ratio from viscosity and diffusion, (p2), has a value of 0.74 and 0.86 for the subunit in 4M GdHCl and 0.15M NaCl respectively, and 0.85 for PGA. These values are also significantly below the theoretical minimum value of 1.0 obtained for impermeable hard spheres.
DISCUSSION

In what follows, I: a.) contrast these anomalous findings to literature results of proteoglycans, b.) determine whether sample polydispersity can be the source of the discrepancies noted above in β, p1, and p2, c.) examine if similar anomalies have been reported in the literature results of experiments on synthetically-produced branched macromolecules, and finally d.) discuss the results in light of more recent hydrodynamic theories of model star branch polymer systems.

Review of proteoglycan literature:

A review of proteoglycan literature provides little experimental data on the β parameter. To date, only three published studies have determined all three parameters Mw, [η], and D0t independently of each other. The first of these, by Pastemack et al. [13] report for BNS in 4M GdHCl a Mw of 2.30x10^6 using static light scattering; a [η] of 141 ml/gm; and a sedimentation coefficient determined by sedimentation velocity of 21.3 S; Rg=57 nm. Using these values, one is able to calculate β= 2.58x10^6, Rh/η/Rhf= 1.22, and Rg/Rhf=1.69. The β value is larger than the hard-sphere value, and would correspond to an axial ratio of over 200 to 1 for an impermeable prolate ellipsoid.

In a second study by Kitchen and Cleland [14] a series of BNS proteoglycan fractions was characterized in 4M GdHCl by static light scattering, viscometry, and sedimentation velocity which gave
varying results. Their findings for a series of BNS fractions with increasing molecular weight in the range 1.0-2.4 \times 10^6 daltons gave a corresponding systematic increase of $\beta$ from 1.51-2.57 \times 10^6. Likewise, the measured ratio of the hydrodynamic radii, $R_{\eta}/R_{f}$, increased with molecular weight from 0.66 to 1.21 respectively. It is of interest to note that the results obtained in Pasternack's work are practically identical to the values of Kitchen's values for the highest molecular weight fraction. The increasing values of the $\beta$ parameter was duly noted by Kitchen and Cleland, but left unexplained.

Lyon et al. [15] using static light scattering obtained an absolute molecular weight of bovine femoral-head cartilage of 0.96 \times 10^6 in 4M GdHCl. In addition, both the sedimentation velocity and dynamic laser light scattering were used to determine a sedimentation coefficient of 12.0 S, and diffusion coefficient of $D^0t=5.41 \times 10^{-9}$ cm$^2$/sec at 20C respectively. Unfortunately, no intrinsic viscosity values were determined or reported. By combining an estimated intrinsic viscosity value of 120 ml/gm from Kitchen and Cleland's data on a BNS PGS of $M_w=1.0 \times 10^6$, with Lyon's value for $D^0t$ and $S_0$ we estimate $\beta$ of 1.74 \times 10^6 and 1.95 \times 10^6 respectively. Note that this calculation can give only an estimated $\beta$ value. The agreement between the reported molecular weights (1.0 \times 10^6 and 0.96 \times 10^6 for Kitchen's and Lyon's samples respectively) and radius of gyration (43 nm for Kitchen and 42 nm for Lyon) indicate that the two PGS species are essentially structurally
identical. In addition, the hydrodynamic radii are also in close agreement (41 nm for Kitchen and 37 nm for Lyon). Therefore, although the $[\eta]$ value is borrowed from Kitchen's data to calculate $\beta$ in Lyon's study, the error is small in light of the structural similarities. The ratio of hydrodynamic radii, $R_{\eta}/R_h$, is 0.76, also well below the limit of conventional theory.

In unpublished work by Shogren [16], an intrinsic viscosity value of 160 ml/gm was obtained for chick limb bud proteoglycan in 4M GdHCl, with a $D_0t$ of $5.5 \times 10^{-8}$ cm$^2$/sec at 20 C determined using dynamic light scattering, as well as Mw of $1.25 \times 10^6$ from static light scattering measurements. Using these values, $\beta = 1.72 \times 10^6$ and $R_{\eta}/R_h$ is 0.81 which is consistent with findings from this work as well as the work of Lyon et al. and some of Kitchen and Cleland's data. The $R_g/R_h \approx 1.74$.

Recently, Soby characterized both the subunit (A1A1D1D1) and aggregate (A1A1) isolated from bovine articular cartilage using dynamic and static light scattering, as well as viscometry. Results in 0.15M NaCl gave a Mw of $2.08 \times 10^6$ for PGS and $59.4 \times 10^6$ for PGA. $\beta = 1.87 \times 10^6$ and $1.31 \times 10^6$ for PGS and PGA respectively. In addition, $R_{\eta}/R_h = 0.845$ for PGS and 0.871 for PGA respectively [30]. Results from these independent studies are summarized in TABLE 4.2.

The range in $\beta$ values from 1.31 to $2.58 \times 10^6$ for proteoglycans is difficult to explain, particularly values that fall below the theoretical
Table 4.2 Molecular parameters of cartilage proteoglycan subunit from literature sources.

<table>
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<tr>
<th>Author</th>
<th>Sample</th>
<th>Solvent</th>
<th>$M_w \times 10^{-6}$</th>
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<th>$[\eta]$</th>
<th>$D_t$ or $S^0$</th>
<th>$R_g/R_h$</th>
<th>$R_h/R_f$</th>
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<tr>
<td>Pasterнак et al. [6]</td>
<td>BNS-DI</td>
<td>4MGd</td>
<td>2.3</td>
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<td>21.3 $S^0$</td>
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* estimated from Kitchen's data
lower limit for hard spheres. Sedimentation and intrinsic viscosity data on certain globular proteins indicate [12] β values slightly smaller than the hard sphere value, 2.11 x10^6. To rationalize this result, McCammon et al. [12] presented a theoretical analysis which leads to prediction of β values below 2.11 x10^6 based on a porous sphere hydrodynamic model. However, the magnitude of the discrepancy manifested in the PGS solutions (Tables 4.1 and 4.2) is much larger than that observed for globular proteins or predicted by the porous sphere theory [12]. The uniquely high ionic character and branching structure of proteoglycans could influence hydrodynamic properties to such an extent as to cause significant deviations from conventional hydrodynamic theory. To investigate this further, we have examined synthetically produced branched polymers as models for comparison. Unfortunately, synthetic models have not yet been developed which can reproduce the complex structure of the proteoglycan molecule. However, synthetic systems can provide an idea of the direction of hydrodynamic behavior as the branching functionality increases from a linear molecule to that approaching that of the proteoglycan subunit (200-250) [17].

Review of Synthetic Star Branched Polymers:

Experimental synthesis and physical characterization has been performed for a number of various star- and comb-branched polymers. Calculation of β, R_h/ R_f, and Rg/Rh have allowed
comparison of these values with predicted values based on the conventional hydrodynamic models of linear chains. Roovers et al. [18, 19] found β values for 4-, 6-, 12-, and 18-arm polystyrene star polymers in the theta solvent cyclohexane (T=35 C) of 2.17, 2.08, 2.03, and 2.06 x10^6 respectively. This is substantially smaller than the theoretical value of β for linear chains of 2.7 x10^6, and slightly below the predicted hard-sphere limit of 2.11 x10^6. In a good solvent such as toluene, β = 2.14 x10^6, and 2.02 x10^6 for the 12-, and 18-arm star polymers respectively. The ratio of the hydrodynamic radii R_h/\eta/\eta/\eta was found to be 0.96 and 0.98 for the 12-, and 18- arm stars in cyclohexane respectively, while in the good solvent toluene the values were 1.02 and 0.98 respectively (recall: R_h/\eta/\eta/\eta=1 for hard spheres). The p1 (Rg/R_h) values for the 12- and 18-arm stars in cyclohexane were 0.81 and 0.79 respectively (recall: p1=0.775 for hard spheres, and 1.27 and 1.57 for linear polystyrene in cyclohexane and toluene respectively). In Toluene, Rg/R_h=0.93 and 0.88 for the 12- and 18-arm stars respectively. Therefore, even in the good solvent the 12-, and 18- arm stars are significantly compacted having values comparable to the hard sphere limit.

Characterization of 3-arm polyisoprene stars in good and theta solvents in which the arm molecular weights were varied 12 times gave a range of β values from 1.53 to 2.02 x10^6 [20]. In addition, p2 ranged from 0.73 to 0.96.
Polybutadiene stars with functionalities as high as 278 were produced and characterized by Roover et al. [17]. In the theta solvent, dioxane, $\beta$ values ranged from 1.79 to $2.18 \times 10^6$ for stars of functionalities of 263 to 267 respectively, while $\rho_2$ values ranged from 0.81 to 1.02, and $\rho_1$ values ranged from 0.72 to 0.84. In the good solvent, cyclohexane, similar results were obtained. They concluded that this indicates hard-sphere hydrodynamic behavior consistent with the Daoud-Cotton model [21]. A summary of results obtained from synthetic star-branched polymers is given in TABLE 4.3.

Recent Hydrodynamic Theories and Models:

Based on experiment and theory, the ratio $\rho_1$ may vary from 1.504 for a linear chain in a good solvent (gaussian non-draining chain), to a value of 1.27 in a theta solvent, whereas a hard sphere would have a value of 0.775 [32]. We note that, even in the case of the linear chains, a complete understanding of the hydrodynamic behavior is not yet available. However, under specific levels of draining, it is clear that the effect of chain branching is to shift the hydrodynamic behavior towards that typical of non-draining hard spheres.

The scaling theory of regular star-branched polymers by Daoud and Cotton [21] uses blob concepts for polymers in good solvents, and a geometric model for average chain dimensions. Their model proposes a monotonically decreasing function of blob
<table>
<thead>
<tr>
<th>Author</th>
<th>Sample</th>
<th>f</th>
<th>$\beta \times 10^{-6}$</th>
<th>$R_{h}/R_{hf}$</th>
<th>$R_{g}/R_{hf}$</th>
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<tr>
<td>Roovers et al.</td>
<td>Polystyrene</td>
<td>4</td>
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<td>Bauer et al.</td>
<td>Polybutadiene</td>
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<td>Roovers et al.</td>
<td>Polybutadiene</td>
<td>267</td>
<td>2.18</td>
<td>1.02</td>
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<td>263</td>
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Table 4.3 Molecular parameters of synthetic star branch polymers from literature sources.
size, and therefore subchain concentration, as a function of radial distance from the center of the star. Based on these assumptions, three distinct regimes of subchain concentration were developed: (1) a close-packed core of constant subchain density, (2) a high subchain concentration zone of intermediate density, where excluded volume interaction is screened giving subchains Gaussian behavior, and (3) an outer regime of lower subchain concentration, where excluded volume effects may occur between the blobs. Drawbacks to this theory include assumptions made about the geometry of the blob model, as well as the need for this scaling theory to use asymptotic limits of chain length as well as excluded volume effects. In addition, the theory does not describe adequately the crossover between Gaussian behavior and excluded volume effects. However, experimental results [22, 17], as well as molecular dynamic simulation [23] have shown good agreement with this theory.

Using the rigorous chain conformation space renormalization group theory, Miyake and Freed [24] were able to address the deficiencies of the Daoud-Cotton theory. In contrast to the close-packed regime of the core proposed by Daoud and Cotton, the renormalization group method finds local expansion becomes more significant as the functionality of the star increases. In addition, this local expansion is found to diminish in the outer region of the star polymer. However, the Miyake-Freed theory is only applicable to
stars with functionalities (f) less than or equal to 7. It is therefore likely that the Daoud-Cotton theory is better suited for star polymers of larger f.

Monte Carlo studies [25, 26], work well in describing chain behavior as Gaussian for stars with less than 6 branches. However, above 6 branches deviations begin to occur. Hydrodynamic and radius of gyration properties have calculated the β parameter using various Leonard-Jones interaction potentials for non-neighboring units. Rey et al. [27] calculated β values ranging from 2.28 to 2.10 x10^6 for Gaussian branches as the functionality increases from 2 to 18. Monte Carlo calculations by Zimm [28] of frictional coefficients and viscosity numbers for stars with functionalities of 2, 4, and 6 indicate that deviations from the Kirkwood-Riseman theory may be due in part to preaveraging the Oseen interaction tensor, as well as some wormlike character and excluded volume effects as the chains emanate from the branch point. Douglas et al. compared experimental and Monte Carlo data for branched polymers with renormalization group calculations and found good agreement [33]. A limiting hard-sphere behavior was found to set in with f>20.

The complexity of the branch systems limits the analysis of these newer theories. Calculations based on the relatively simple branched structures of low functionality, have indicated that the observed hydrodynamic parameters that deviate from conventional
theory may not be due to error, but may reflect the unique hydrodynamic behavior of branched systems.

Effects of Proteoglycan Polydispersity:

Since the intrinsic viscosity depends on the weight-average molecular weight, and the diffusion coefficient depends on the higher z-average moment, the ratio of \( \text{Rh}_\eta/\text{Rh}_f \) can be numerically altered by changes in sample polydispersity. The effect of polydispersity can be evaluated by determining the molecular weight distribution by gel permeation chromatography. The GPC profile of PGS in 4M GdHCl is shown in Figure (4.12). The profile indicates a relatively narrow distribution of molecular size. The number-average molecular weight can be calculated from:

\[
\overline{M}_n = \frac{\Sigma c_i M_i}{\Sigma c_i} \quad (4.15)
\]

In addition, the weight-average molecular weight can be computed from:

\[
\overline{M}_w = \frac{\Sigma c_i M_i}{\Sigma c_i} \quad (4.16)
\]

The molecular weight of each fraction, \( M_i \), was evaluated using the Kav-Mw relationship derived by Ohno et al. [29] for various proteoglycan species:

\[
\text{Log}\overline{M}_i = -(1.65 \pm 0.27)K_{av} + (6.58 \pm 0.08) \quad (4.17)
\]
Figure 4.12  GPC profile of bovine nasal septum PGS A1D1 in 4M GdHCl on a Sepharose CL-2B column. Fraction size: 0.445 ml, Void volume, V₀: 13.8 ml, Total volume, Vₜ: 33.7 ml. Isolated sample labeled PGS A1D1-2B.
As shown in the following calculations, the influence of polydispersity on the ratios \( \rho_1 \) and \( \rho_2 \) may be estimated by explicit expressions for the molecular weight averages measured in \( R_{hf} \), \( R_{Hf} \), and \( R_g \) in terms of the 'monodisperse' ratios \( k = \frac{R_{hf}}{R_{Hf}} \) and \( k' = \frac{R_g}{R_{hf}} \). Then, assuming that the molecular weight dependence of \( R_{Hf} \), \( R_{hf} \), and \( R_g \) each follow the power-law behavior reported by Ohno et al. [29]: \( R_i \propto M_i^{0.38} \). We obtain expressions relating the experimental ratios \( \rho_1 \) and \( \rho_2 \) to \( k \) and \( k' \) via expressions which can be evaluated from the molecular weight distribution. Using the molecular weight distribution shown in Figure (4.12), we estimate that the 'monomeric' value of \( \rho_1 = k' = 1.07 \), while the 'monomeric' value of \( \rho_2 = k = 0.84 \). When we compare these corrected values of \( \rho_1 \) and \( \rho_2 \) with our experimental values of 1.24 and 0.74 respectively we see that \( \rho_1 \) is still greater than the hard sphere value and \( \rho_2 \) is still less than unity.

Thus it appears that even after correction for sample polydispersity is made, the ratio \( \rho_2 = 0.84 \) is still smaller than the theoretical impermeable sphere limit of unity. However, the corrected ratio \( \rho_1 = 1.07 \) is larger than the hard-sphere value [\( \rho_1(HS) = 0.775 \)], but is much smaller than the smallest values typically observed for linear flexible chains. While the value of \( \rho_2 \) is rather smaller than expected, our results for \( \rho_1 \) and \( \rho_2 \) quantitatively are comparable to those reported for synthetic branched macromolecules with high branch density (Table 4.3). The source
Effect of PGS Polydispersity on P1 (Rg/Rf) and P2 (Rn/Rf)

For individual chromatography fractions, the "monodisperse"

results are given as:
\[ R_{\eta_i} = kR_{rf} \]
where \( R_{rf} = KM_i^{0.38} \). Therefore, \( R_{\eta_i} = kKM^{0.38} \)

\[ \hat{R}_{\eta} = \frac{3 \Sigma c_i M_i}{\Sigma c_i} \cdot \frac{\Sigma c_i R_{\eta_i}}{\Sigma c_i} = \frac{3 \Sigma c_i M_i}{\Sigma c_i} \cdot \frac{\Sigma c_i (R_{c_i} M_i)}{\Sigma c_i} \]

and since \( R_{rf} \) is computed from the z-average translational
diffusion coefficient, \( D_t \), via the Stokes-Einstein equation,

\[ \hat{R}_{rf} = \frac{\Sigma c_i M_i R_{rf}}{\Sigma c_i M_i} \]

Therefore:

\[ (R_{\eta}/R_c)^3 \exp = \frac{\Sigma c_i M_i}{\Sigma c_i} \cdot \frac{\Sigma c_i kKM^{0.14}}{\Sigma c_i} \cdot \left(\frac{\Sigma c_i M_i^{0.62} K^{-1}}{\Sigma c_i M_i}\right)^3 \]

\[ = k^3 \frac{\Sigma c_i M_i^{0.14}}{(\Sigma c_i)^2} \cdot \left(\frac{\Sigma c_i M_i^{0.62}}{(\Sigma c_i M_i)^2}\right)^3 \]

\[ = k^3 (0.76) \]

Since
\[ (R_{\eta}/R_c)^3 \exp = 0.77 \quad k = \left[\frac{(0.77)^3}{0.76}\right]^{1/3} = 0.84 \]
or
\[ (R_{\eta}/R_c) \text{corrected for polydispersity} = 0.84 \]
\[ R_g = \left( \frac{\Sigma c_i M_i R_{gi}^2}{\Sigma c_i M_i} \right)^{1/2} \]

Therefore,

\[ (R_g/R_f)^{\text{EXP}} = 1.24 \quad k' = \frac{1.24}{1.15} = 1.07 \]

or

\[ (R_g/R_f) \text{ corrected for polydispersity} = 1.07 \]

Note that from the chromatogram, the second moment of the autocorrelation function of light scattered by PGS:

\[
\frac{\mu_2}{\Gamma} = \frac{(\Gamma)^2}{2} - 1 \quad \frac{\mu_2}{\Gamma} + 1 = \frac{\Sigma c_i M_i R_{fi}^{-2}}{\Sigma c_i M_i} \cdot \frac{\left(\Sigma c_i M_i\right)^2}{(\Sigma c_i M_i R_{fi}^{-1})^2}
\]

Thus the calculated second moment is slightly smaller than the experimental value.

\[ \frac{\mu_2}{\Gamma}^{\text{calc}} = 0.10 \quad \frac{\mu_2}{\Gamma}^{\text{EXP}} = 0.16 \]
of the discrepancy in $\rho_2$ is unclear, but may be due to: a.) inadequate correction for polydispersity, b.) effects not accounted for in the theory due to ionic character and/or rigidity of polysaccharide chains.
CONCLUSIONS

Bovine nasal septum subunit and aggregate proteoglycans have been characterized in 4M GdHCl, and 0.15M NaCl using viscosity measurements, and static and dynamic scattering techniques. Results from 0.15M NaCl indicate that some of self-association occurs. This seems to be influenced in the manner in which the PGS is dissolved in this solvent. When PGS is initially dissolved in the chaotropic solvent 4M GdHCl and then dialyzed to 0.15M NaCl, the molecular weight is found to be $2.46 \times 10^6$, compared to a value $3.01 \times 10^6$ for PGS dissolved in 0.15M NaCl.

Calculation of the MFS parameter, $\beta$, as well as the ratio of the hydrodynamic radii $R_{Hr}/R_{Hf}$ for PGS in both 4M GdHCl and 0.15M NaCl and PGA in 0.15M NaCl gives values significantly below expected theoretical results based on linear chains. Review of literature results of proteoglycans permitted estimation of the $\beta$ values for comparable PGS preparations. Results from all but one of these works gives values consistent with our data.

It was established that the sample polydispersity is responsible to a significant extent for these anomalous $\beta$ values. However, even after correcting for polydispersity, data for PGS in 4M GdHCl gives a $\beta$ value smaller than the hard-sphere result.

Review of theoretical and experimental results from synthetically-produced star-branch and comb-branch polymers indicate $\beta$ and $p$ ratios which tend to the hard sphere values as
branch density increases, and even falls below the lower limit in many studies.

Although quantitatively impossible to compare proteoglycan results with results from monodisperse nonionic branch polymers, it has been possible to qualitatively explain the hydrodynamic behavior of the proteoglycan subunit as being consistent with its highly branched architecture. After correction for the effects of molecular weight polydispersity, however, we find that \( p_2 \) lies below, whereas, \( p_1 \), lies above the value for an impermeable hard sphere. The origin of this anomaly is not clear. However, it may be related to the highly ionic and rigid nature of the polysaccharide side-chains. It is also noted that a decrease of \( p_2 \) and \( \beta \) below the hard-sphere value is predicted for homogeneous or concentric spheres of varying permeability. However, these deviations are smaller than the relatively large discrepancies reported in this work.
REFERENCES

15. Lyon, M., Greenwood, J., Sheehan, J.K., Nieduszynski, I. A.
CHAPTER 5
THERMAL STABILITY OF CARTILAGE PROTEOGLYCANS

INTRODUCTION

Proteoglycans are a major component in many different types of connective tissues such as bone, skin, and cartilage [1]. Although their structure and size are quite variable depending upon the source of isolation, they all have in common a protein core with one or more covalently-attached pendant glycosaminoglycan side chains [2].

The focus of this chapter is the proteoglycan isolated from bovine nasal septum cartilage. Previous characterization of this molecule has shown a polydispersity in size, with a molecular weight ranging from 1-4 x10^6 daltons [3,4,5,6]. The protein core, which has covalently bound side chains of keratin sulfate (M = 12,000) and chondroitin sulfate (M = 20,000) has a molecular weight that ranged from 2-3 x10^5. In addition, the protein core also has a globular protein region devoid of glycosaminoglycans and interacts non-covalently with hyaluronic acid (HA) enabling it to form huge aggregates with molecular weights up to 200 x10^6 daltons [7]. This non-covalent association is further stabilized by a separate protein moiety, called link protein, that non-covalently binds to both the HA and the proteoglycan subunit (PGS). Schematic illustrations of the aggregate structure are shown in Figures 5.1 and 5.2, and an electron micrograph of the aggregate is exhibited in Figure 5.3.
Figure 5.1 Schematic showing the dimensions and structure of the cartilage proteoglycan aggregate. Ref. [33, Chap. 1].
Figure 5.2 Schematic model of the structure of the cartilage proteoglycan aggregate. Ref. [1].
Figure 5.3  Electron micrograph of bovine nasal septum proteoglycan aggregate. Ref. [Rosenberg et al. (1975) J. Biol. Chem., 250, 277].
The thermal stability of the aggregate species has been previously investigated using dynamic light scattering methods [8,9]. These studies demonstrated that the thermal stability of link-stabilized aggregates are significantly greater when contrasted with link-free aggregates. The link-free aggregate displayed an abrupt increase in the diffusion coefficient at about 45-55 °C, indicating onset of dissociation of the PGS from the HA backbone.

The analysis of the light scattering data is complicated by the fact that the aggregate species is a much stronger scatterer than the subunit since the scattering amplitude $A$ is directly proportional to molecular weight [10]. As an approximation, we may use the Guinier expression:

$$ A_i = c_i M_i \exp(-q^2 R_g^2/3) $$

(5.1)

where $c_i$ = weight concentration of species $i$ of molecular weight $M_i$ and radius of gyration $R_g$. Thus, the scattering amplitude, in addition to its dependence on $M$, is also proportional to the interference factor, $P_i = \exp(-q^2 R_g^2/3)$, where $q$ is the scattering vector given as:

$$ q = \frac{4\pi n_0}{\lambda} \sin \theta/2 $$

(5.2)
Therefore, the ratio of scattering amplitudes A1/A2 for two molecular species of different sizes, e.g. PGA aggregate and PGS subunit, can be arranged to directly reflect the concentration ratio C1/C2 by selecting the appropriate scattering angle such that M1/M2= P2/P1. At such an angle, M1P1/M2P2 will equal 1 and the scattering amplitudes will accurately reflect the ratio of weight concentrations of aggregate to subunit: A1/A2=C1/C2.

Since the proteoglycan aggregate and subunit monomer have widely different diffusion coefficients, we said that the electric-field correlation function is fitted by two well-separated distributions of exponential decays, each representing the aggregate and subunit species, respectively.

In this study, we use a program (GEXPSAM) developed by Brookhaven Instrument Corp. that provides a spectrum of relaxation rates G(Γ)ΓdΓ. We examined solutions of the proteoglycan subunit at identical experimental conditions to that of aggregate solutions, at various angles and temperatures, and used the PGS results to provide a baseline when looking at the more complex aggregate preparations. This proved to be very helpful since the aggregate has many types of molecular relaxations, ranging from translation diffusion of PGA to that of the subunit as it thermally dissociates from the HA backbone. In addition, internal relaxations of both the
aggregate and subunit, as well as the effects of polydispersity complicate the definitive interpretation of the relaxation spectrum.

In the two previous studies [8,9] that examined the thermal stability of PGA, the status of the aggregate structure as a function of temperature was evaluated using the apparent diffusion coefficient, Dt. This parameter is calculated from the mean relaxation frequency, $\Gamma$, (Equation 5.19) and represented a sum of two exponential relaxations, i.e. PGA and PGS. As discussed previously, this method requires a specific scattering angle such that the intensity of scattered light from the aggregate and subunit accurately reflect their weight-concentration in solution. Scattering angles below this optimum angle will exaggerate the contribution due to the aggregate, while higher angles will accentuate the contribution by the subunit. The method of light scattering analysis used in this work provides a distribution of relaxation frequencies, as well as the mean relaxation frequency. We are therefore not restricted to use a specific optimum scattering angle in order to correctly measure the relative proportions of PGA and PGS in solution, but rather can obtain such information directly from the distribution.

EXPERIMENTAL

Sample Preparation: Bovine nasal septum proteoglycan subunit and aggregate were isolated and prepared as previously
described [15]. The subunit material is designated A1D1-CL2B. This indicates a specimen which has been purified by density gradient centrifugation under associative conditions, followed by dissociative conditions, and subsequently isolated as an excluded fraction following gel permeation chromatography on a cross-linked Sepharose 2B analytical column. The link-stabilized aggregate sample was obtained as the void volume fraction on a Sepharose 2B column and is thus designated A1-2B. It was originally isolated under dissociative conditions and reassembled with its link proteins.

Lyophilized samples of A1D1-CL2B and A1-2B were weighed on a Perkin-Elmer Autobalance AD-2 to the nearest 10th of a milligram. Stock solutions of each material in 0.4M GndHCl, 0.05M NaCl, 0.2% NaN3, pH 7 were made, each at a concentration 3.0 mg/ml. PGS and PGA solutions were filtered, respectively, through 0.22 μ and 0.8 μ Millipore filters, and centrifuged just prior to light scattering.

Light Scattering: Static and dynamic light scattering measurements were carried out using a Brookhaven BI-240 goniometer, and a BI-30S2 Multi-Tau correlator with 264 channels (Brookhaven Instruments, Ronkonkoma, N.Y.). The light source used was a 15 mW Spectra-Physics He-Ne laser (6328 Å). Static light scattering measurements on the aggregate and subunit were performed previously, and are reported in the previous chapter.
The Multi-Tau correlator computes the correlation function simultaneously in four head-to-tail linked groups of 64 channels each, at four different sample time increments. This is advantageous when analyzing dynamic light scattering from a specimen with a broad spectrum of relaxations. The correlator was interfaced with an IBM PC-AT computer for curve fitting.

Data Analysis: The first-order electric field autocorrelation function of a polydisperse sample consist of a sum or integral of exponential decays:

\[
\left| g^{(1)}(\tau) \right| = \int_{0}^{\infty} G(\Gamma) \exp(-\Gamma \tau) d\Gamma
\]  

(5.3)

with

\[
\int_{0}^{\infty} G(\Gamma) d\Gamma = 1
\]

(5.4)

The distribution function of the decay rates, \( G(\Gamma) \), can be modelled as broad and continuous, or as a series of delta functions. \( G(\Gamma) d\Gamma \) is the fraction of total integrated intensity scattered, on average, by particles whose individual contribution to the total correlation function is an exponential decay with characteristic relaxation factor
$\Gamma = D_0 q^2$, where $D_0$ is the translational diffusion coefficient, and $q$ is the magnitude of the scattering vector (Equation 5.2). The hydrodynamic radius, $R_h$, can be determined using the Stokes-Einstein relation:

$$D_0^{0.5} = \frac{k_B T}{6\pi \eta_0 R_h}$$

(5.5)

where $D_0^{0.5}$ is the translational diffusion coefficient extrapolated to infinite dilution and zero angle, $k_B$ is Boltzmann's constant, $T$ is the absolute temperature, and $\eta_0$ is the solvent viscosity.

Several methods of analysis of $g_1^{(1)}(\tau)$ are available. Using the method of cumulants [13], the exponential term $\exp(-\Gamma'\tau)$ can be expanded in a Taylor series around the mean value $\Gamma$:

$$\exp(-\Gamma'\tau) = \exp(-\bar{\Gamma} \tau) \exp[-(\Gamma - \bar{\Gamma})\tau]$$

(5.6)

Therefore the electric field autocorrelation function $g_1(\tau)$ can be expressed as:

$$\left| g_1^{(1)}(\tau) \right| = \exp(-\bar{\Gamma} \tau)[1 - \frac{\mu_2}{2!} \tau^2 + \frac{\mu_3}{3!} \tau^3 - \ldots]$$

(5.7)

or:
\[ \ln |g^{(1)}(\tau)| = -\bar{\Gamma}\tau + \frac{1}{2!}\left(\frac{\mu_2}{\bar{\Gamma}}\right)^2 - \frac{1}{3!}\left(\frac{\mu_3}{\bar{\Gamma}}\right)^3 + \ldots \]  

(5.8)

where by definition:

\[ \frac{\mu_i}{\bar{\Gamma}} - \frac{1}{i!} \int (\bar{\Gamma} - \bar{\Gamma})^i G(\Gamma) d\Gamma \]  

(5.9)

is the normalized \(i\)th moment about the mean of \(G(\Gamma)\). The second cumulant \((i=2)\) corresponds to the variance in \(\Gamma\).

A newer method involving inversion of equation (5.3) by Laplace transform can be used to obtain \(G(\Gamma)\) [14]. This approach leads to a simple linear least-squares fit, based on the eigenfunctions \(\psi_w(\Gamma)\), and the eigenvalue \(\lambda_w\) of the Laplace transform of \(G(\Gamma)\), which must satisfy the eigenvalue relation:

\[ \int_0^\infty \psi_\infty(\Gamma) \exp(-\Gamma\tau) d\Gamma = \lambda_w \psi_w(\Gamma) \]  

(5.10)

The distribution function \(G(\Gamma)\) vs. \(\Gamma\) may then be expanded as a continuous complete set of its eigenfunctions:

\[ G(\Gamma) = \int_{-\infty}^{\infty} a_w \psi_w(\Gamma) dw \]  

(5.11)
where $a_w$ is a set of coefficients. Inserting equation (5.11) into equation (5.3) and using equation (5.10), one obtains for the correlation function:

$$g^{(1)}(\tau) = \int_{-\infty}^{\infty} \lambda_w a_w \psi_w(\Gamma) dw$$

(5.12)

This equation can be rewritten in terms of a band-limited Fourier transform, and an interpolation formula to give:

$$G(\Gamma) = \sum_j P_j \delta(\Gamma - \Gamma_j)$$

(5.13)

where $P_j$ are the weighting factors of the delta function and

$$\sum_j P_j = 1 \text{ (normalization condition)}$$

(5.14)

Substituting for $G(\Gamma)$ into equation (5.3) yields:

$$\left| g^{(1)}(\tau) \right| = \sum_j P_j \exp(-\Gamma_j \tau)$$

(5.15)
The software program for this method (GEXPSAM) provides the data in a histogram form on a semi-log plot of $G(\Gamma)$, the intensity, vs. $\Gamma$, the relaxation frequency. An example of this can be seen for PGS at $T=20$ C, $\theta = 30$ in Figure 5.4. The mean relaxation frequency, $\Gamma$, is computed from the following equation:

$$\Gamma = \frac{\Sigma G(\Gamma)\Gamma}{\Sigma G(\Gamma)}$$  \hspace{1cm} (5.16)

Resolution of the relaxation frequency spectrum into two major components can be done by finding the point where $G(\Gamma) = 0$ between the two distribution peaks. The lower portion of the relaxation frequency spectrum is arbitrarily called $\Gamma_1$, where:

$$\Gamma_1 = \frac{\Sigma G(\Gamma_1)\Gamma_1}{\Sigma G(\Gamma_1)}$$  \hspace{1cm} (5.17)

Conversely, the set of relaxation frequencies above the minimum, $G(\Gamma)=0$, are called $\Gamma_2$, where:

$$\Gamma_2 = \frac{\Sigma G(\Gamma_2)\Gamma_2}{\Sigma G(\Gamma_2)}$$  \hspace{1cm} (5.18)
Figure 5.4 Relaxation frequency distribution curve $G(\Gamma)$ vs. $\Gamma$(sec$^{-1}$) of BNS PGS in 0.4M GdHCl. $c = 3.0$ mg/cc. Scattering angle: 30. $\Gamma_1$ represents multimers of PGS, $\Gamma_2$ represents the monomeric form of PGS.
Both $\Gamma_1$ and $\Gamma_2$ are related to the total mean relaxation frequency by the relation:

$$\bar{\Gamma} = a_1 \Gamma_1 + a_2 \Gamma_2$$  \hspace{1cm} (5.19)

where,

$$a_1 = \frac{\Sigma G(\Gamma_1)}{\Sigma G(\Gamma_1) + \Sigma G(\Gamma_2)}$$

and

$$a_2 = \frac{\Sigma G(\Gamma_2)}{\Sigma G(\Gamma_1) + \Sigma G(\Gamma_2)}$$  \hspace{1cm} (5.20)

RESULTS

Proteoglycan Subunit:

The thermal stability of proteoglycan subunit (PGS) was examined using dynamic light scattering at three scattering angles $\theta = 30, 55, 90$, and eight temperatures, $T = 20, 25, 30, 35, 45, 55, 65, 70$ C for each angle respectively. The temperature dependence of the mean relaxation frequency ($\Gamma$) for PGS as the temperature is increased from 20 to 70 C is shown for the three scattering angles in Figure 5.5. Here, $\Gamma$ is normalized with respect to $T$ and $\eta_0$ at 20 C. In addition, the relaxation frequency distribution curves $G(\Gamma)$ vs. $\Gamma$ can be seen in Figures 5.6-5.13 for the different temperatures.
Figure 5.5. Plot of the mean relaxation frequency, $\bar{f}$, vs. temperature $T$ for BNS PGS in 0.4M GdHCl. Scattering angles: 30, 55, 90. $c = 3.0$ mg/cc. $\bar{f}$ is calculated from Equation (5.16).
Figure 5.6 Plot of the relaxation frequency curve \(G(\Gamma)\) vs. \(\Gamma (\text{sec}^{-1})\) for BNS PGS in 0.4M GdHCl. Scattering angles: (A) 30, (B) 55, (C) 90. Temperature 20 C.
Figure 5.7  Plot of the relaxation frequency curve $G(\Gamma)$ vs. $\Gamma(\text{sec}^{-1})$ for BNS PGS in 0.4M GdHCl. Scattering angles: (A) 30, (B) 55, (C) 90. Temperature 25 C.
Figure 5.8  Plot of the relaxation frequency curve $G(\Gamma)$ vs. $\Gamma$(sec$^{-1}$) for BNS PGS in 0.4M GdCl$_3$. Scattering angles: (A) 30, (B) 55, (C) 90. Temperature 30 C.
Figure 5.9  Plot of the relaxation frequency curve $G(\Gamma)$ vs. $\Gamma$ (sec$^{-1}$) for BNS PGS in 0.4M GdHCl. Scattering angles: (A) 30, (B) 55, (C) 90. Temperature 35 C.
Figure 5.10  Plot of the relaxation frequency curve $G(\Gamma)$ vs. $\Gamma$(sec$^{-1}$) for BNS PGS in 0.4M GdCl. Scattering angles: (A) 30, (B) 55, (C) 90. Temperature 45 C.
Figure 5.11  Plot of the relaxation frequency curve $G(\Gamma)$ vs. $\Gamma$(sec$^{-1}$) for BNS PGS in 0.4M GdHCl. Scattering angles: (A) 30, (B) 55, (C) 90. Temperature 55 C.
Figure 5.12  Plot of the relaxation frequency curve $G(\Gamma)$ vs. $\Gamma$ (sec$^{-1}$) for BNS PGS in 0.4M GdHCl. Scattering angles: (A) 30, (B) 55, (C) 90. Temperature 65 C.
Figure 5.13 Plot of the relaxation frequency curve $G(\Gamma)$ vs. $\Gamma$ (sec$^{-1}$) for BNS PGS in 0.4M GdHCl. Scattering angles: (A) 30, (B) 55, (C) 90. Temperature 70 C.
respectively. Also shown in Figure 5.5 is the \( \Gamma \) value for PGS at 25°C after cooling from 70°C for \( \theta = 30 \) and 55.

The results indicate that the subunit is relatively thermally stable even at a temperature of 70. As seen in Figure 5.5, \( \Gamma \) changes by less than 4 and 6% for angles 30 and 55 respectively. The difference in \( \Gamma \) approaches 12% at \( \theta = 90 \), which differs from the behavior at the smaller angles. This finding will be addressed later.

The question arises as to which of the two major sets of peaks in the bimodal relaxation spectrum of \( \Gamma \) represents the translational diffusion relaxation of the subunit. To determine this, we looked at the relaxation spectrum for PGS at \( T = 20 \) and \( \theta = 30 \). The maximum in \( G(\Gamma) \) from the lower portion of the spectrum, \( \Gamma_1 \text{max} \), was found to equal 94 sec\(^{-1}\), while in the upper set of \( \Gamma \), \( \Gamma_2 \text{max} \) equals 287 sec\(^{-1}\). Using the relation \( \Gamma = q^2D \) and equations (5.2) and (5.5), the hydrodynamic radius can be computed for both of these relaxations. Results show a \( R_h \) of 106 nm for \( \Gamma_1 \text{max} \) and an \( R_h \) of 35 nm for \( \Gamma_2 \text{max} \). This latter value is more consistent with the \( R_h \) obtained from the translational diffusion coefficient of the PGS species in 4M GdHCl. We therefore used the higher frequency component of the relaxation spectrum to obtain the contributed due to the translation diffusion of the monomeric PGS. The width of the upper portion of the relaxation spectrum probably represents the polydispersity in size of the subunit. The lower frequency component of the relaxation spectrum most likely represents contributions from subunit dimers and higher multimers. This is a reasonable assumption since PGS is known to self-associate in lower ionic-strength
solvents [3, 16]. Reihanian et al. demonstrated using dynamic light scattering that the apparent diffusion coefficient decreased with increasing PGS concentration for A1D1D1 PGS from bovine nasal septum. This indicated self-association of PGS in 0.15M NaCl [3]. Harper et al. demonstrated using computer simulations of dynamic light scattering that small quantities of self-associating subunits (< 5%) can decrease the apparent diffusion coefficient of PGS by 20 to 30%. This finding supported the results of Reihanian. It is therefore probable that the concentration-dependent aggregation of PGS, either in the form of a loose aggregate, or a liquid-crystal type association is a property of proteoglycans. The presence of self-association within a PGS solution are, for the first time, clearly evident as seen in bimodal frequency distribution for PGS.

Proteoglycan Aggregate:

The thermal behavior of the proteoglycan aggregate (PGA) was examined using dynamic light scattering at scattering angles and temperatures identical to those used for PGS. Results of the temperature dependence of Γ for PGA is shown in Figure 5.14 for the three scattering angles respectively. Evidently, a rather different picture of the apparent change in particle size is observed at each angle. At θ=30°, only a slight increase is observed in Γ as the temperature approaches 65°C, at which point a sharp increase is observed. At θ= 55°, the mean relaxation, Γ, is relatively constant as the temperature is increased from 20°C to about 55°C, at which point a sharp increase is observed. At θ=90°, Γ slowly increases up
Figure 5.14. Plot of the mean relaxation frequency, $\bar{T}$, vs. temperature $C$ for BNS PGA in 0.4M GdCl$_3$. Scattering angles: 30, 55, 90. $c = 3.0$ mg/cc. $\bar{T}$ is calculated from Equation (5.16).
to $T=65\,^\circ C$ and then a sharp increase occurs. This behavior at $\theta = 90\,^\circ C$ arises because at larger scattering angles the subunit makes a relatively larger contribution to the detected intensity (review equation 5.1). It is interesting to observe (see Figure 5.15) that for all three scattering angles, $\Gamma$ (PGA) tends to $\Gamma$ (PGS) as the temperature approaches 70 $^\circ C$. This demonstrates conclusively that link-stabilized PGA is thermally stable up to a critical temperature ($T = 55-65\,^\circ C$) at which point thermal dissociates into PGS occurs.

The distribution of $G(\Gamma)$ vs $\Gamma$ for PGA is shown in Figures 5.16-5.23 for the various temperatures and scattering angles. Again, a bimodal distribution of $\Gamma$ is observed. The slow mode has an average decay rate that is smaller than that of the subunit and is therefore presumed to be due to scattering from intact PGA. The faster component of the distribution has decay rates comparable to that observed for subunit multimers at lower temperatures, which become increasing more consistent with the decay rates attributed to the monomeric species as the temperature approaches 70 $^\circ C$.

The contribution to the PGA relaxation spectrum due to the monomeric form of PGS is seen to increase as the temperature approaches 70 $^\circ C$. The magnitude of the PGA-monomer component is reflected by $G(\Gamma)$. Because we have established, from the PGS study, the relaxation decay rate for the monomeric PGS species, we can discern within the PGA relaxation spectrum which relaxation decays are contributed by the PGS monomer. The relative
Figure 5.15. Plot of the mean relaxation frequency, $\bar{\Gamma}$, vs. temperature C for both BNS PGA and PGS. Scattering angles: 30, 55, 90. $c = 3.0 \text{ mg/cc}$ $\bar{\Gamma}$ is calculated from Equation (5.16).
Figure 5.16. Plot of the relaxation frequency curve $G(\Gamma)$ vs. $\Gamma (\text{sec}^{-1})$ for BNS PGA in 0.4M GdHCl. Scattering angles: (A) 30, (B) 55, (C) 90. Temperature 20°C.
Figure 5.17. Plot of the relaxation frequency curve $G(\Gamma)$ vs. $\Gamma (\text{sec}^{-1})$ for BNS PGA in 0.4M GdHCl. Scattering angles: (A) 30, (B) 55, (C) 90. Temperature 25 C.
Figure 5.18. Plot of the relaxation frequency curve $G(\Gamma)$ vs. $\Gamma$ (sec$^{-1}$) for BNS PGA in 0.4M GdHCl. Scattering angles: (A) 30, (B) 55, (C) 90. Temperature 30°C.
Figure 5.19. Plot of the relaxation frequency curve $G(\Gamma)$ vs. $\Gamma$ (sec$^{-1}$) for BNS PGA in 0.4M GdHCl. Scattering angles: (A) 30, (B) 55, (C) 90. Temperature 35 C.
Figure 5.20. Plot of the relaxation frequency curve $G(\Gamma)$ vs. $\Gamma$ (sec$^{-1}$) for BNS PGA in 0.4M GdHCl. Scattering angles: (A) 30, (B) 55, (C) 90. Temperature 45°C.
Figure 5.21. Plot of the relaxation frequency curve $G(\Gamma)$ vs. $\Gamma$(sec$^{-1}$) for BNS PGA in 0.4M GdHCl. Scattering angles: (A) 30, (B) 55, (C) 90. Temperature 55 C.
Figure 5.22. Plot of the relaxation frequency curve $G(\Gamma)$ vs. $\Gamma$(sec$^{-1}$) for BNS PGA in 0.4M GdHCl. Scattering angles: (A) 30, (B) 55, (C) 90. Temperature 65 C.
Figure 5.23. Plot of the relaxation frequency curve $G(\Gamma)$ vs. $\Gamma(\text{sec}^{-1})$ for BNS PGA in 0.4M GdCl$_3$. Scattering angles: (A) 30, (B) 55, (C) 90. Temperature 70°C.
contribution is reflected by the magnitude of $G(T)$. It is observed that at lower temperatures, $G(T)$ is negligible for the relaxation decays due to PGS monomer. However, as the temperature approaches 70 C the relative contribution of the relaxation spectrum, as reflected by $G(T)$, increases for PGS monomer. A plot of relative contribution to the PGA frequency distribution spectrum by PGS as a function of temperature is shown in Figures 5.24-5.26 for the scattering angles 30°, 55°, and 90° respectively.

As with the PGS, the aggregate was cooled to T=25°C following equilibration at 70°C. The average relaxation decay is shown in Figure 5.14, while Figure 5.25 shows the contribution of PGS to the PGA relaxation spectrum.

The relationship between the frequency distribution profiles for PGS and PGA as a function of temperature are shown more concisely in Figures 5.27 and 5.28, which show the composite plots of the frequency distribution spectrums for PGS and PGA as a function of temperature at scattering angles 30 and 55 respectively. An interpretation of these results will be discussed in the following section.
Figure 5.24. Plot of the fraction subunit that exist in an aggregate solution as the temperature is increased from 20 to 70 C. Solvent 0.4M GdHCl. c= 3.0 mg/cc. Scattering angle 30.
Figure 5.25. Plot of the fraction subunit that exist in an aggregate solution as the temperature is increased from 20 to 70 °C. Solvent 0.4M GdHCl. c = 3.0 mg/cc. Scattering angle 55°.
Figure 5.26. Plot of the fraction subunit that exist in an aggregate solution as the temperature is increased from 20 to 70 C. Solvent 0.4M GdHCl. c= 3.0 mg/cc. Scattering angle 90.
DISCUSSION

The analysis of the proteoglycan aggregate as it thermally dissociates into its subunit components is a complex dynamical process. We have used a newer method of light scattering analysis that gives a distribution of the relaxation frequencies. Contributions to these distributions are made from the aggregate form, as well as smaller multimer complexes of subunit. The proteoglycan aggregate, by nature, is a polydisperse species, and therefore will cause broadening of the relaxation spectrum. Therefore, in an attempt to simplify the interpretation of this complex system we have chosen to confine our attention to the translational diffusion relaxation frequency and ignore the contributions from the other order modes, i.e. internal and rotational relaxations.

To gain further insight into what is occurring to the link-stabilized aggregate species as thermal disruption takes place, we first performed the experiment with the proteoglycan subunit at identical experimental conditions. Since this is the species to which the aggregate dissociates into when heated, we can determine what peak(s) within the relaxation spectrum is(are) due to the subunit, and therefore know the subunit's relative contribution.

In Figures 5.5 and 5.14, the mean relaxation frequencies ($\Gamma$) for PGS and PGA are seen as a function of temperature at the three scattering angles used in this study. For the subunit, the normalized
curves are relatively constant at \( \theta = 30 \) and 55 and indicate thermal stability. Even upon cooling to 25 C from 70 C there is good agreement seen in \( \Gamma \), which supports the contention that no irreversible thermal changes take place in the subunit. At \( \theta = 90 \), \( \Gamma \) appears to slowly increase as the temperature is elevated. This change is only about 12%, and may indicate that subunit multimer dissociations are occurring, the effects of which are more sensitive at this higher scattering angle.

In contrast to the \( \Gamma \) vs. \( T \) behavior of the subunit, the aggregate shows a sharp increase in \( \Gamma \) at the higher temperatures, after showing similar behavior to the subunit at the lower temperatures. The behavior at \( \theta = 90 \) is less consistent, with \( \Gamma \) showing a slow increase with temperature up to \( T = 65 \) C. However, it does show a sharp increase at this upper temperature which is consistent to that seen at the other two angles.

At this point, the question arises as to whether this represents a true disruption of the subunit from the hyaluronic acid backbone, or possibly a chain-scission of HA with the link-stabilized subunit still attached. To distinguish which of the two mechanisms is present, the subunit data provides a helpful baseline. If Figures 5.5 and 5.14 are overlapped, (see Figure 5.15), one sees that \( \Gamma \) of the aggregate, approaches that of the subunit as the \( \Gamma \) approaches 70 C, supporting the assertion that the aggregate is indeed dissociating into the subunit form. This result supports earlier results on the
thermal stability of PGA, which also indicated relative thermal stability of the link-stabilized PGA up to approximately 65 to 70 C, at which point a large increase in the apparent diffusion coefficient (Dt) occurred [8,9]. This higher value of Dt which occurred at 70 C approached the Dt of the subunit measured at 20 C, and thus this result was interpreted to indicate that the aggregate does indeed dissociates into the subunit form [8,9]. However, the apparent diffusion coefficient of the subunit was determined only at T = 20 C, and extrapolated to the higher temperatures. In this study, the thermal stability of the subunit was determined at 5-10 degree increments up to 70 C, and definitively demonstrates that the aggregate dissociates into the subunit form as the solution temperature approaches 70 C.

If the subunit is indeed dissociating from the HA backbone, then we should observe the relative proportion of the subunit increase as the temperature approaches 70 C. This is exactly what is seen, as evident in Figures 5.24-5.26 for the three scattering angles respectively. As can be seen, as the temperature increases a point is reached where the proportion of the subunit, G(Γ), begins to increase and approach unity as the temperature approaches 70 C. This observation further supports the contention that the increase in Γ observed for PGA in Figure 5.14 is attributable to a corresponding increase in the fraction of free subunit.
An interesting observation is noted in Figure 5.25, which shows the proportion of the subunit in the PGA solution vs. temperature at \( \theta = 55 \). Upon cooling to 25\(^\circ\)C after heating to and equilibration at 70 \(^\circ\)C it is seen that the fraction of monomeric subunit remains at approximately 60 \%. This implies that following heating, an irreversible change has occurred in the binding capacity of either the subunit, the HA backbone, or both. This irreversible behavior in the binding capacity has been previously observed by Jamieson et al. [9]. In that study, the translational diffusion coefficient of the BNS PGA was followed as the temperature was increased to 70\(^\circ\)C using dynamic light scattering. A modest increase in \( D_t \) was observed as the PGA was heated through to 65\(^\circ\)C at which point, a sharp increase occurred. Upon cooling to 25\(^\circ\)C, \( D_t \) did not return to its original value at this temperature, but remained elevated at approximately 70\% of the maximum value attained at 70\(^\circ\)C. This fractional increase agrees well with our fractional increase of 65\%. It was concluded by Jamieson et al., that an irreversible denaturation of the link protein, or the hook region of the subunit protein core, or both occurs. Our results support this conclusion.

An interesting and unexpected finding from this study was the bimodal distribution of relaxation frequencies observed in both the PGS (Figures 5.6-5.13) and PGA (Figures 5.16-5.23) profiles. This bimodal behavior persisted throughout the temperature range 20-70 \(^\circ\)C and the three scattering angles 30, 55, 90, for both PGS and
PGA. To further appreciate this phenomenon, and to facilitate comparison of the PGS and PGA relaxational frequency distributions, an overlap composite of the PGS and PGA distributions are shown as a function of temperature for scattering angles 30 and 55 in Figures 5.27 and 5.28 respectively. Observe the upper left distribution profile (PGS:PGA at $T = 20, \theta = 30$) in Figure 5.27. Three distribution peaks are seen: the lower distribution peak belongs to the intact aggregate species, while the upper most distribution peak is due to the monomeric form of the subunit. The middle distribution peak has contributions from both the PGS and PGA profiles, and probably represents multimers of the self-associating subunit. This profile pattern is relatively consistently maintained as the temperature is increased from 20 to 45 C. At $T = 55$ C the PGA profile begins to shift towards higher relaxation frequencies (i.e., to smaller particles). As the temperature approaches 70 C, the PGA's multimer peak moves to the right to become superimposed on the PGS's monomer peak, while at the same time the PGA's aggregate peak moves to the right to become superimposed on the PGS's multimer peak. Note that the PGS's monomeric peak remains relatively constant at 500 sec$^{-1}$ throughout the temperature range. This latter observation is consistent with the findings in Figure 5.5 which demonstrates the thermal consistency of $\Gamma$, and probably represents the subunit's thermal stability. It is interesting to note that the PGS's multimer
Figure 5.27. Composite plot of $G(\Gamma')$ vs. $\Gamma'(sec^{-1})$ for BNS PGS (green) and PGA (blue) as a function of temperature C. Scattering angle 30.
Figure 5.28. Composite plot of $G(\Gamma)$ vs. $\Gamma(\text{sec}^{-1})$ for BNS PGS (green) and PGA (blue) as a function of temperature C. Scattering angle 55.
peak also remains relatively constant as the temperature varies from 20 to 70°C. Therefore, with the subunit showing relative thermal stability of both its monomeric and multimeric forms, the aggregate species is seen to migrate, as a function of temperature, to higher relaxational frequencies consistent with those observed for the subunit. This result is in complete agreement with the results demonstrated in Figure 5.15. It appears that the thermal dissociation of the aggregate does not occur in a one-step process, but possibly a two-step process in which the aggregate form may exist in equilibrium with the multimer complex. As the temperature reaches a critical point, the aggregate multimer equilibrium is shifted towards aggregate multimer subunit, and finally to a multimer subunit equilibrium. Similar findings are shown in Figure 5.28 and 5.29 for scattering angles 55 and 90° respectively.

The existence of multimeric subunit complexes may be due to the fact that the experimental solution concentrations (3.0 mg/cc) are very close to the overlap concentration $C^*$, where the lower and upper concentration bounds are given by $3M/4kT < C^* < MkT/4kT$. Taking $M = 1.93 \times 10^6$, and $R_g = 60$ nm, gives $3.3 \text{ mg/cc} < C^* < 14.5 \text{ mg/cc}$. The relatively low ionic strength of the solvent (0.4M GdnHCl) and the polyionic character of the proteoglycan subunit provide conditions for subunit interaction. The physical nature of these subunit interactions is unknown, but evidence of concentration-dependent associations in the form of loose
Figure 5.29: Composite plot of $G(T)$ vs. $I(\sec^{-1})$ for BNS PGSS (green) and PGA (blue) as a function of temperature C. Scattering angle 90°.
networks, or liquid-crystals is a general property of poly electrolytes, and may explain the observations in the light scattering data [17,18].

CONCLUSIONS

For the first time, the thermal stability of both the proteoglycan aggregate and subunit were investigated using multi-tau correlation dynamic light scattering and analyzed by a modified Laplace transform of the autocorrelation function. This newer method of data analysis has allowed the distribution of relaxational frequencies to be demonstrated in a histogram profile. The utility of this method allows for the interpretation of various frequency relaxations at various scattering angles that otherwise would be difficult to interpret using the cumulant method of analysis.
REFERENCES


Rays, Wiley, N.Y., Chap. 2.


CONCLUSIONS

Dynamic and static laser light scattering methods have been used to characterize the structure of proteoglycans. Two distinct species of proteoglycans were studied in this work: the large, HA aggregating type isolated from bovine nasal cartilage, and the smaller proteodermatan sulfate (PDS) found in bovine skin.

The light scattering study of bovine skin PDS is the first time this molecule has been characterized by this method. Results show a relatively low molecular weight of 62,000 for the intact PDS, while its constituent core protein was found to have a Mw of 39,000 in 4M GndHCl. The Mw for both the PDS and core protein increased significantly in 0.15M NaCl, indicating a great degree of self-association in this solvent medium. In contrast, DS in 0.15M NaCl had a Mw of 24,000 indicating a lack of self-association. Therefore, the locus of self-association of PDS is probably on the core protein. The fact that core protein was found to self-associate in 0.15M NaCl supports this contention. Corrections in light scattering data were made to account for the block-copolymer nature of PDS. Results show that the copolymer nature did not influence the light scattering results significantly. The abnormally large radii of gyrations for both the core protein and PDS were determined to be the result of trace amounts of self-associating aggregates in 4M GndHCl. Finally, the
PDS was hydrodynamically modelled as a "lollipop" structure. Calculated hydrodynamic results were found to be in excellent agreement with experimental observations.

The hydrodynamic values obtained from light scattering of the cartilage proteoglycan were found to deviate from expected theoretical values. In fact, the results gave values below the theoretical lower limit, i.e. a hard sphere conformation. When a review of the proteoglycan literature was performed, similar results were noticed. A hypothesis that the large branch functionality of the cartilage PGS may be responsible for these observed hydrodynamic deviations was tested by looking at other branched structures. In this case, a literature review of synthetically produced star- and comb-branched structures gave results that were in qualitative agreement with the proteoglycan results. At this point, a review of some newer hydrodynamic theories provided models which would qualitatively explain the deviations of hydrodynamic results.

Lastly, a newer method of light scattering analysis from polydisperse samples was applied to the thermal dissociation of the link-protein stabilized proteoglycan aggregate. The subunit was also investigated, and shown to be thermally stable. In contrast, aggregate became thermally unstable at about 55°C and approached a subunit size distribution profile as T=70°C. Prior studies of this phenomenon had to rely on choosing an appropriate
scattering angle to make the interpretation of the relative amount of subunit/aggregate unambiguous. With this newer method, any scattering angle will give information about the relative amounts of subunit and aggregate that co-exist in solution.
BIBLIOGRAPHY

CHAPTER 1


Chem., 240, 1005.


223, 587.


CHAPTER 2

1. Huglin

3. Lord Rayleigh (1871) Phil. Mag., 41, 447.


CHAPTER 3


Wiley, New York.


CHAPTER 4


CHAPTER 5


2. Hardingham, T.E., Beadmore-Gray, M., Dunchan, D.C., Ratcliffe,


