SOLID PHASE ASSAYS IN GLYCOCONJUGATE RESEARCH

Demitrios H. Vynios

Glycoconjugates

The term glycoconjugates describes a large family of biologic macromolecules composed of two moieties, an oligosaccharide and a protein (glycoprotein or proteoglycan) or lipid (glycosphingolipid).

The oligosaccharide portion is covalently linked to the protein or lipid.

Glycoconjugates

The building blocks for the oligosaccharide portion of a glycoconjugate are monosaccharides

- Glucose
- Galactose
- N-acetylglucosamine
- N-acetylgalactosamine
- Glucuronic acid, etc

Glycoconjugates

- The oligosaccharide portion of a glycoconjugate is structurally complex due in part to the structural variations of its building blocks
- This is similar to the situation with other macromolecules such as proteins which derive structural diversity from the variation in the structure of amino acids that are used as building blocks

Glycoconjugates

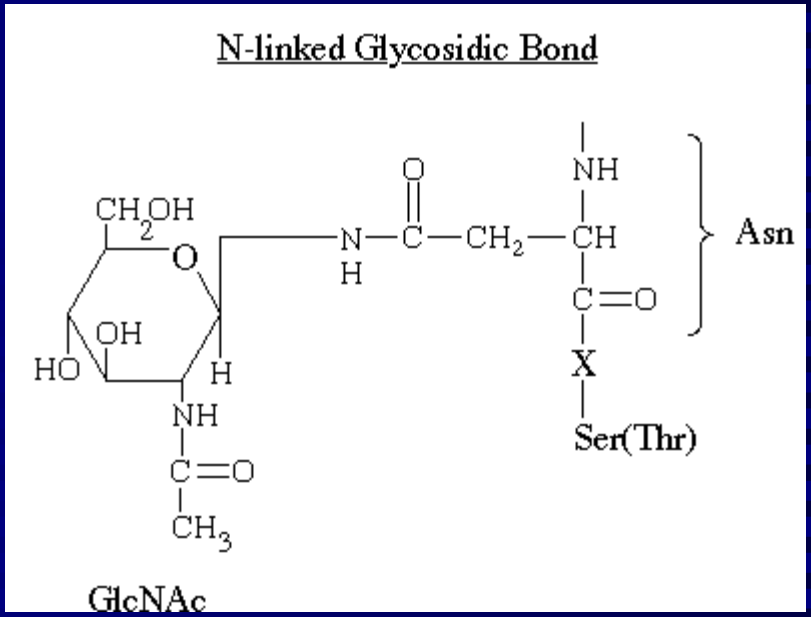
Glycoconjugates may be

- Glycoproteins
- Proteoglycans / Glycosaminoglycans
- Glycolipids

Proteoglycans / Glycosaminoglycans and Glycoproteins are the main glycoconjugates of connective tissue

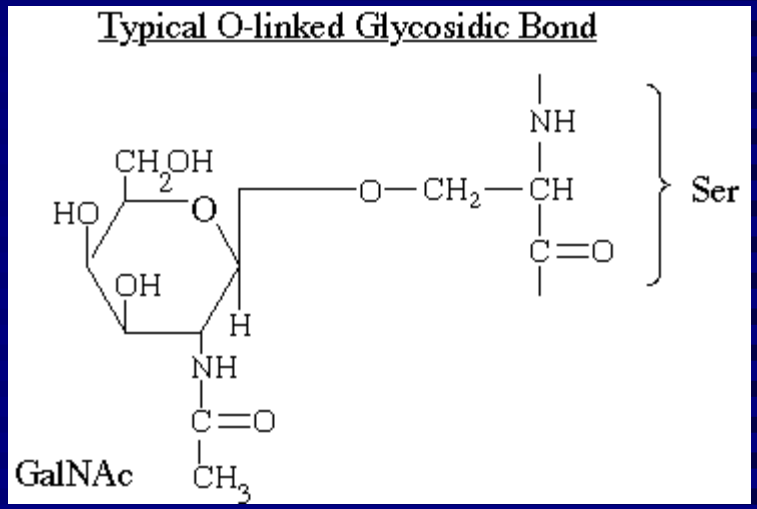
What types of glycosylations occur in proteins?

- Asparagine or N-linked
  - occurs in about 1/3 of all Asn-X-Ser(Thr) sites of glycoproteins
  - linkage occurs between Asn and GlcNAc
  - core structure composed of GlcNAc and Man residues



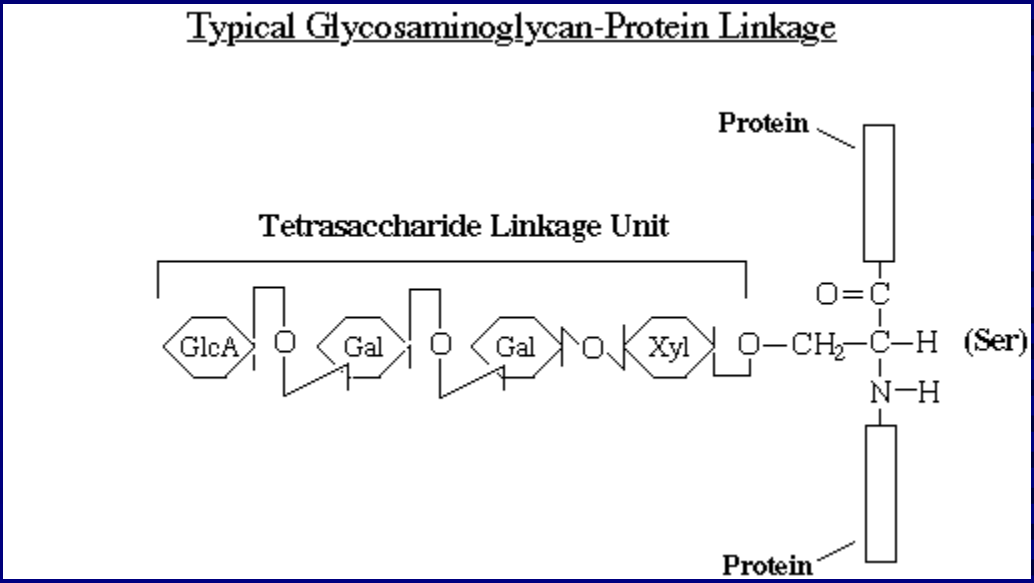
What types of glycosylations occur in proteins?

- Serine / Threonine or <u>O-linked</u>
  - occurs on variable number of Ser/Thr residues of glycoproteins
  - increased frequency of O-linked glycosylation when amino acids at positions -1 and +3 relative to the Ser/Thr are Pro residues
  - linkage occurs between Ser/Thr and GalNAc



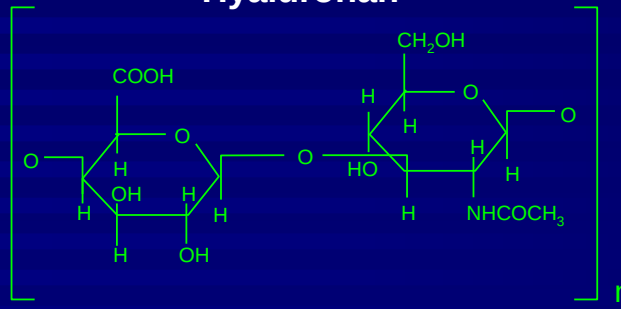
What types of glycosylations occur in proteins?

- Glycosaminoglycans linked to glycoproteins
  - occurs on variable number of Ser residues of glycoproteins
  - linkage occurs between Ser and Xyl
  - core structure composed of Xyl, Gal and GlcA

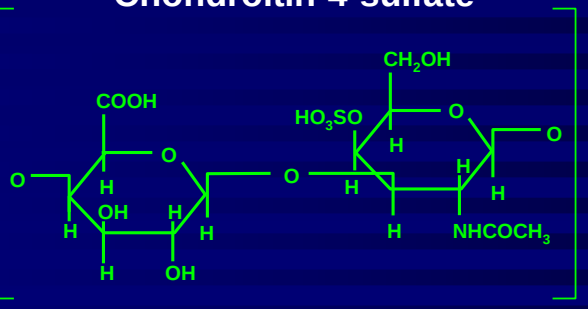


Glycosaminoglycans disaccharide structure

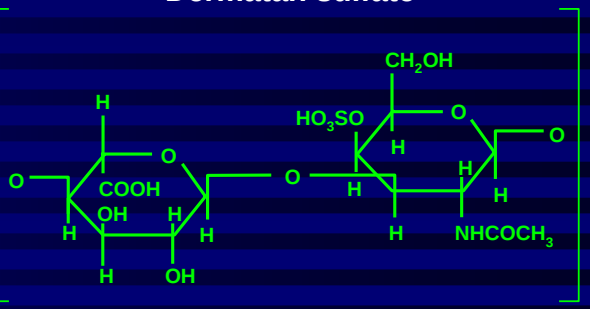
Hyaluronan



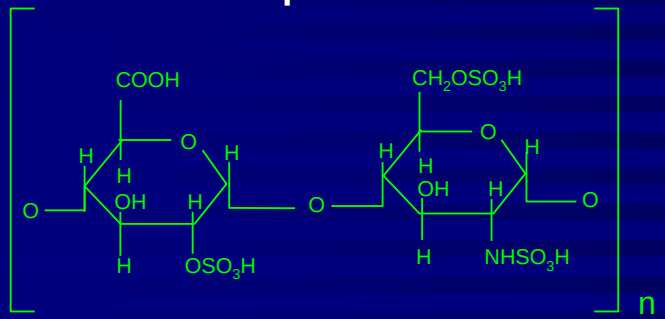
Chondroitin-4-sulfate



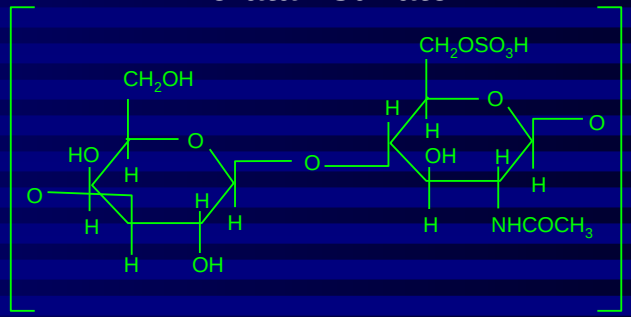
Dermatan sulfate



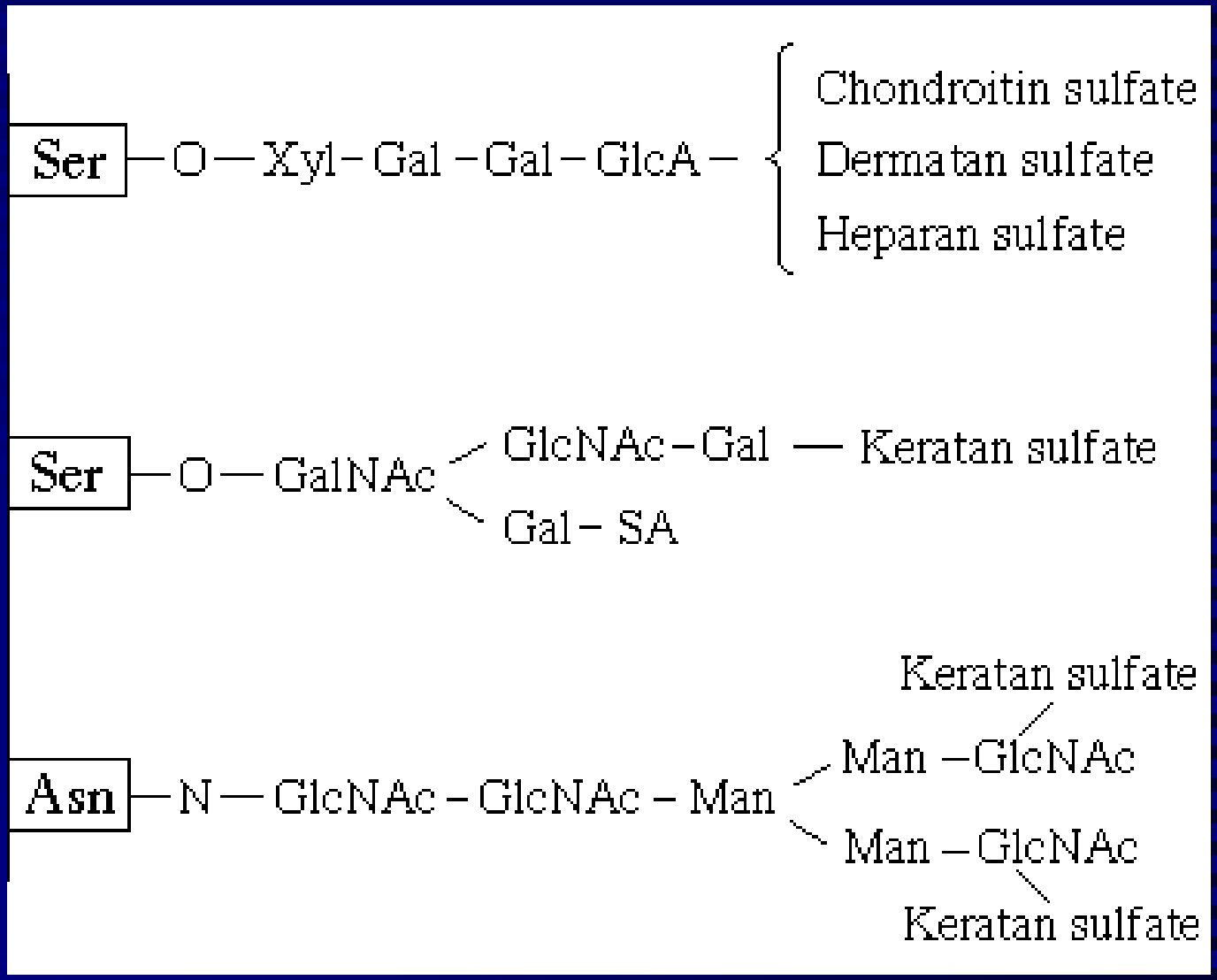
Heparin



keratan sulfate



Glycosaminoglycan - Protein linkages



Glycosylation. Why?

- Trafficking of proteins
- Properties of proteins (specific immunogenicity, specific interactions)
- Directed binding of small ions
- Maintainance of extracellular water
- Spring-like properties of glycosaminoglycans

Glycosylated macromolecules of connective tissue

- Proteoglycans
- Collagens
- Glycoproteins (stuctural glycoproteins, enzymes)

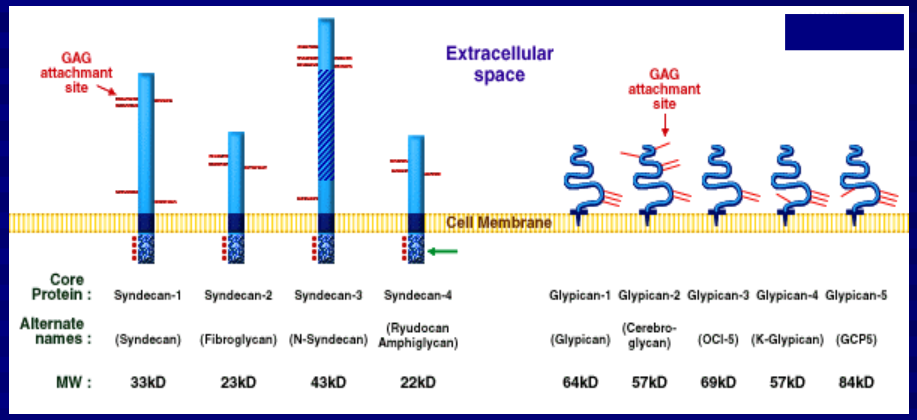
Connective tissue proteoglycans

- Proteoglycans are complex macromolecules with a core protein onto which variable number (up to 100) of glycosaminoglycans are covalently linked

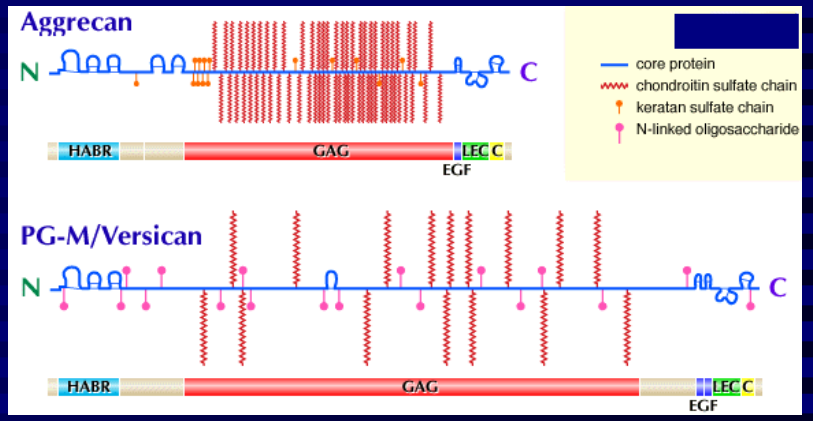
- Depending to their structure and role they are separated to:
  - Hyalectans
  - Small and leucine - rich proteoglycans
  - Basement membrane proteoglycans
  - Cell - surface proteoglycans

Proteoglycans: many forms and many functions

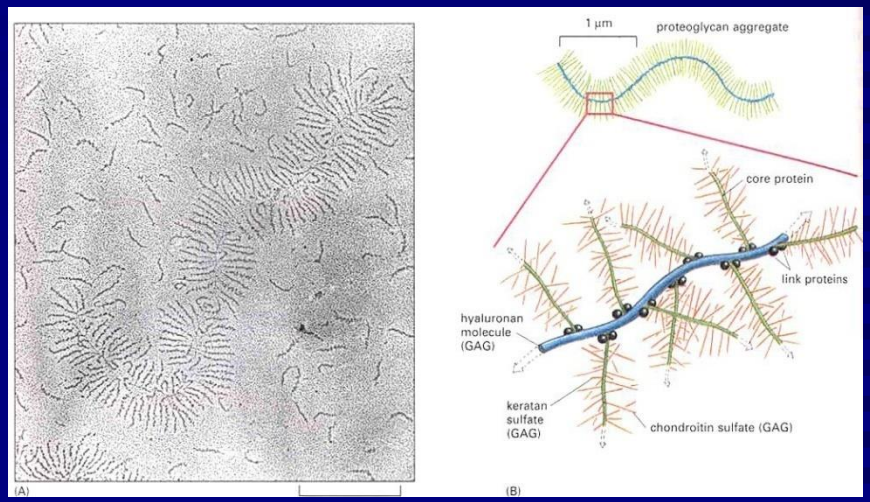
Membrane-bound PGs

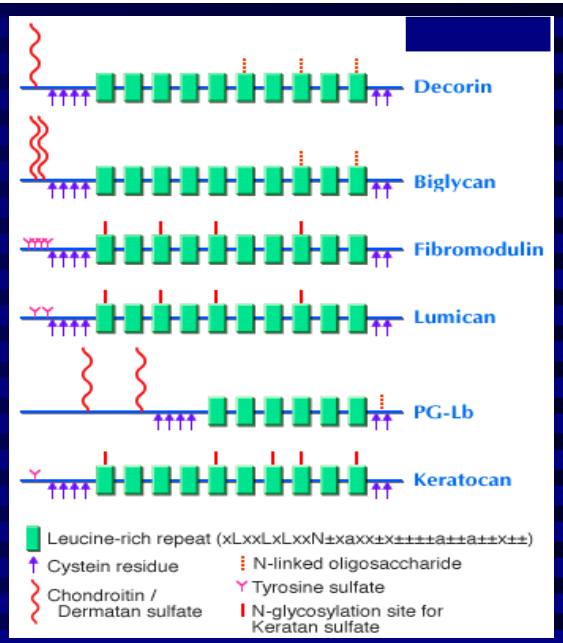


Extracellular PGs



Proteoglycan aggregates





Interactions of proteoglycans

- Hyalectans
- Small MW
- Basement membrane
- Cell-surface

- Hyaluronan
  - Link protein
- Collagen
  - Growth factors
- Laminin
  - Growth factors
- Fibronectin
  - Integrins
  - Cytoplasmic proteins

Interactions of glycosaminoglycans

- Heparin
  Heparan sulphate
  Chondroitin sulphate
  Dermatan sulphate

- Protease inhibitors
  Plasma lipoproteins
  Growth factors
  Lipolytic enzymes
  ECM proteins
  PF4
  Viral coat proteins
  Prion proteins

- Hyaluronan

- ECM proteins
  Cell surface receptors

Role of proteoglycans

- Aggrecan
  - Regulation of hydroxyapatite growth
    Resistance of tissue compression
    Development / Differentiation
- Versican
  - Morphogenesis / Cell migration
    Tumor invasion / metastasis
- Biglycan
  - Regulation of growth factors?
- Decorin
  - Decoration of collagen fibrils
    Regulation of growth factors
    Regulation of oncogenes

Role of proteoglycans

- Syndecans
- Perlecan

- Cell adhesion
  Cell signalling
- Restoration of growth factors
  Tumour invasion
  Tumour metastasis

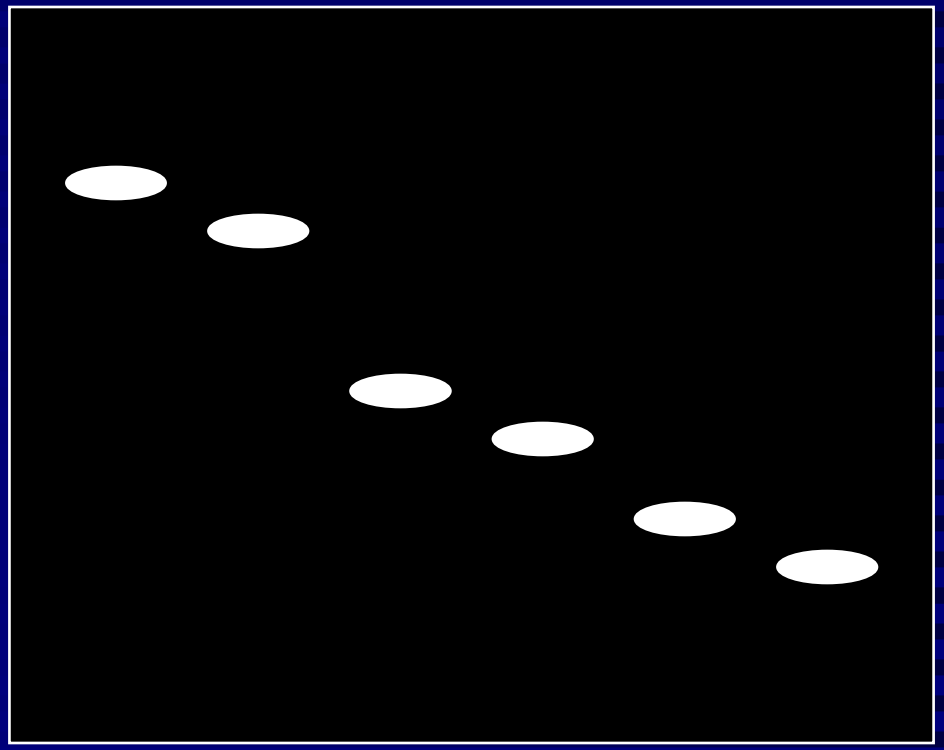
Tools in Proteoglycan / Glycosaminoglycan analysis

- High Performance Liquid Chromatography
- **Fluorophore - assisted carbohydrate electrophoresis**
- Capillary electrophoresis
- Electrospray - ionization mass spectrometry
- **Specific enzymatic treatment**
- **Enzyme linked immunosorbent assays**
- **Molecular Biology Techniques**
- **Zymography**
- Electron microscopy

Fluorophore assisted carbohydrate electrophoresis

- Degradation of glycoconjugates with specific enzymes (i.e. chondroitinases, heparitinases)
- Derivatisation with a fluorophore (i.e. AMAC, 2-ABA, ANDSA)
- Separation by electrophoresis (gradient or dense polyacrylamide gels are required)

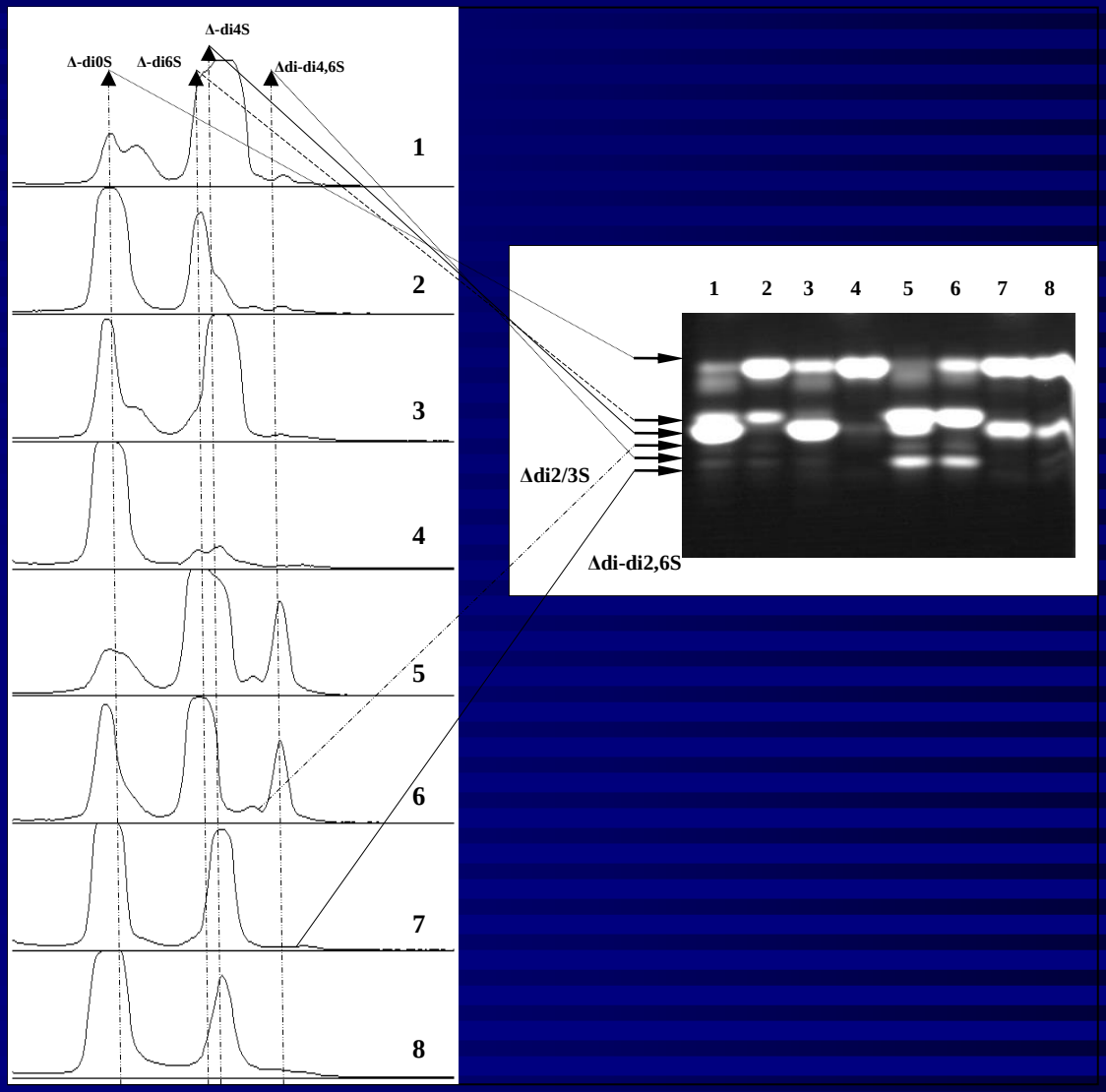
FACE result in 35% polyacrylamide



HA C0S C6S C4S CdiS CtriS

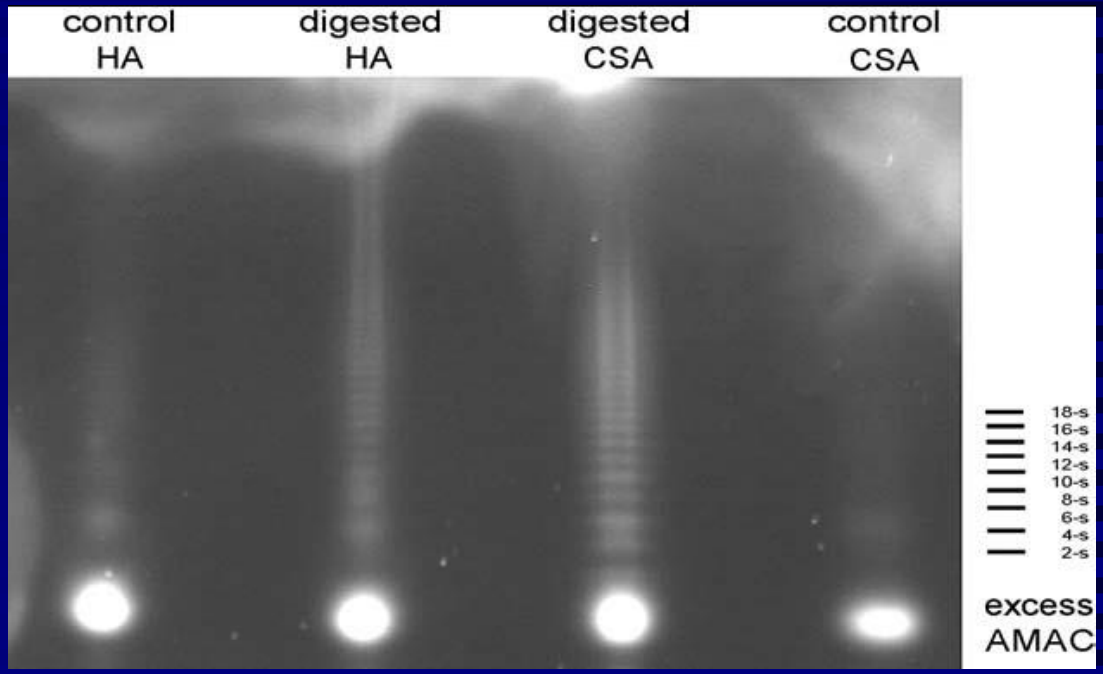
FACE result in 35% polyacrylamide

Assouti et al. BBA (2006) 1762, 54-58



Identification of hyaluronidase activity products

Tsilemou et al. Biochimie (2004) 86, 579-586



Zymography experiments for the detection of enzymes

- By entrapping the substrate into the electrophoresis gel

  Detection of metalloproteinases and hyaluronidases

- By copolymerising the substrate with the acrylamide

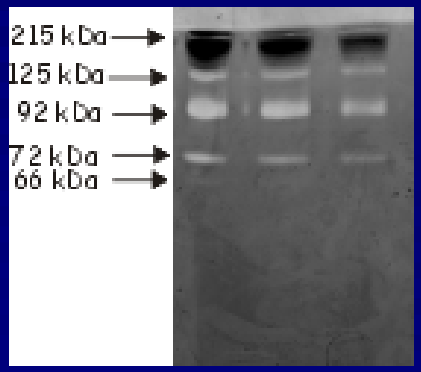
  Detection of hyaluronidases

- By incubating the gel onto which the enzymes are separated with the specific substrate in a suitable buffer

  Detection of phosphatases, cellulases, etc.

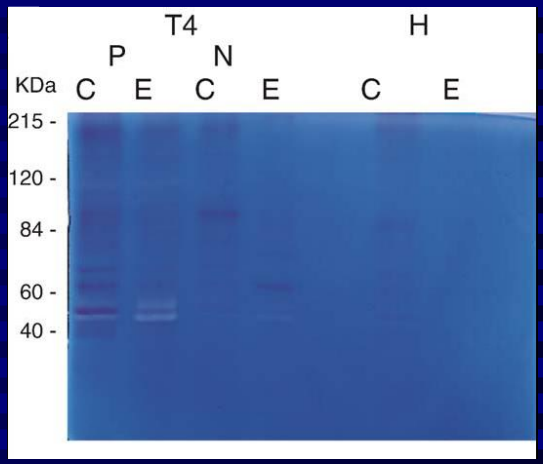
Zymography result

Serum gelatinases

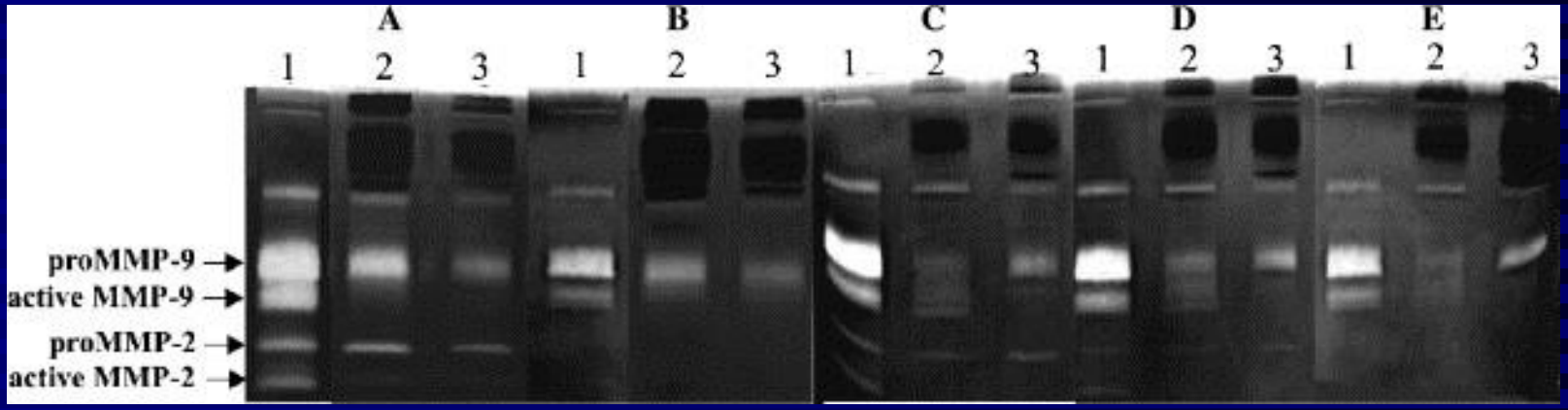


Ziouti et al. J Pharmaceut Biomed Anal (2004) 34, 771-789

Cancer hyaluronidase



Christopoulos et al. Biochim Biophys Acta (2006) 1760, 1039-1045



Effect of increased concentration of Ba^{2+} in the activity of MMP-2 in the presence of 5 mM Ca^{2+}. Lanes 1–3, reference mixture of MMP-2 and MMP-9 and sera from rheumatoid arthritis and Sjögren's syndrome patients, respectively. Gels were washed and incubated in a buffer containing 5 mM $CaCl_2$ (A), 5 mM $BaCl_2$ (B), 5 mM $CaCl_2$ and 50 mM $BaCl_2$ (C), 5 mM $CaCl_2$ and 100 mM $BaCl_2$ (D), and 200 mM $CaCl_2$ and 5 mM $BaCl_2$ (E).

N. Ziouti, N.S. Mastronikolis, A.P. Andonopoulos, C.D. Georgakopoulos, D.H. Vynios

Selective inhibition of matrix metalloproteinase 2 (gelatinase A) by barium chloride

Analytical Biochemistry, Volume 350, Issue 1, 2006, 159–161

http://dx.doi.org/10.1016/j.ab.2005.12.014

A very promising ELISA technique

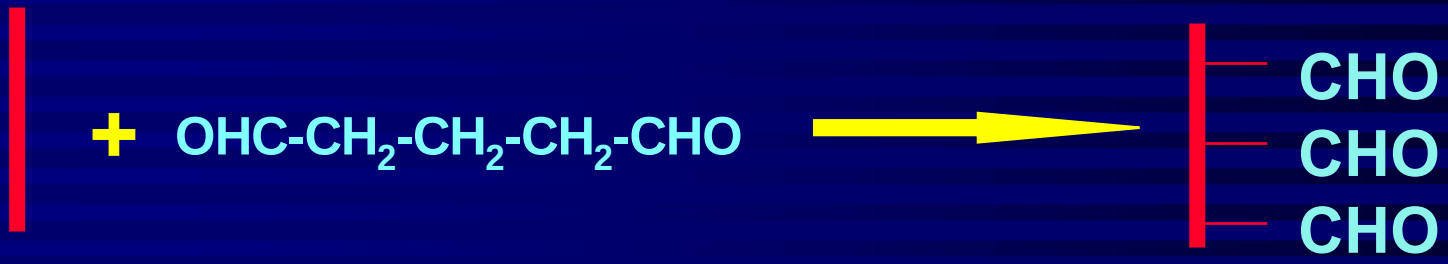
Vynios et al. Anal. Biochem. (1998) 260, 64-70

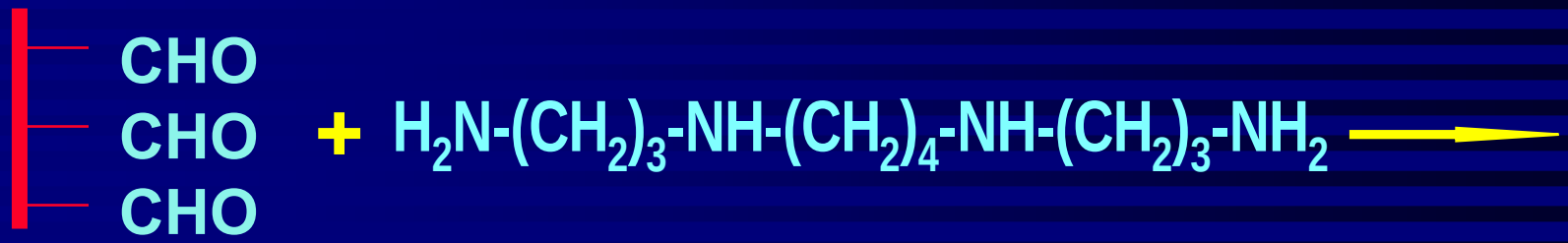
Step 1: Activation of polystyrene

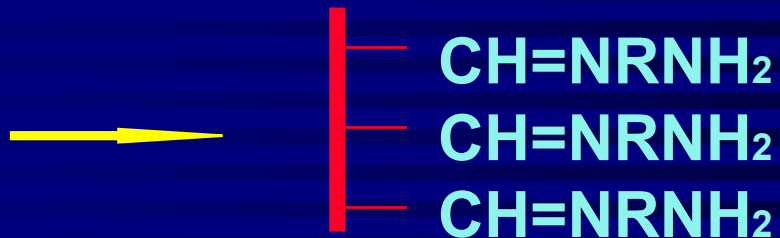
Step 2: Immobilisation of negatively charged molecules

Step 3: Identification of the immobilised molecules

Activation of polystyrene







A very promising ELISA technique

- Quantitation of a proteoglycan when there are antibodies against it
- Study of the interactions of a proteoglycan / glycosaminoglycan
- Detection of antibodies in serum against a proteoglycan
- Quantitation of glycosaminoglycans using a labelled glycosaminoglycan as reference compound

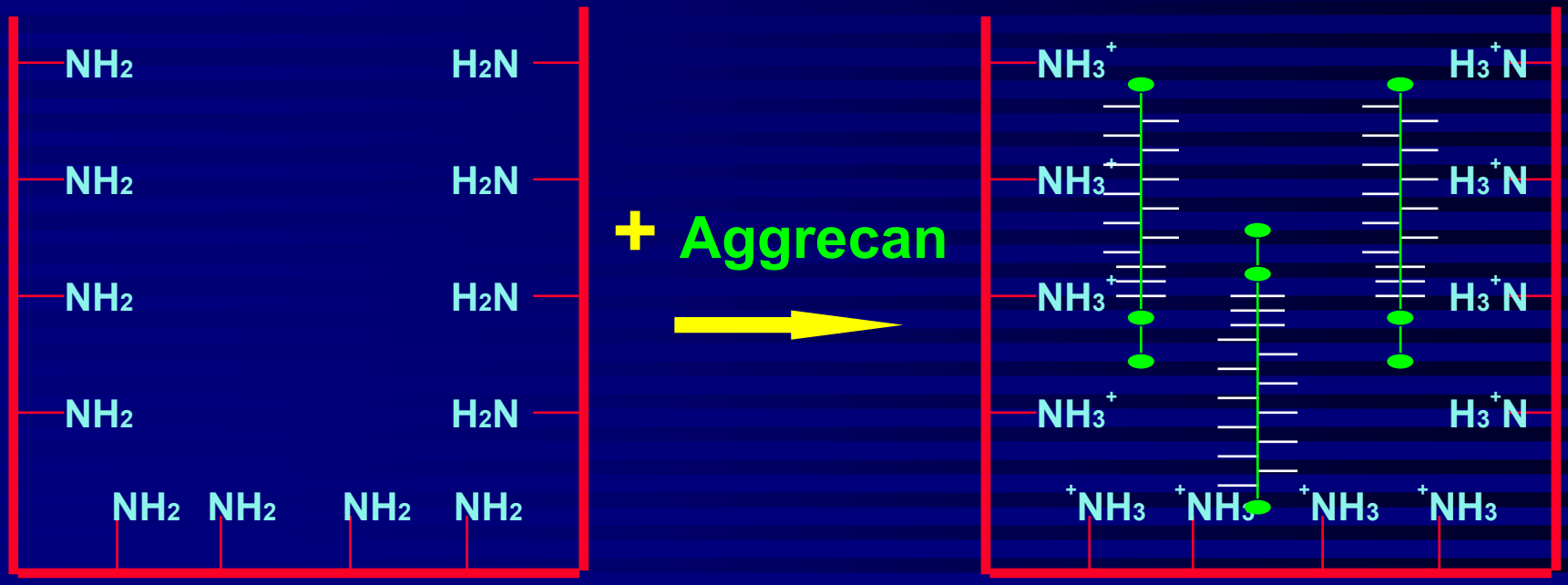
Applications

- Development of analytical / diagnostic methods
- Organisation of connective tissue extracellular matrix
- Alterations of glycoconjugates in pathologic states

Development of analytical / diagnostic methods

- Quantitation of proteoglycans
- Quantitation of glycosaminoglycans
- Quantitation of human prions
- Quantitation of autoantibodies against proteoglycans in systemic diseases

Quantitation of aggrecan

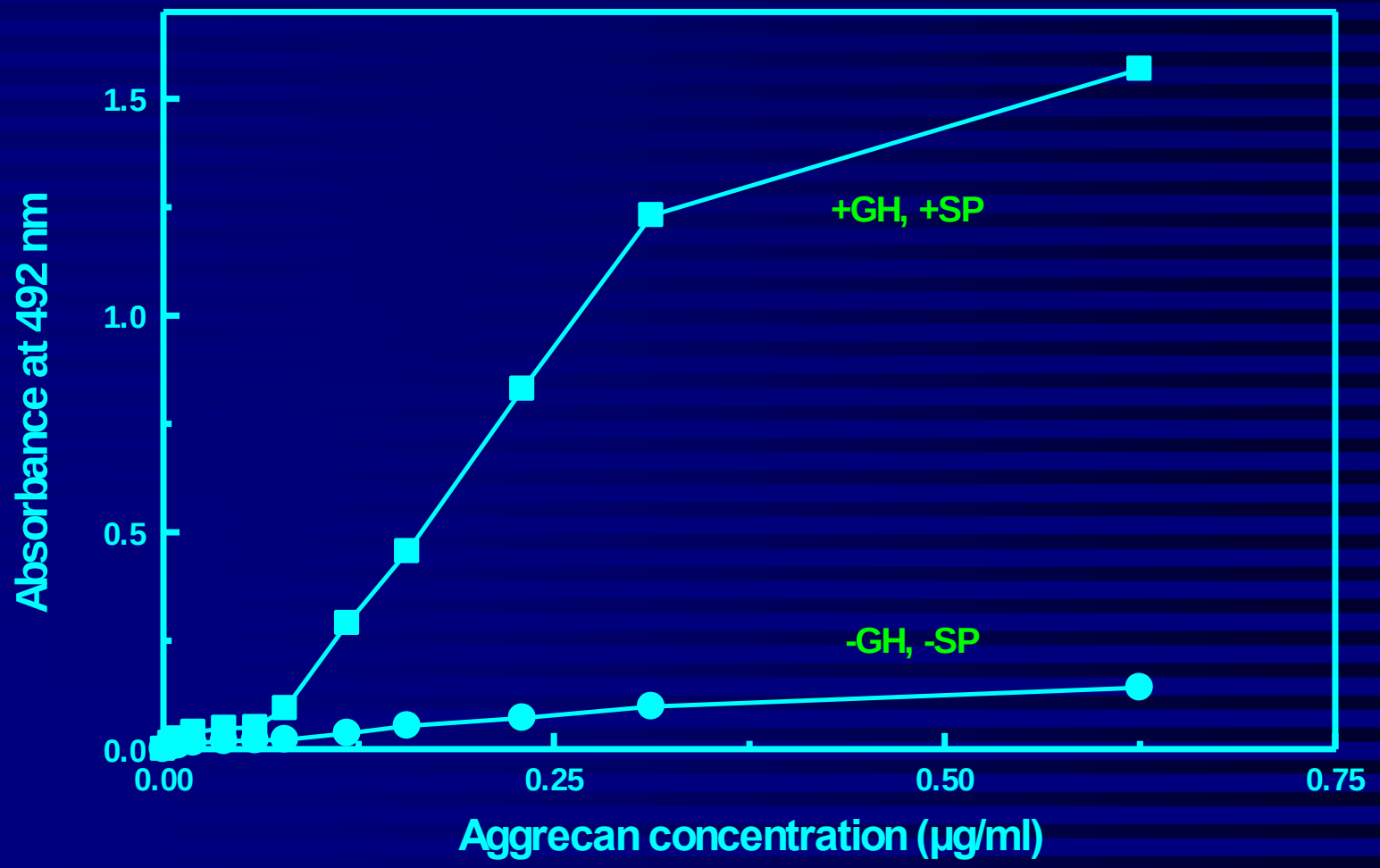


Optimum binding conditions

- C_{GH}: 1.25 mM, T=25^{0}C, t=4h, pH=5.0
- C_{SP}: 50µM, T=37^{0}C, t=4h, pH=9.0
- Aggrecan: T=37^{0}C, t=1h, pH=4.8

Quantitation of aggrecan immunochemically

Vynios et al. Anal. Biochem. (1998) 260, 64-70



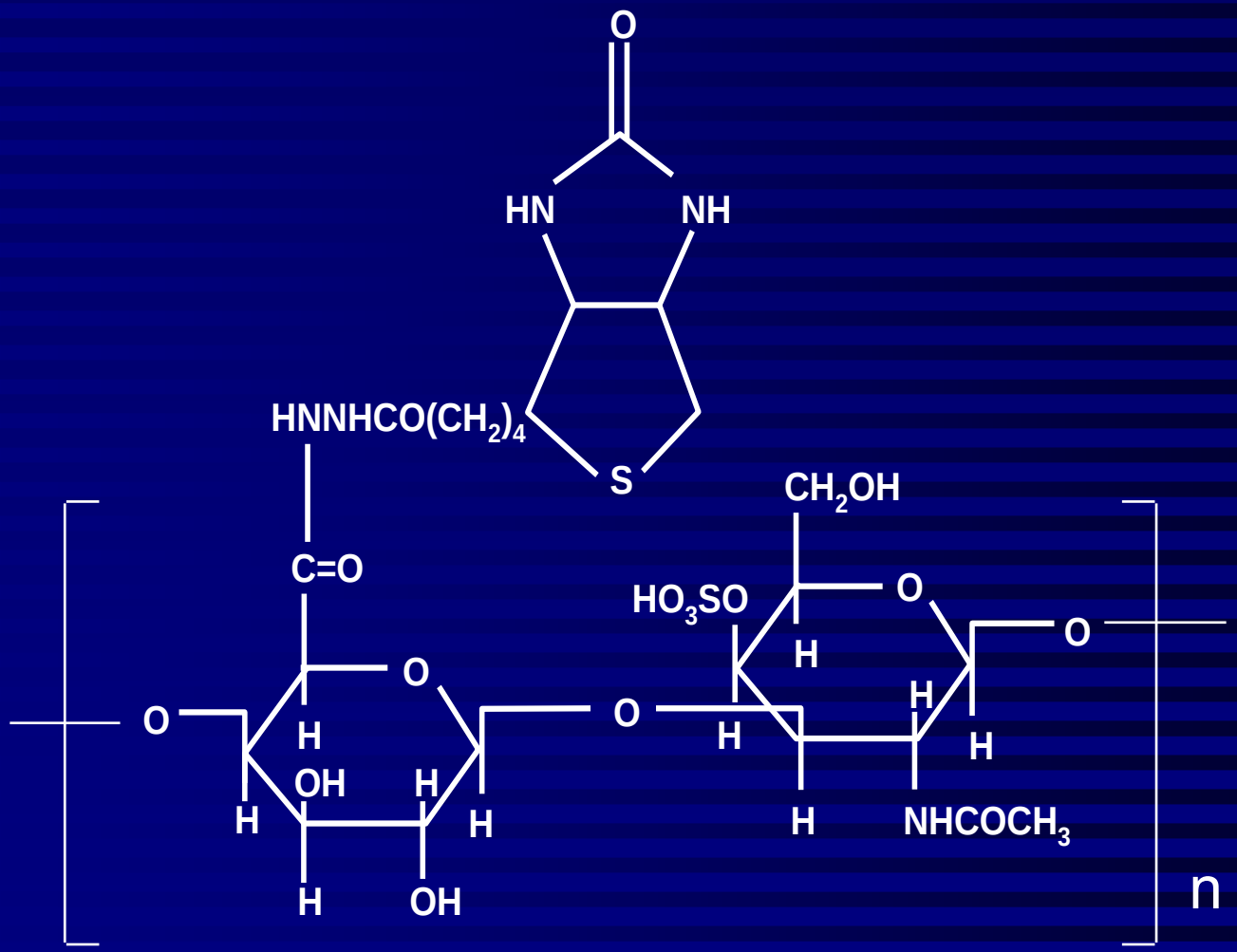
Development of analytical / diagnostic methods

- Quantitation of proteoglycans
- Quantitation of glycosaminoglycans
- Quantitation of human prions
- Quantitation of autoantibodies against proteoglycans in systemic diseases

Quantitation of glycosaminoglycans

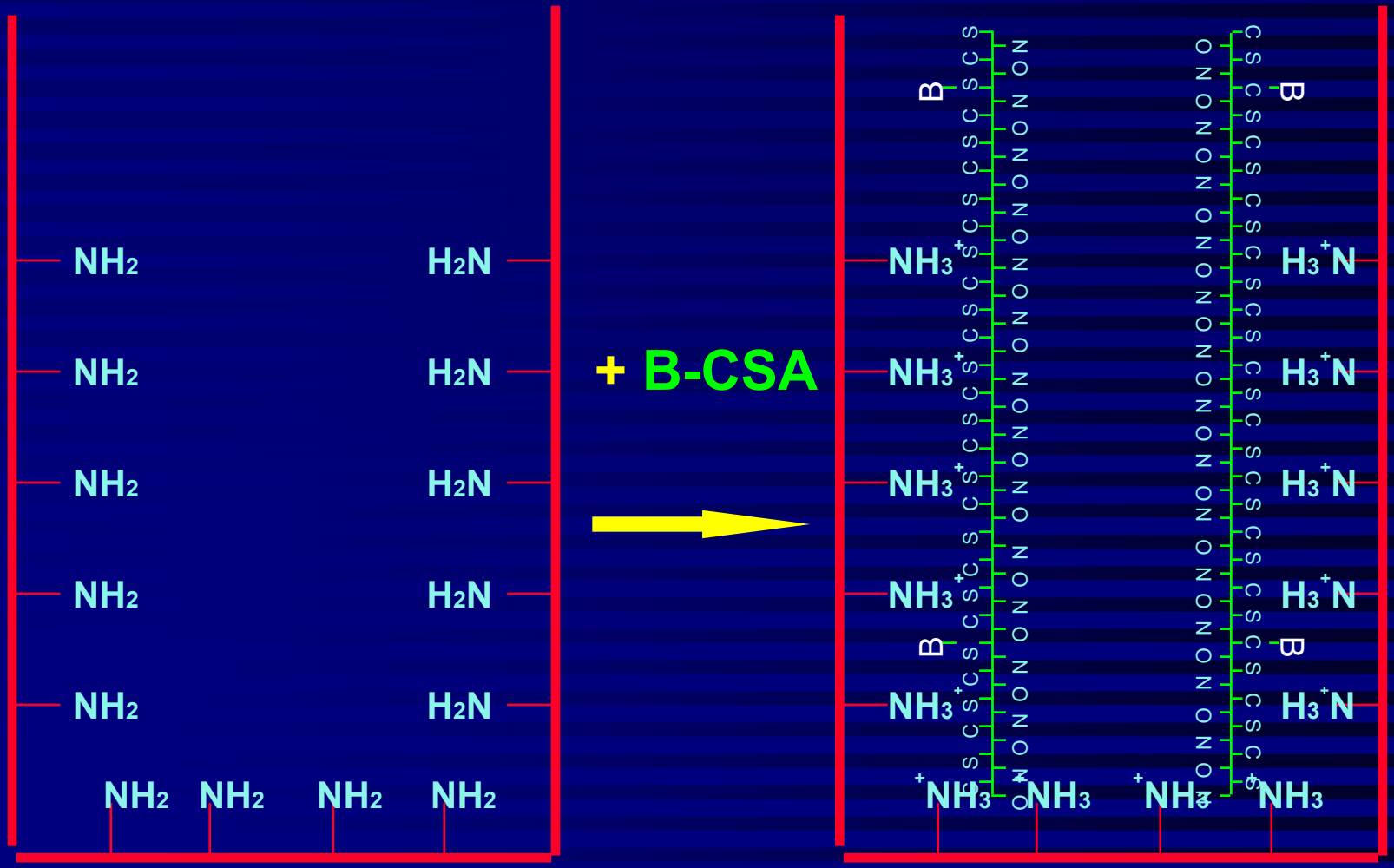
- Labelling of chondroitin sulphate with biotin
- Quantitative analysis by a competitve assay

Labelling of Chondroitin sulphate with biotin



Substitution in 1 to 10 disaccharides

Immobilisation of B-Chondroitin sulphate onto ELISA plates



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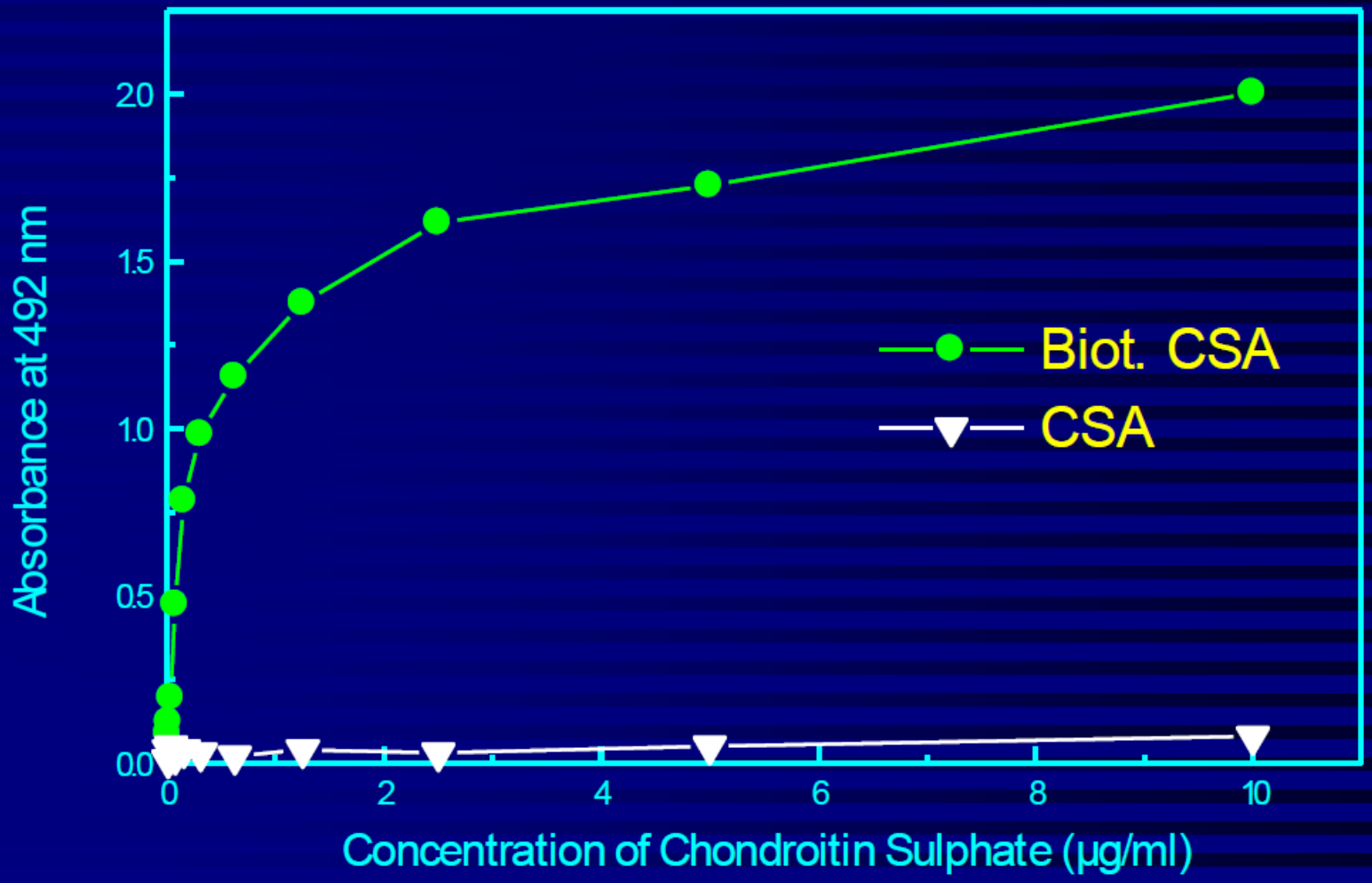
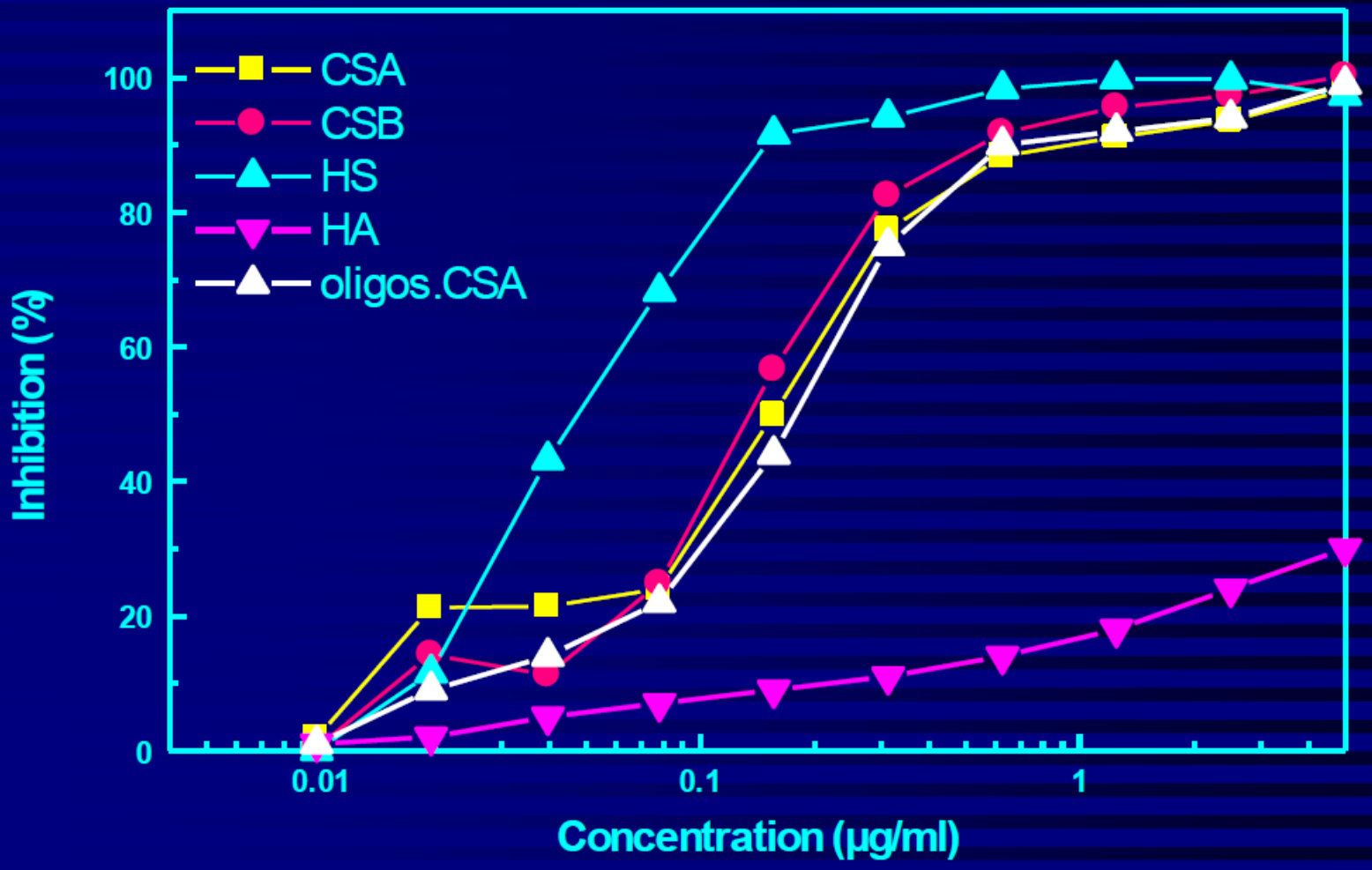
- B-CSA: T=37^{0}C, t=1h, pH=4.3

Immobilisation of B-Chondroitin sulphate onto ELISA plates

Vynios et al. J. Pharm. Biomed. Anal. (1999) 21, 859-865

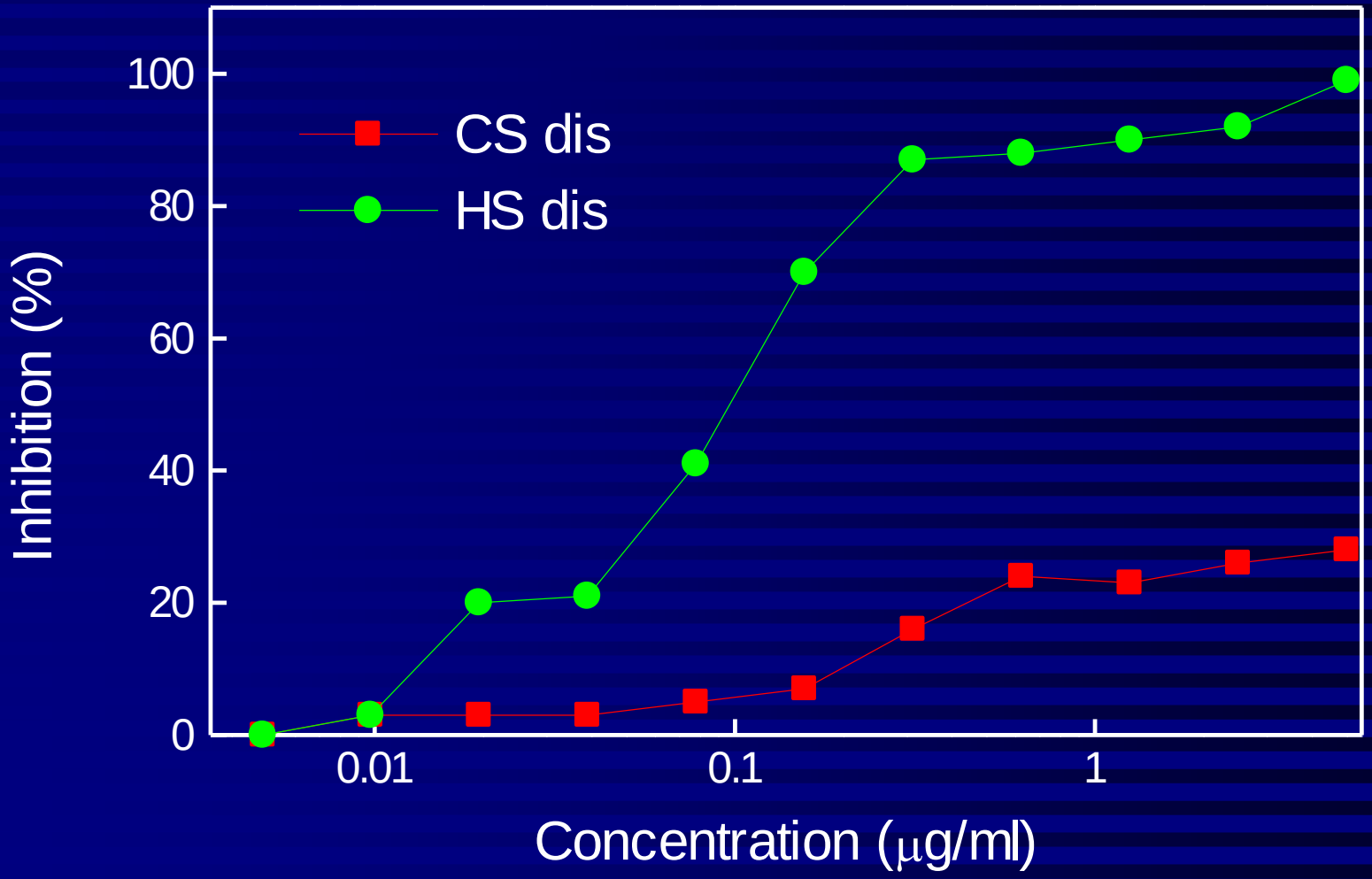


Quantitation of glycosaminoglycans



Quantitation of glycosaminoglycan disaccharides

Vynios et al. J. Immunoass. Immunochem. 22 (2001) 337-351



Quantitation of hyaluronan

- Activation of the plate wells with GH and spermine
- Covalent binding of hyaluronan
- Addition of aggrecan
- Detection of aggrecan by immunochemistry

- Quantitative analysis of hyaluronan by a competitve assay

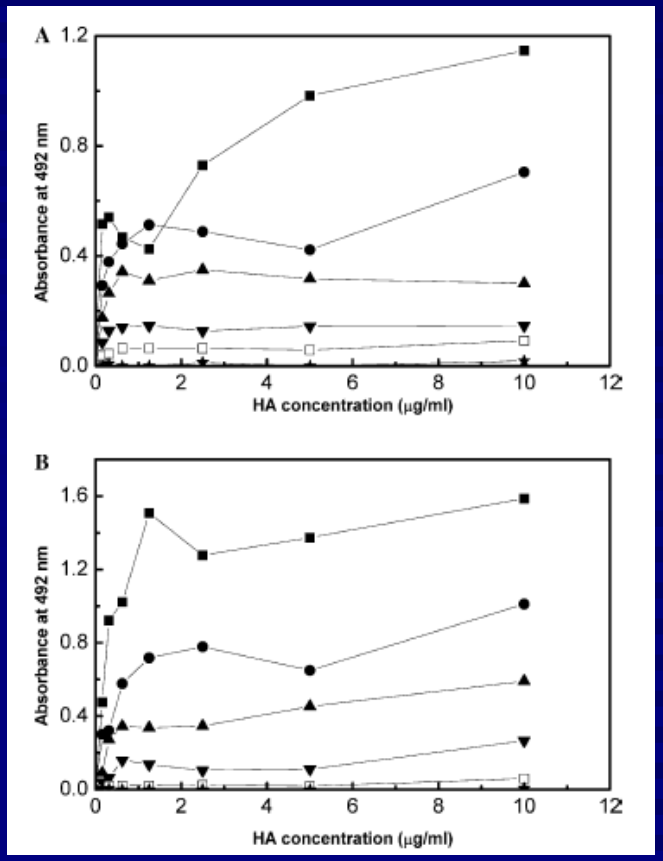
Quantitation of hyaluronan

Grigoreas GHA et al. (2003) Anal. Biochem. 320, 179-184

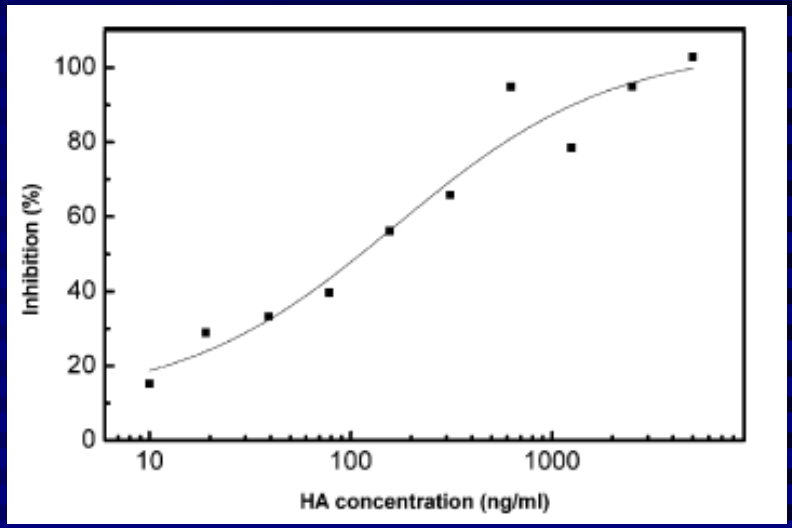
Binding of aggrecan to HA

pH 6.0

pH 9.0



Reference competitive curve



Development of analytical / diagnostic methods

- Quantitation of proteoglycans
- Quantitation of glycosaminoglycans
- Quantitation of human prions
- Quantitation of autoantibodies against proteoglycans in systemic diseases

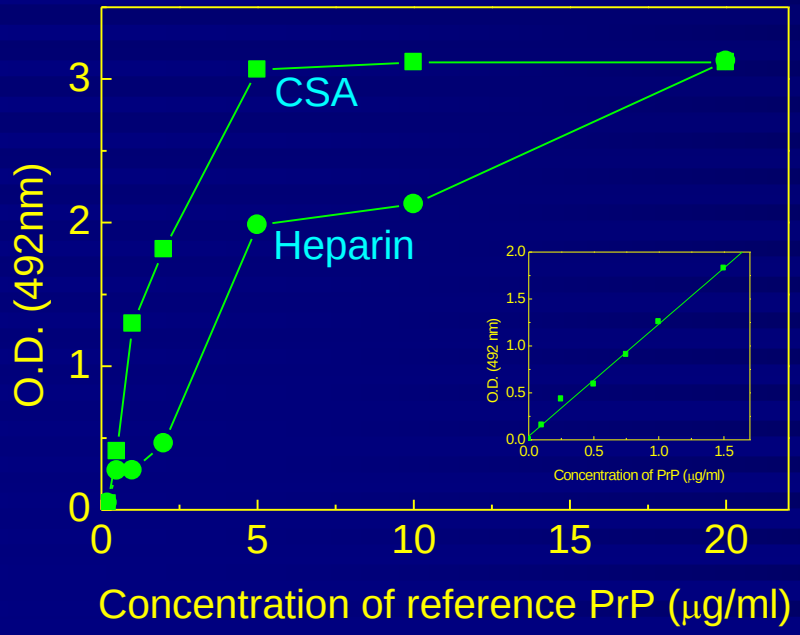
Quantitation of prion protein

- Immobilisation of chondroitin sulphate onto activated polystyrene
- Brief homogenisation of tissue in a detergent - containing solution
- Interaction of prion of the extracts or the reference samples with the immobilised chondroitin sulphate
- Immunodetection of prion

Quantitation of prion protein

Triantaphyllidou et al. (2006) Anal Biochem 359, 176-182

Aim: Detection of both normal and pathologic types of prions



Sample	H-PT	H-NT	S-C	S-SC
Extract	1.18	1.05	0	1.82
Papain digest	0	0	0	0.65
Proteinase K digest	0	0	0	0.17

Development of analytical / diagnostic methods

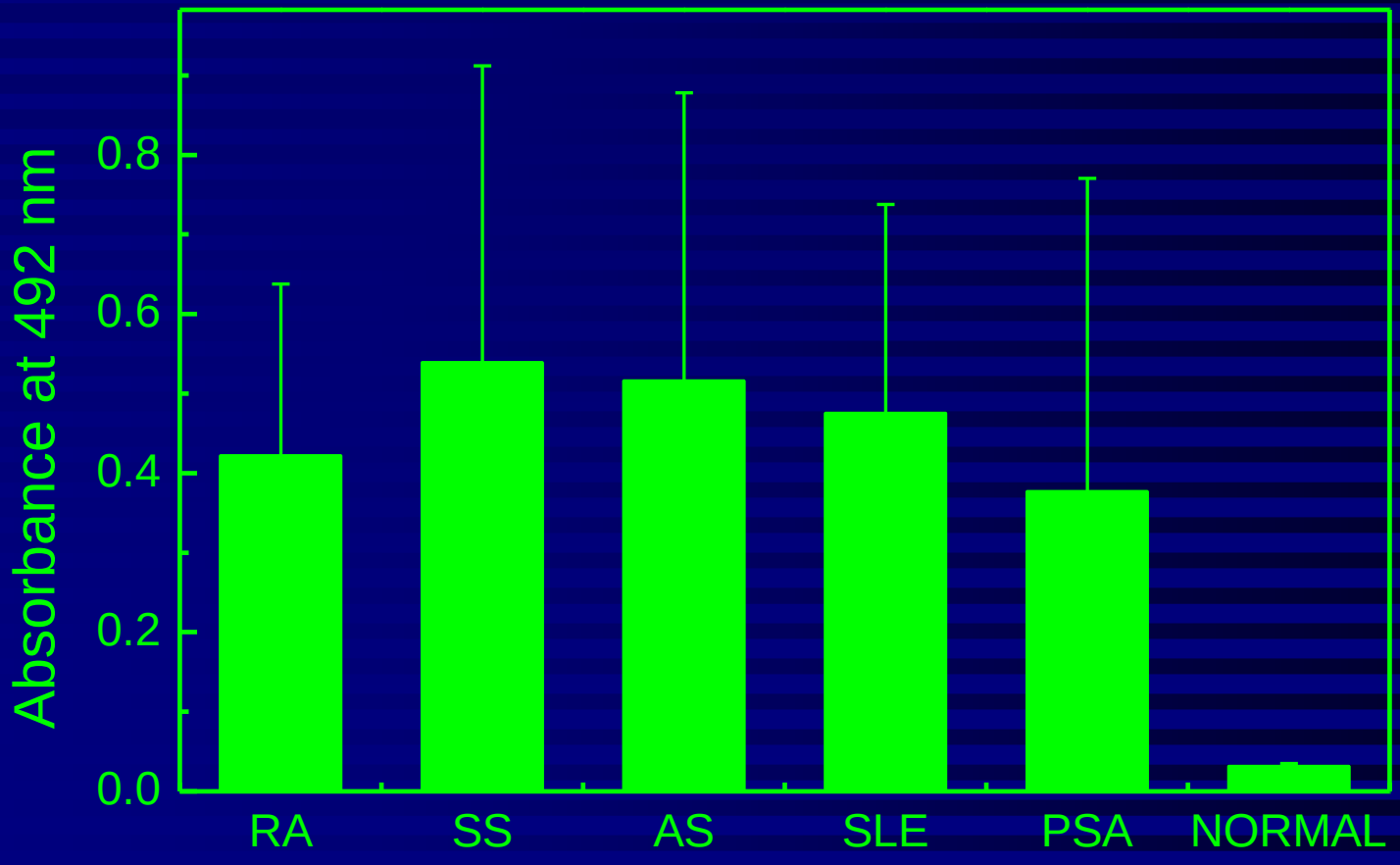
- Quantitation of proteoglycans
- Quantitation of glycosaminoglycans
- Quantitation of human prions
- Quantitation of autoantibodies against proteoglycans in systemic diseases

Autoantibodies against aggrecan in systemic diseases

Vynios et al. (2006) Biochimie 88, 767-773

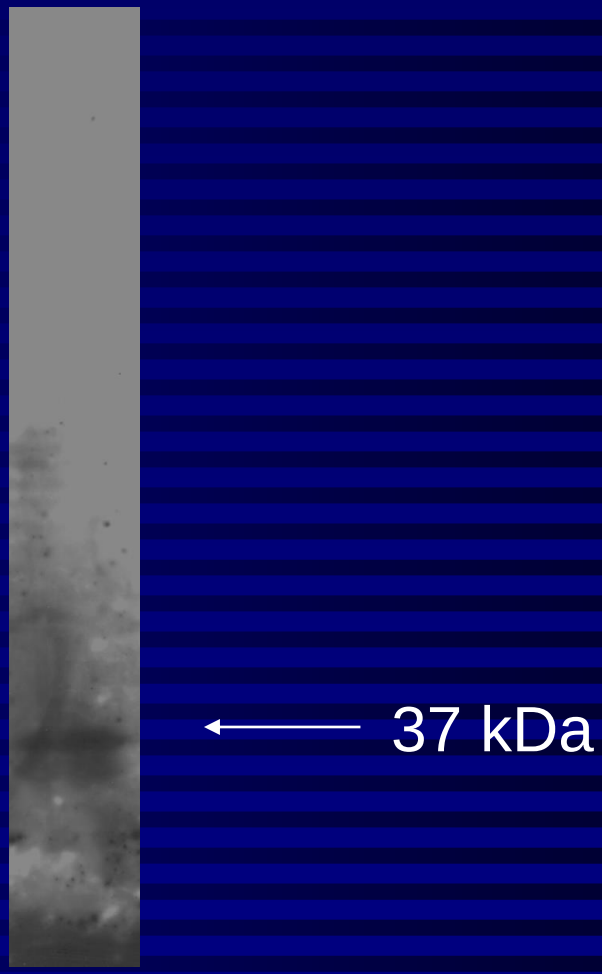
- Immobilisation of aggrecan onto activated polystyrene
- Interaction with human sera

Autoantibodies against aggrecan in human serum

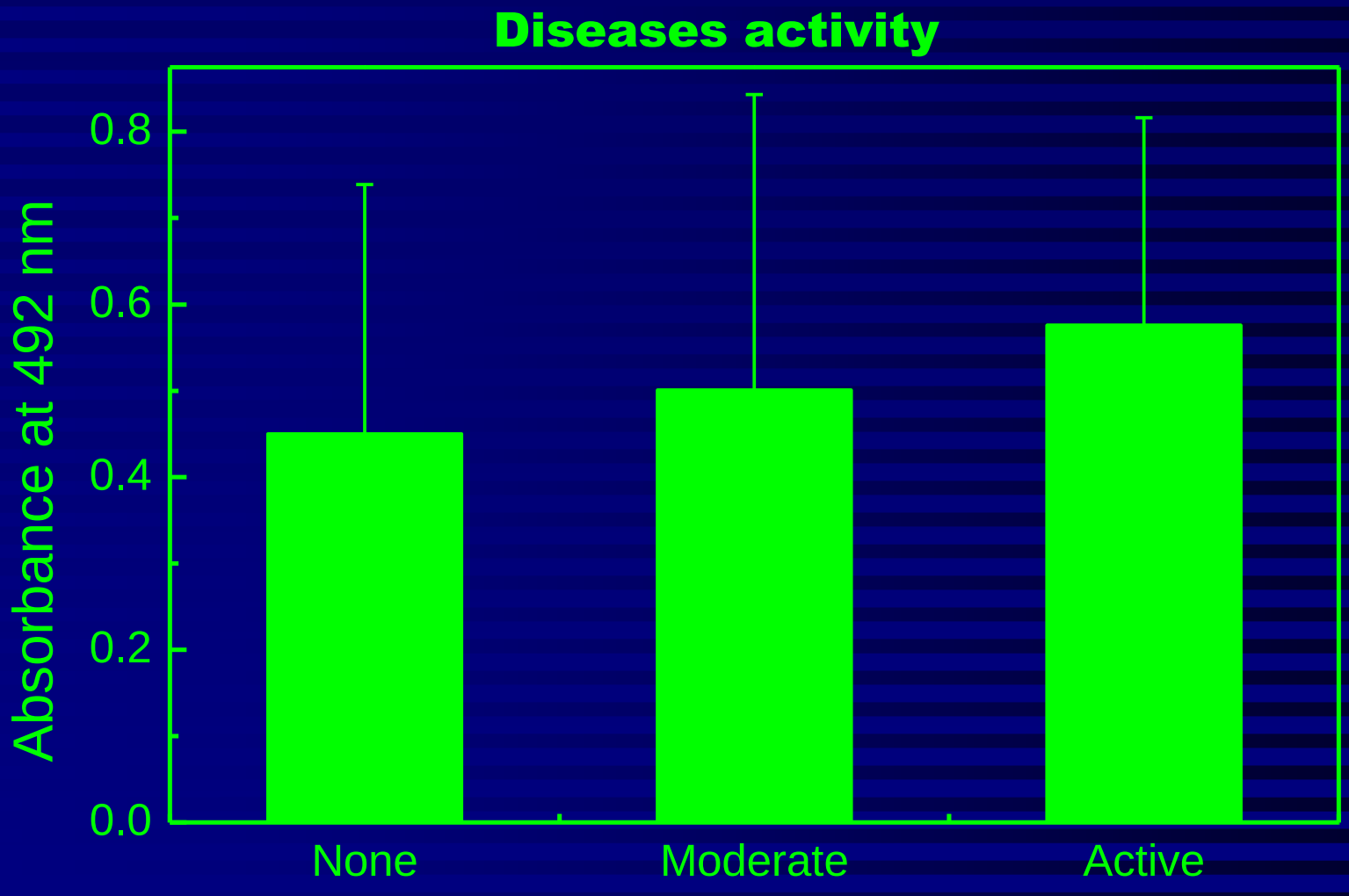


Autoantibodies against aggrecan in human serum

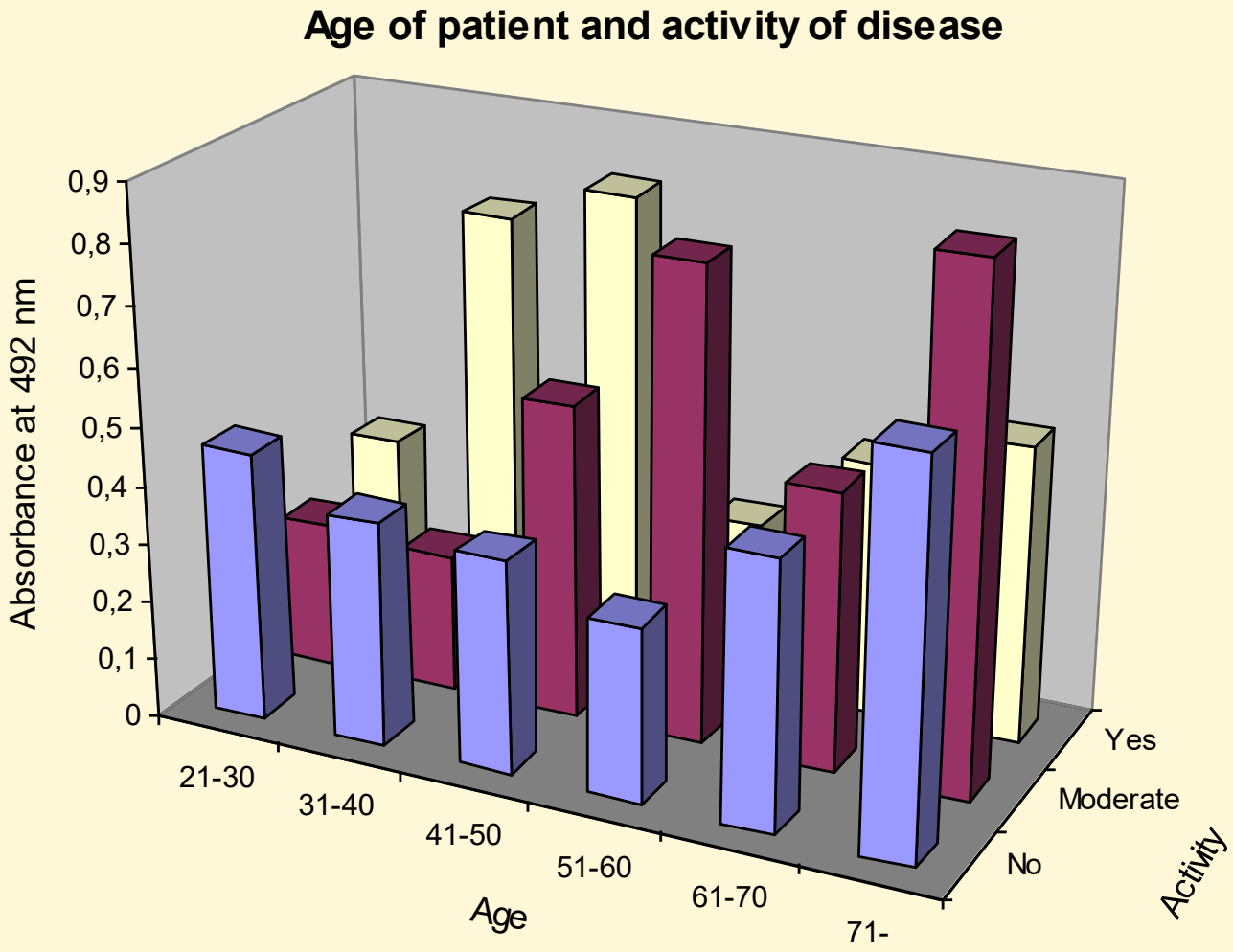
V8 Protease treatment of aggrecan



Autoantibodies against aggrecan in human serum



Autoantibodies against aggrecan in human serum



Organisation of connective tissue extracellular matrix

- Interaction of proteoglycans with collagen
- Interaction of proteoglycans with proteins
- Interaction of hyaluronan with aggrecan and link protein
- Self-interactions of proteoglycans

Interactions of proteoglycans with collagen

Vynios et al. Biochimie 82 (2000) 773-782
Vynios et al. Biochimie 83 (2001) 899-906

Aim

- Examination of the possible different behaviour of collagen types I and II in interacting with the various proteoglycans
- Investigation of the part of proteoglycans involved in the interaction

Methods

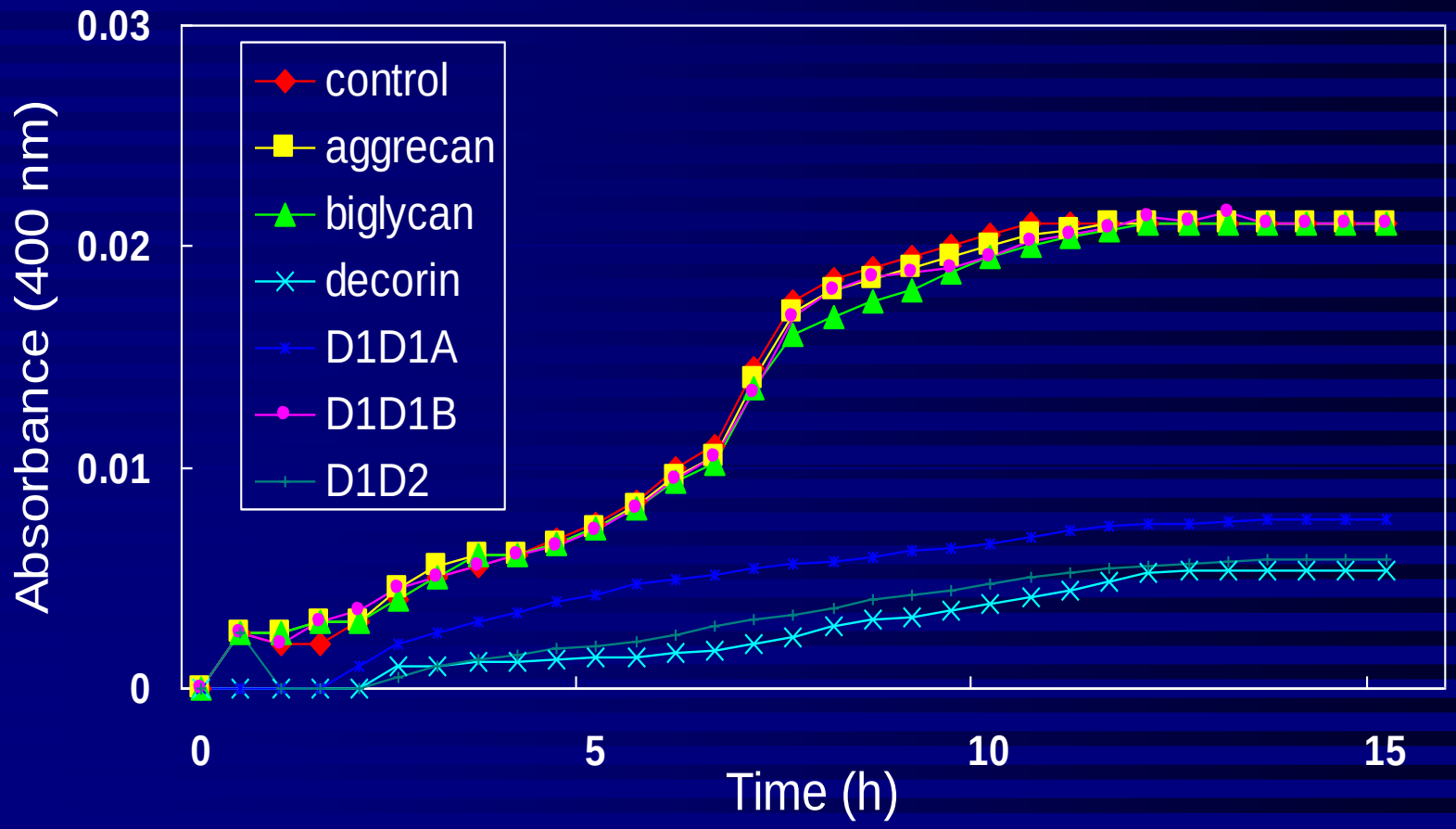
Inhibition of collagen fibril formation

- Incubation of collagen with proteoglycans
- Measurement of fibril formation versus time

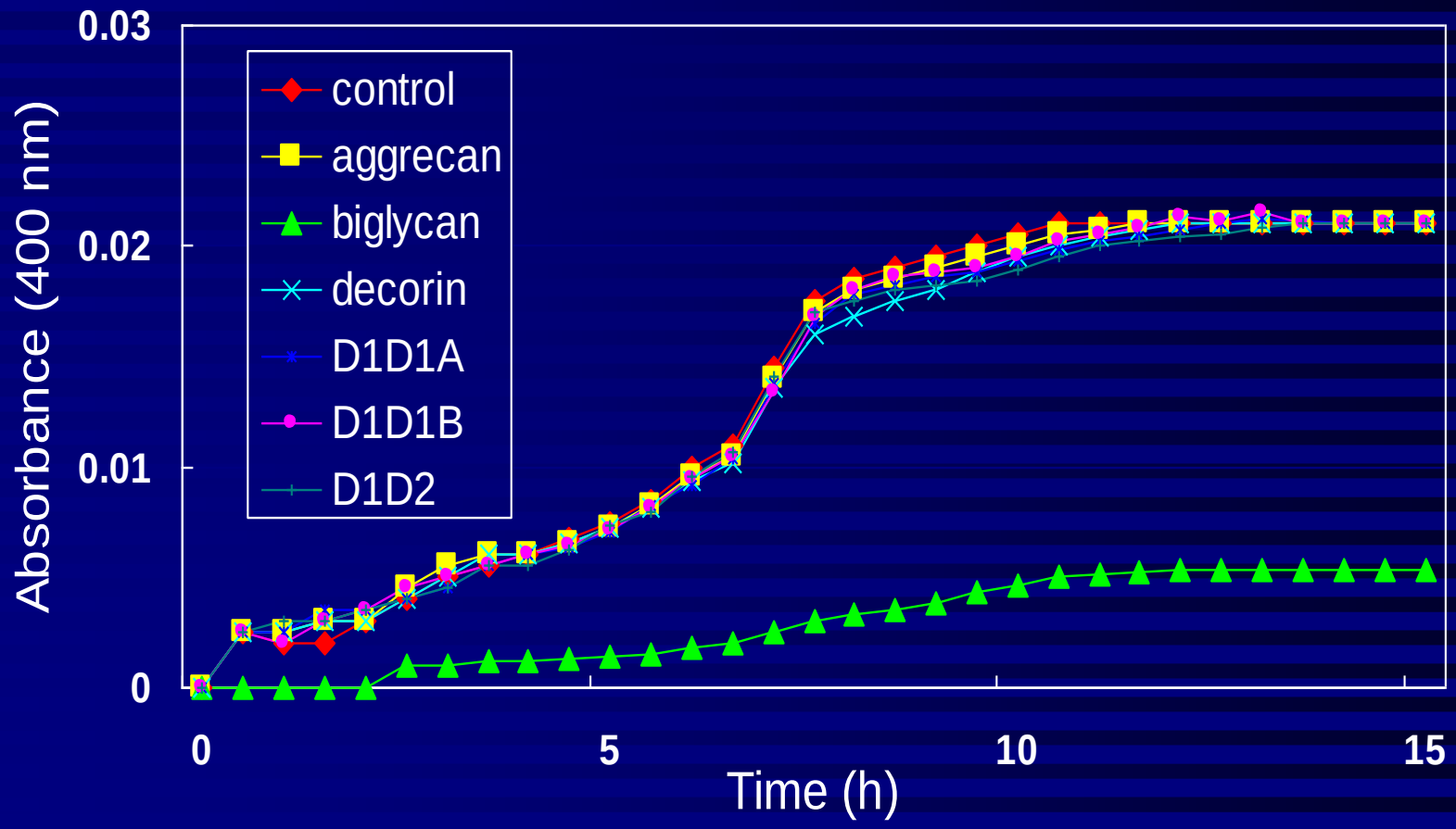
Interaction of collagen with proteoglycans in a solid phase assay

- Immobilisation of collagen
- Blocking with BSA
- Interaction with proteoglycans or fragments thereof
- Immunodetermination of proteoglycans

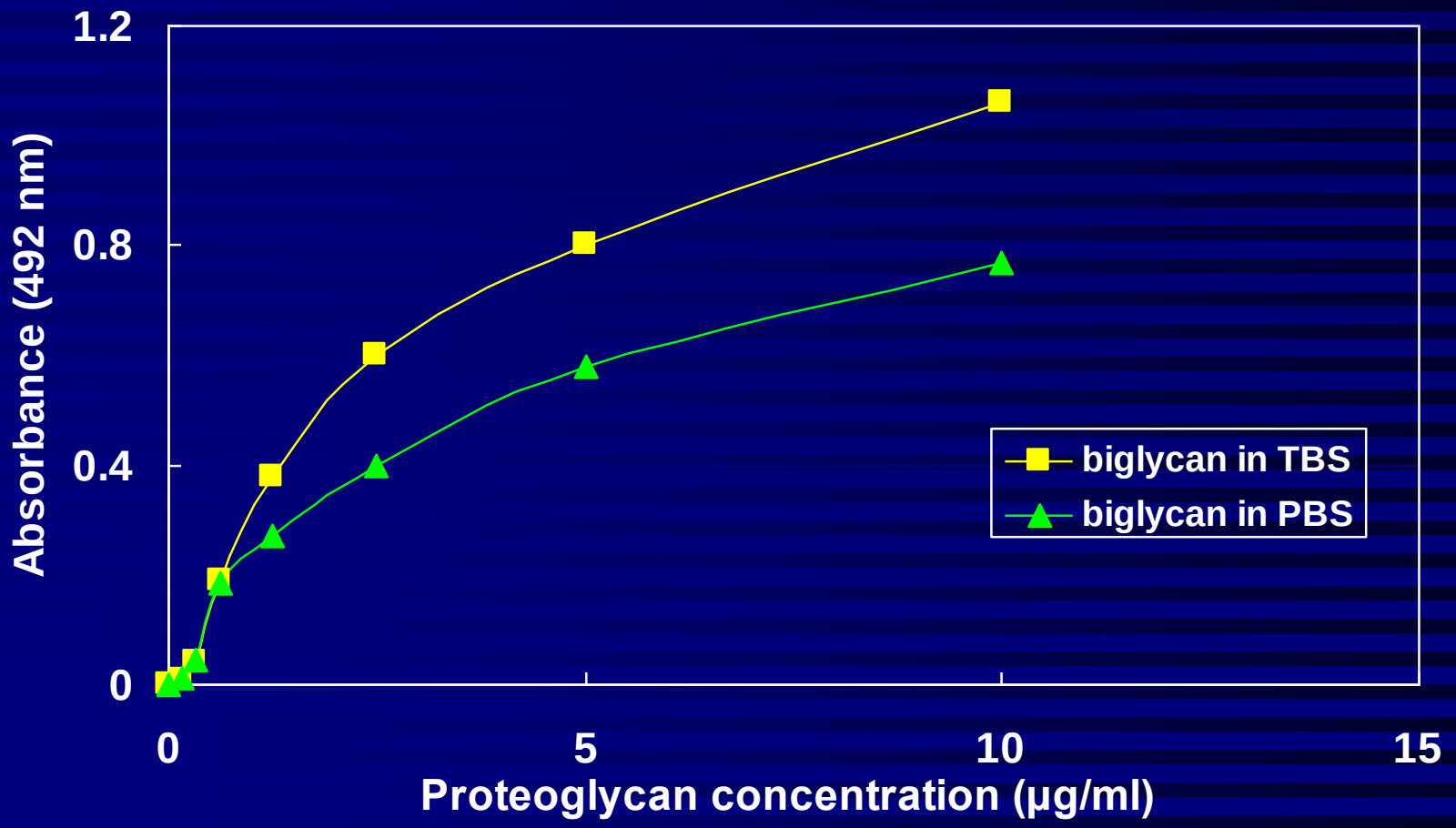
Inhibition of collagen type I fibril formation



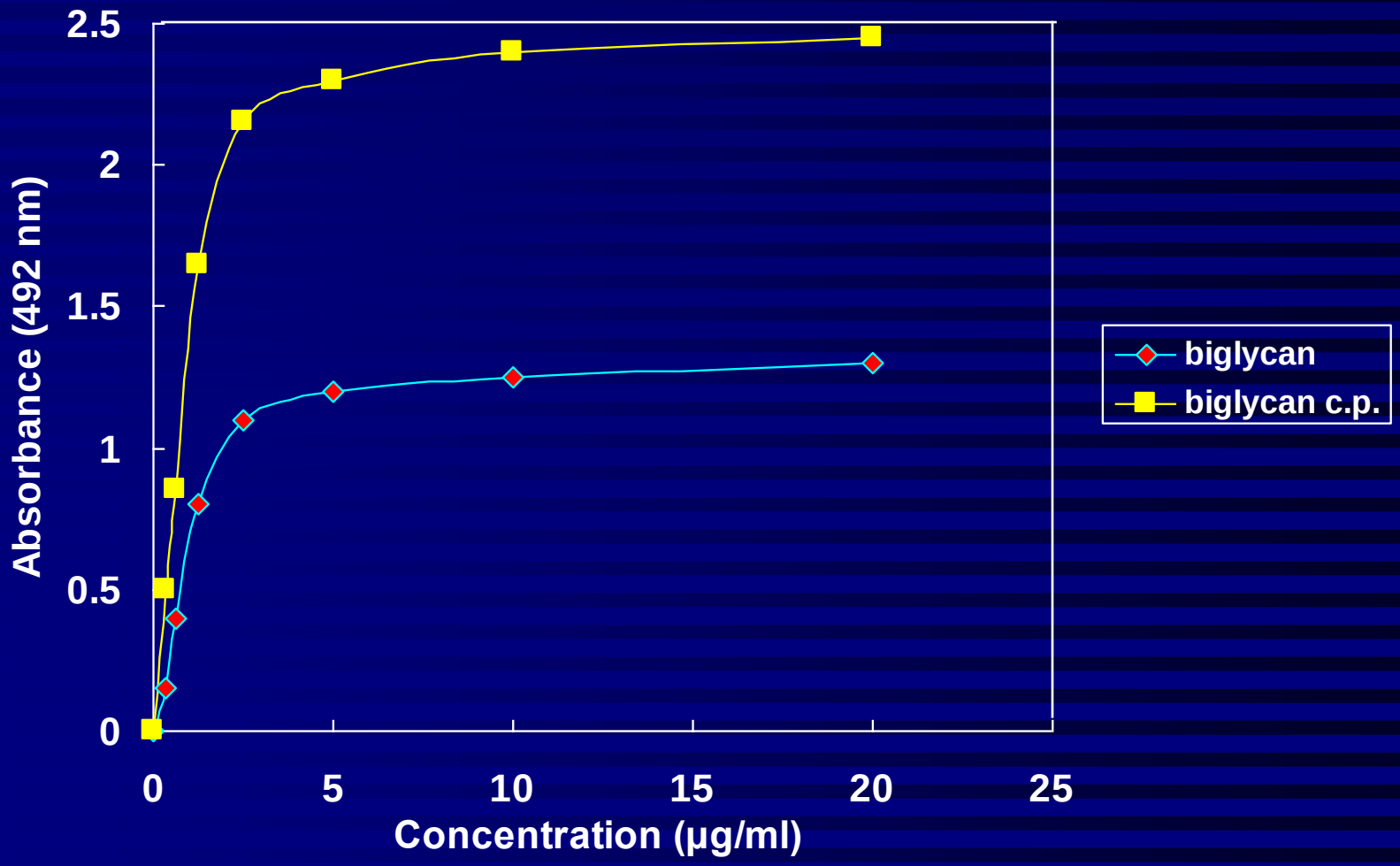
Inhibition of collagen type II fibril formation



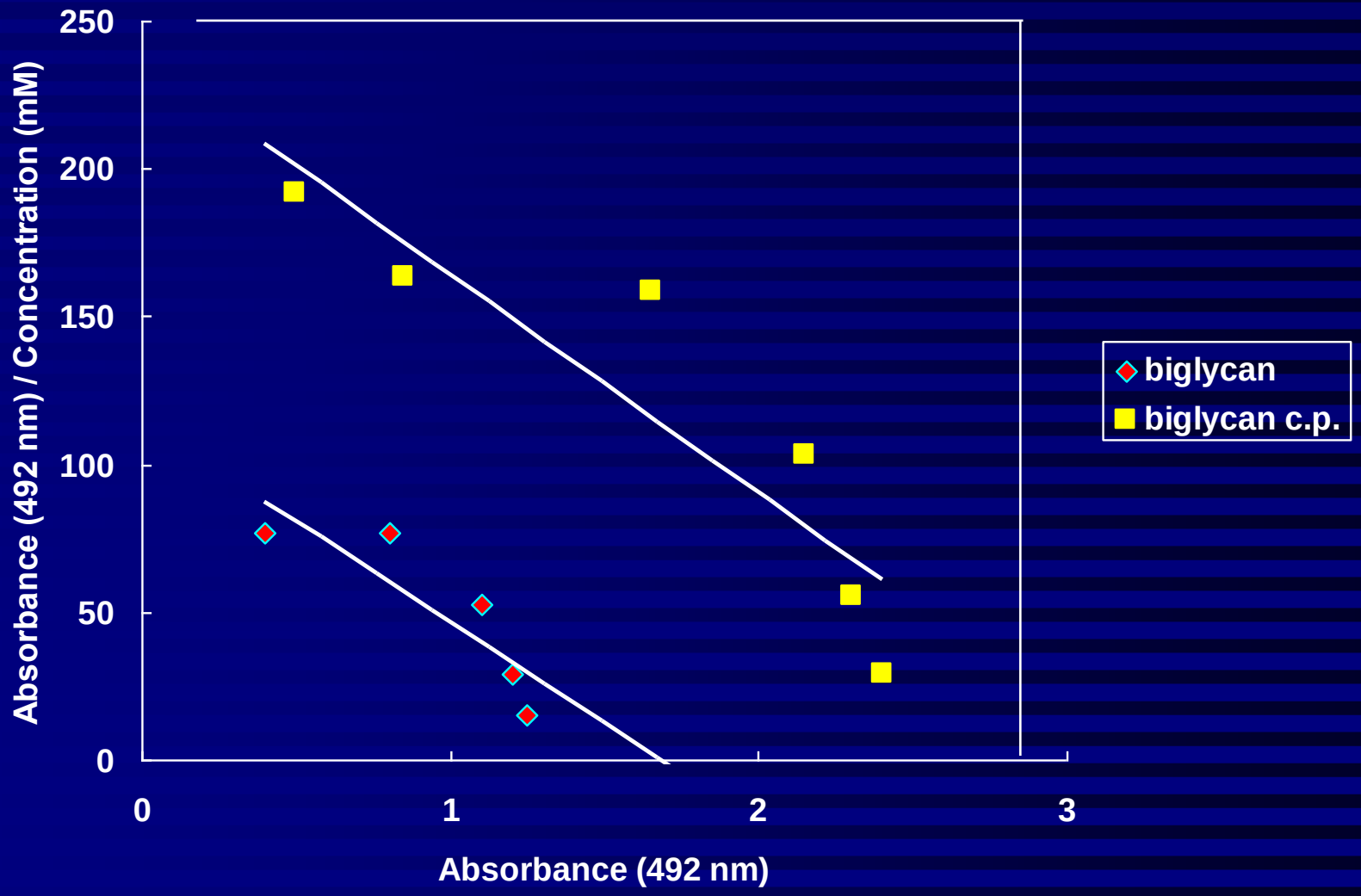
Interaction of biglycan with collagen type II



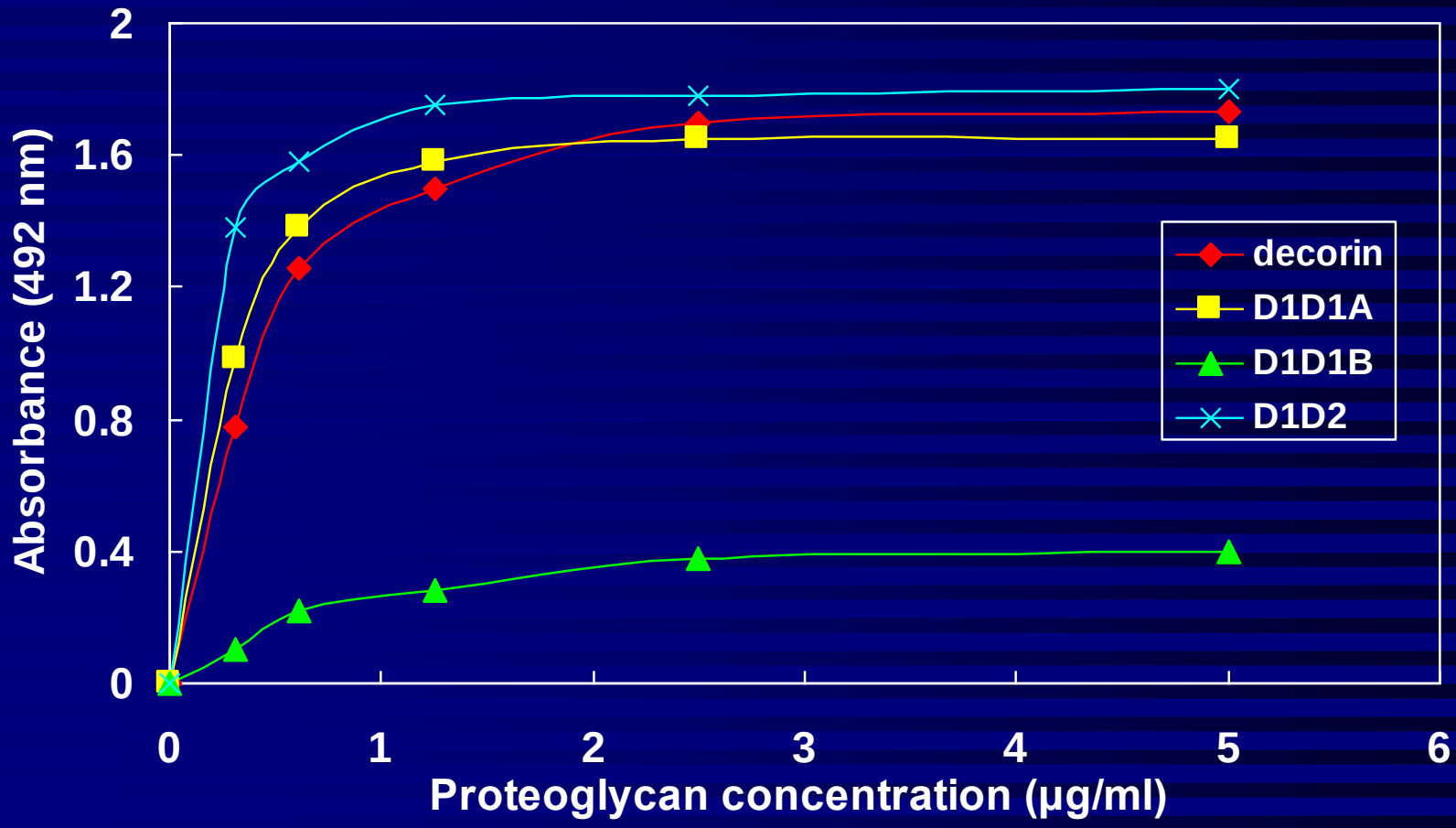
Interaction of biglycan core protein with collagen type II



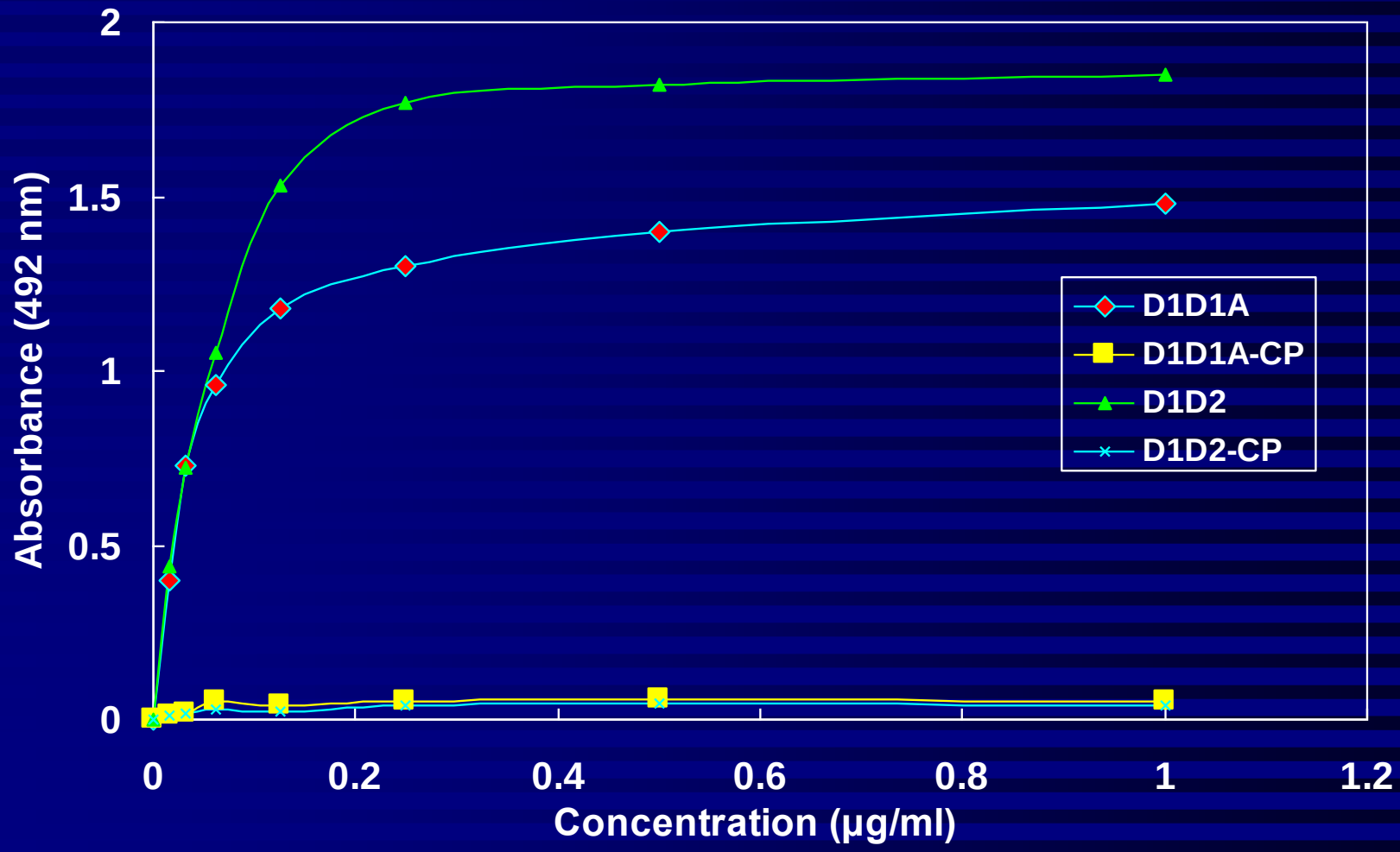
Interaction of biglycan core protein with collagen type II



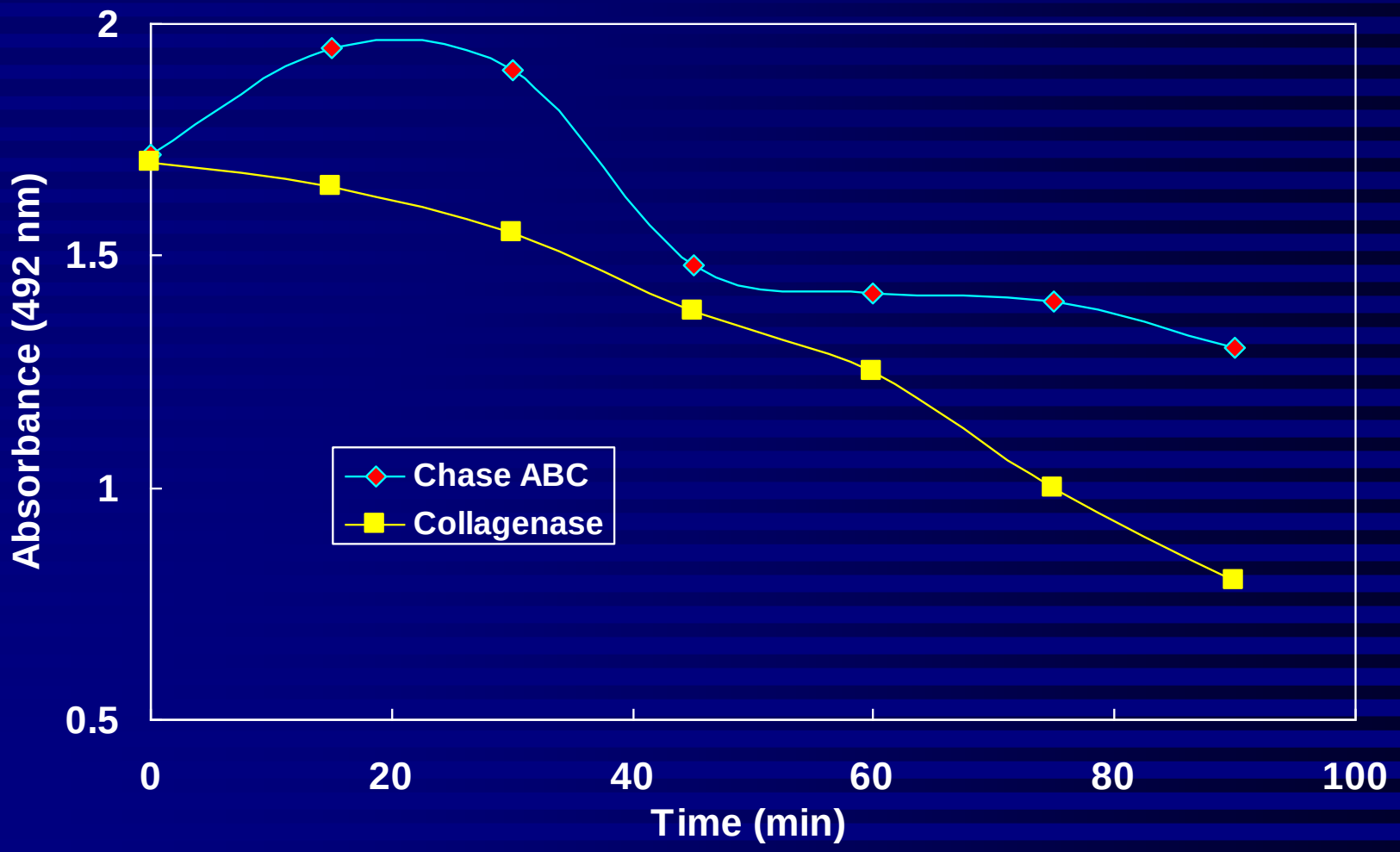
Interaction of proteoglycans with collagen type I



Interaction of D1D1A and D1D2 core protein with collagen type I



Interaction of collagen with D1D2 partially degraded by chondroitinase ABC or collagenase



Organisation of connective tissue extracellular matrix

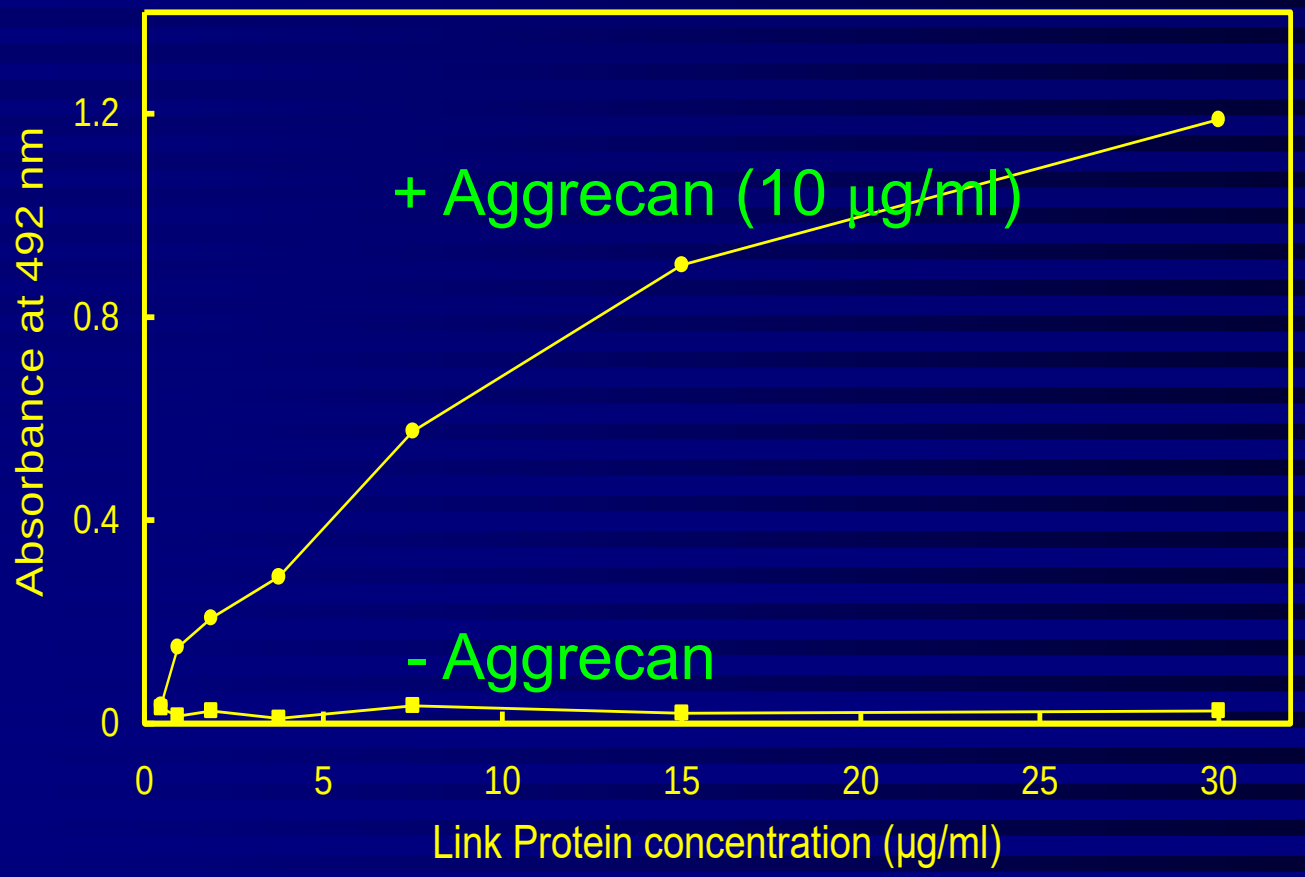
- Interaction of proteoglycans with collagen
- Interaction of proteoglycans with proteins
- Interaction of hyaluronan with aggrecan and link protein
- Self-interactions of proteoglycans

Interaction of aggrecan with link protein from various sources

- Use of the new ELISA assay
- Use of a common ELISA competitive assay

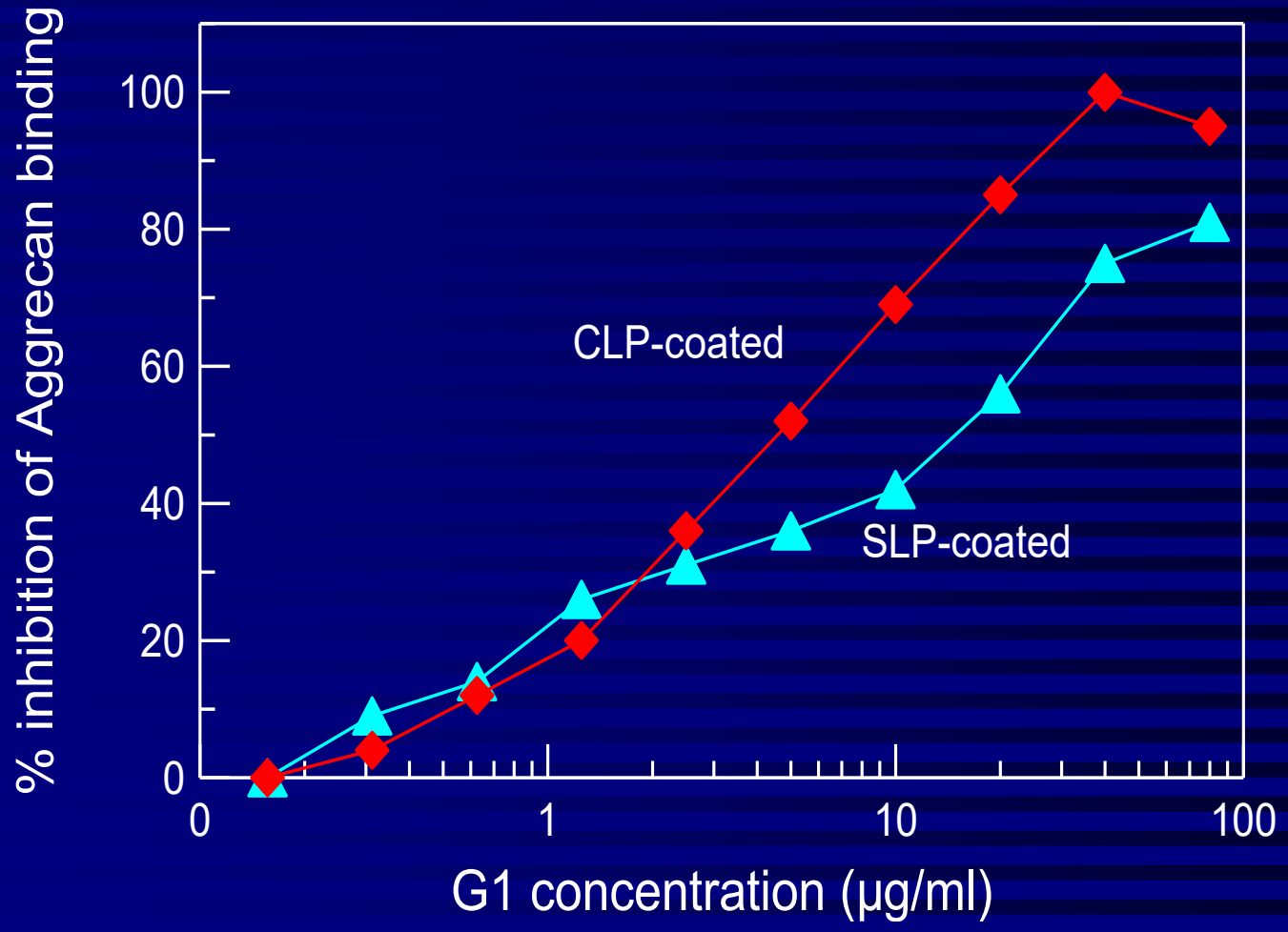
Interaction of aggrecan with cartilage link protein

Vynios et al. Anal. Biochem. (1998) 260, 64-70



Interaction of aggrecan with invertebrate link protein

Vynios et al. Biochimie (1998) 80, 591-594



Organisation of connective tissue extracellular matrix

- Interaction of proteoglycans with collagen
- Interaction of proteoglycans with proteins
- Interaction of hyaluronan with aggrecan and link protein
- Self-interactions of proteoglycans

Interaction of hyaluronan with aggrecan and link protein

Kalpaxis et al. Int. J. biochem. (1985) 17, 61-66

- Immobilisation of hyaluronan on activated cellulose

  Preparation of an affinity chromatography matrix

- Chromatography of tissue extracts or purified macromolecules on this matrix

The affinity chromatography matrix is used for:

- Isolation of macromolecules interacting with hyaluronan
- Study of the interaction of hyaluronan with aggrecan
- Study of the interaction of hyaluronan with link protein

Organisation of connective tissue extracellular matrix

- Interaction of proteoglycans with collagen
- Interaction of proteoglycans with proteins
- Interaction of hyaluronan with aggrecan and link protein
- Self-interactions of proteoglycans

Self-interactions of proteoglycans

- Isolation of the proteoglycans
- Study of possible self-interactions by gel chromatography, ELISA, electron microscopy, etc.

Solid phase assays

Identification of the pathogenesis of a disease

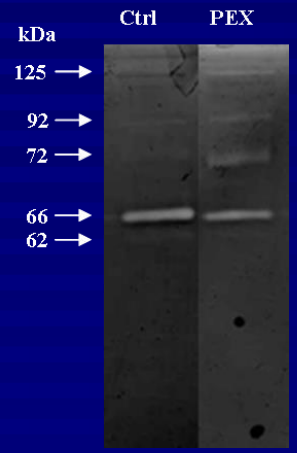
Alterations of glycoconjugates in exfoliation syndrome

- A disease of undetermined etiology and pathogenesis that involves impaired synthesis and degradation of the extracellular macromolecules of the matrix
- Ultrastructural alterations are observed in anterior segment tissues, such as:
- Deposits of typical PEX fibrils on the iris and ciliary epithelia and in the dilator muscle of the iris
- Accumulation of extracellular matrix, including microfibrils and reduplicated basement membrane material in the periphery of iris vessels, in the dilator muscle and in the juxtacanalicular tissue of the trabecular meshwork
- Degenerative changes of the iris pigment epithelium and dilator muscle cells
- **The origin of the exfoliated material remains unknown**

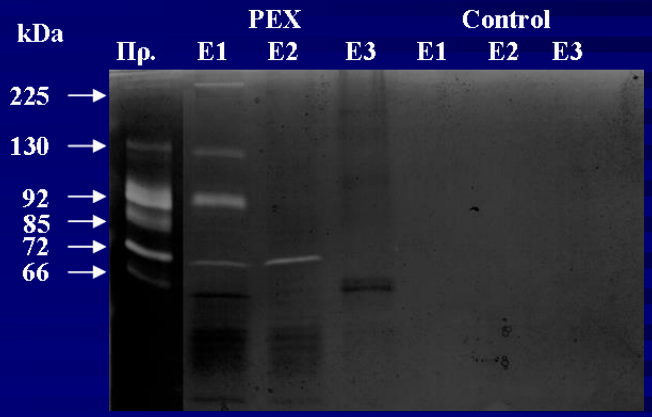
Detection of MMPs in exfoliation syndrome

Gartaganis et al. Ophthalmic research (2002) 34, 165-171

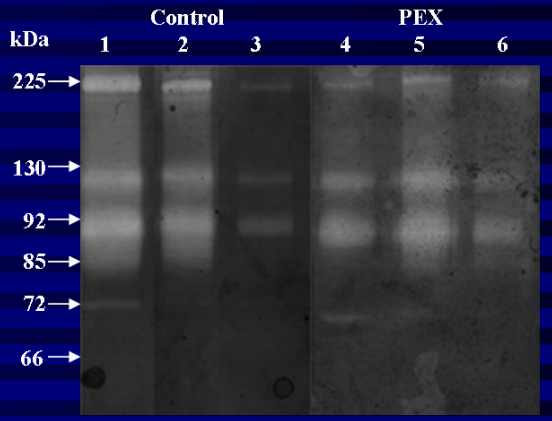
Aqueous humour

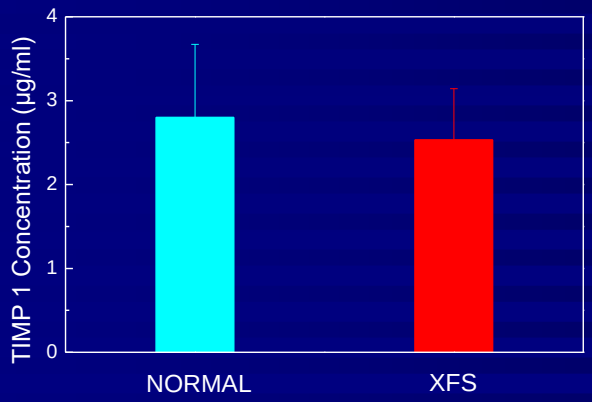


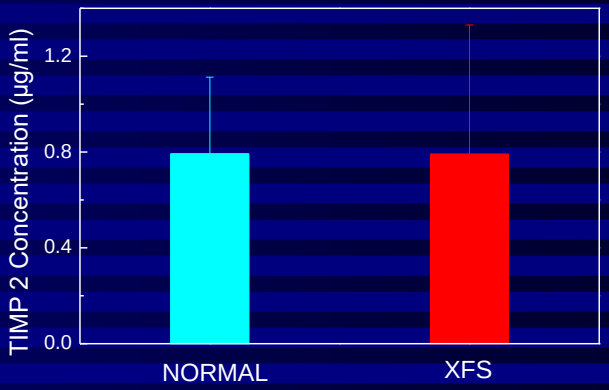
Lens



Tears







Proteoglycans alterations in exfoliation syndrome

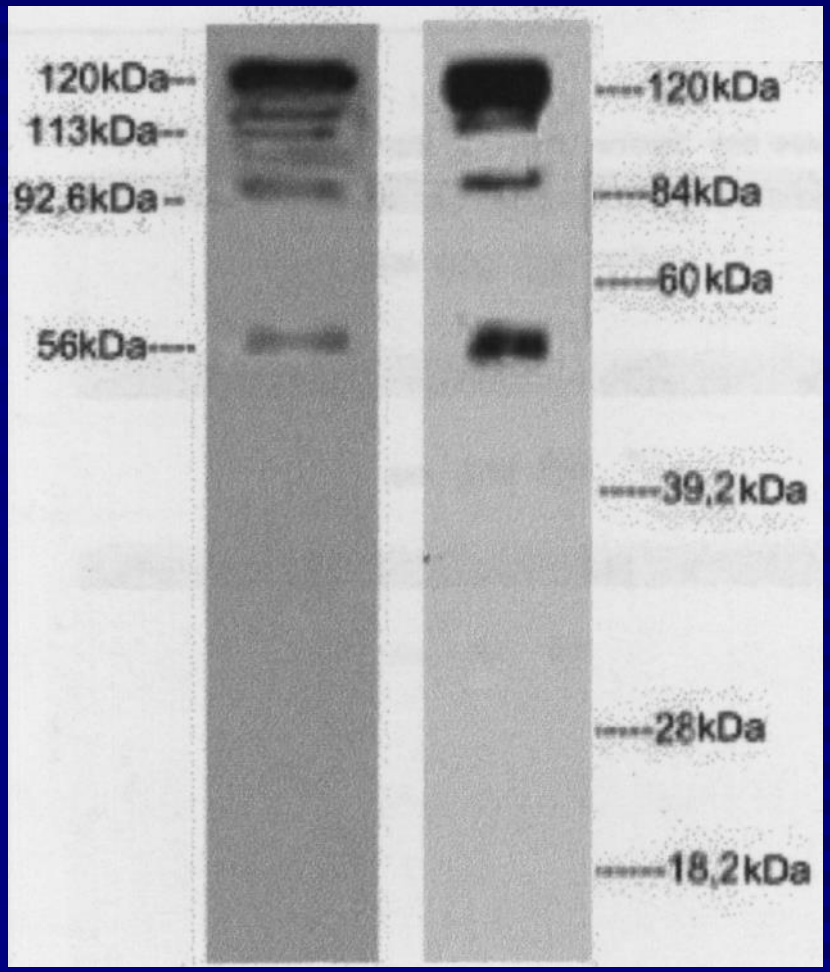
Gartaganis et al. Curr. Eye Research (2004) 28, 5-10

	PRESENCE		TYPE OF GAGS	
	NORMAL	XFS	NORMAL	XFS
Aggrecan	-	-	-	-
Biglycan	+	+	CS	CS/DS
Collagen type IX	+++	+++	CS/DS	DS
Decorin	-	-	-	-
HS PGs	+	-	HS	-
Keratocan	-	-	-	-
Lumican	-	-	-	-
Versican	-	-	-	-
3-S GlcA	+	+++		

HNK1 and collagen type IX in XFS

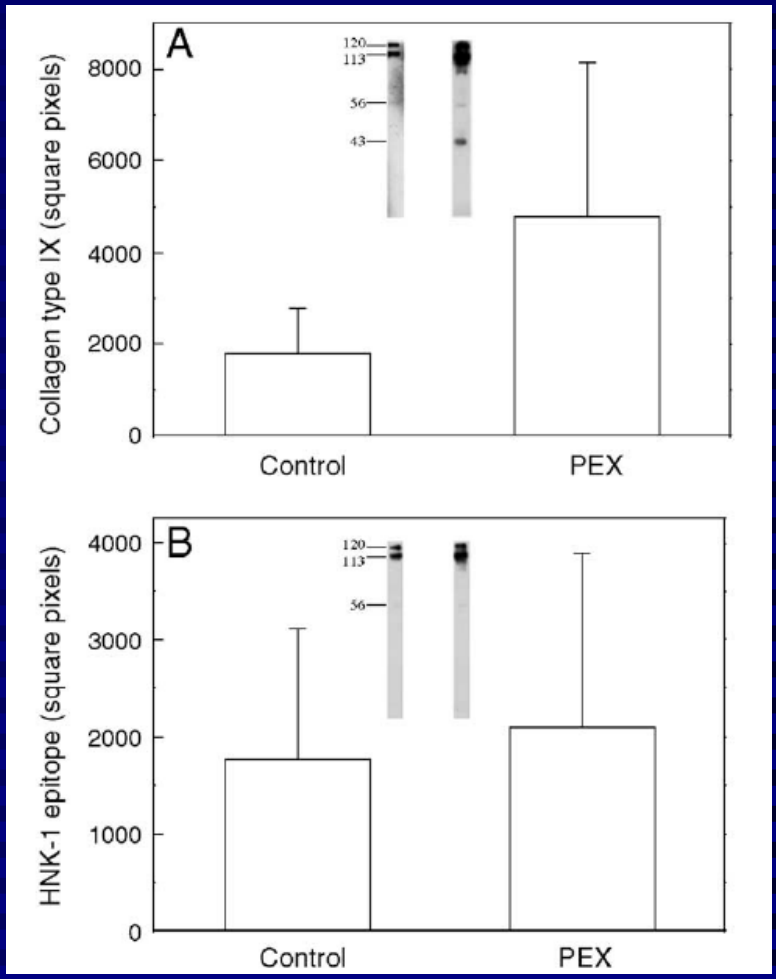
HNK1 B3-1

Gartaganis et al. Curr. Eye Research (2004) 28, 5-10



Analysis in tears

Assouti et al. BBA (2006) 1762, 54-58



General Conclusions

Selection of the method

Selection of the biologic sample

Statistical analysis

Βιβλιογραφία

- http://www.sciencedirect.com/science/article/pii/S0076687910780125
- http://www.sciencedirect.com/science/article/pii/S0076687910800333
- και η υπάρχουσα βιβλιογραφία στις διαφάνειες

ΕΝΔΕΙΚΤΙΚΑ ΘΕΜΑΤΑ ΜΕΛΕΤΗΣ ΑΝΤΙΔΡΑΣΕΩΝ ΣΕ ΣΤΕΡΕΑ ΦΑΣΗ

1: Προτείνατε δύο τουλάχιστο μεθόδους προσδιορισμού των ενζυμικών μονάδων διαλυμάτων υαλουρονιδάσης. Πως θα γίνει ο προσδιορισμός αναστολέων της;

2: Προτείνατε δύο τουλάχιστο μεθόδους προσδιορισμού ενζυμικών μονάδων διαλυμάτων πρωτεασών. Πως θα γίνει ο προσδιορισμός αναστολέων τους;

3: Προτείνετε δύο τουλάχιστο μεθόδους προσδιορισμού θειικής κερατάνης (υπάρχουν εμπορικά διαθέσιμα αντισώματα).

4: Δίδεται μίγμα μακρομοριακών ουσιών (π.χ. γλυκοπρωτεϊνών) με παρόμοια δομή. Πως θα γίνει ποσοτική ανάλυση καθεμιάς; (επιλέξτε εσείς τις ουσίες).

5: Ο υποδοχέας του υαλουρονικού εμφανίζεται σε 17 τουλάχιστον διαφορετικές ισομορφές. Πως θα διαπιστώσετε αν όλες αυτές οι ισομορφές αλληλεπιδρούν με το υαλουρονικό και αν διαφέρει η ισχύς της αλληλεπίδρασης;

6: Πως θα τροποποιηθεί μια μέθοδος ποσοτικού προσδιορισμού σε στερεά φάση ώστε να εφαρμοστεί αυτή για να μελετηθούν οι φυσικοχημικές σταθερές μιας αλληλεπίδρασης; Θεωρήστε την αλληλεπίδραση αγγρικάνης με υαλουρονικό οξύ ή με συζευκτική πρωτεΐνη.

7: Κατά τη μελέτη αλληλεπίδρασης δύο πρωτεϊνικών μορίων με αντίδραση στερεάς φάσης υποψιάζεσθε ότι το αποτέλεσμα που λαμβάνετε (θετικό ή αρνητικό) οφείλεται στη μετουσίωση που υφίσταται η μια (ή η άλλη, αν γίνει με ανάποδη σειρά η μελέτη) πρωτεΐνη κατά τη διαδικασία της ακινητοποίησης. Πως θα προσπαθήσετε να το διορθώσετε;

8: Αναλύοντας τις ζελατινάσες ενός βιολογικού παρασκευάσματος, σας δημιουργείτε η υποψία ότι μια ζώνη λύσης αντιπροσωπεύει δύο διαφορετικές ζελατινάσες. Πως θα κινηθείτε για να το επιλύσετε;