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Isolation and purification of proteoglycans

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Abstract. Purification of a protein typically involves development of a quantitative assay to track protein integrity (e.g. enzyme activity) during subsequent isolation steps. The generalized procedure involves choosing the source of the protein, defining extraction conditions, developing bulk purification methods followed by refined, more selective methods. The purification of proteoglycans is often complicated by a) limited source quantities, b) necessity of chaotropic solvents for efficient extraction, c) their large molecular size and d) lack of defined functions to enable purity (i.e. activity, conformation) to be assessed. Because the usual goal of proteoglycan purification is physical characterization (intact molecular weight, core protein and glycosaminoglycan class and size), the problems of a suitable assay and/or native conformation are avoided. The 'assay' for tracking proteoglycan isolation typically utilizes uronic acid content or radiolabel incorporation as a marker. Once extracted from their cellular/extracellular environment, proteoglycans can be isolated by density gradient centrifugation and/or column chromatography techniques. Recent advances in the composition of chromatographic supports have enabled the application of ion-exchange, gel permeation, hydrophobic interaction and affinity chromatography resins using efficient high-pressure liquid chromatography to proteoglycan purification.
Key words. Proteoglycans; assays; purification; extraction; ultracentrifugation; chromatography.

Introduction

In general, techniques for isolation and purification rely upon the physical characteristics imparted to biological compounds by their composition and structure. Amino acid composition and primary structure, together with the carbohydrate and lipid content can have profound effects upon size, ionic nature, solubility and stability. To discuss methods of proteoglycan (PG) isolation efficiently, a brief overview of PG structure needs to be given, since the various techniques take advantage of PG composite structure. The term PG defines a family of biomolecules that possess a core protein and one or more covalently attached glycosaminoglycan chains. PGs are characterized as having a polyanionic nature conferred for the most part by sulfate substituents on carbohydrate residues and by the carboxyl group of the hexuronic acid moieties. These glycosaminoglycan chains define the class of the PG by a general repeating disaccharide backbone. For heparin, heparan sulfate (HS), chrondroitin sulfate (CS) and dermatan sulfate (DS) PGs the backbone consists of [hexuronic acid-N-acetylhexosamine]$_n$ which can have sulfate ester substituents on either the uronic acid or hexosamine or both[118]. For keratan sulfate (KS) PGs, [galactose-N-acetylglucosamine]$_n$ defines the carbohydrate backbone with the polyanionic character conferred by the presence of sulfate groups on the galactose and hexosamine residues. These GAG modifications result in the production of mature PGs whose biophysical properties would be expected to differ based upon the extent of sulfate substitutions and hexuronic acid epimerization (table 1).

The 'other half' of PGs, namely their core proteins, have profound effects on the physical properties of PGs. As with integral membrane proteins, membrane intercalated PGs possess a hydrophobic nature which must be taken into account in isolation procedures. Some membrane PGs possess a phosphatidylinositol moiety as linkage to the membrane, which will further contribute hydrophobic characteristics[157]. Certain freely soluble secreted PGs such as biglycan, decorin and fibromodulin possess core proteins whose structure (a duplicated leucine-rich repeat) confers an amphipathic nature to the intact PGs[40, 70, 99, 104]. Other PGs, such as aggrecan or versican, have peptide sequences that confer lectin-like properties and have EGF-like repeats that have the potential to influence cellular metabolism[5, 30, 71]. These large CSPGs also have very big core proteins ($M_r > 200{,}000$ daltons) which affect their solution behavior and isolation yields. It is the core protein sequence that determines the identity, and hence the 'name' of a PG, such as for the large CSPGs aggrecan[31] and versican[163], the chondroitin sulfate proteoglycan type IX collagen[51, 155], the HSPGs syndecan[127], perlecan[101], betaglycan[2], glypican[27], fibroglycan[83], and the small CS/DSPGs biglycan[40] and decorin[120].

Two recent reviews have appeared on methods for PG purification[35, 160]. Farach-Carson and Carson[35] review potential strategies for both glycoprotein and

Table 1. Glycosaminoglycan structure in proteoglycans

Glycosaminoglycan class	[Backbone structure][a] Common disaccharides	Sulfate content (per disacch. unit)	Carboxylate content (per disacch. unit)
Chondroitin sulfate	[β1,4 GlcA-β1,3 GalNAc]$_n$ GlcA-GalNAc(4-SO_4) GlcA-GalNAc(6-SO_4)	0 to 1 SO_4	1
Dermatan sulfate	[β1,4 IdoA-α1,3 GalNAc]$_n$ IdoA-GalNAc(4-SO_4) IdoA-GalNAc(6-SO_4) IdoA(2-SO_4)-GalNAc(4-SO_4)	0 to 2 SO_4	1
Heparan sulfate (heparin)	[α1,4 GlcA-β1,4 GlcNAc]$_n$ GlcA-GlcNSO_4 GlcA(2-SO_4)-GlcNSO_4 IdoA-GlcNSO_4 IdoA(2-SO_4)-GlcNSO_4 IdoA(2-SO_4)-GlcNSO_4(6-SO_4)	0 to 3 SO_4	1
Keratan sulfate	[β1,3 Gal-β1,4 GlcNAc]$_n$ Gal(6-SO_4)-GlcNAc	1 SO_4	0

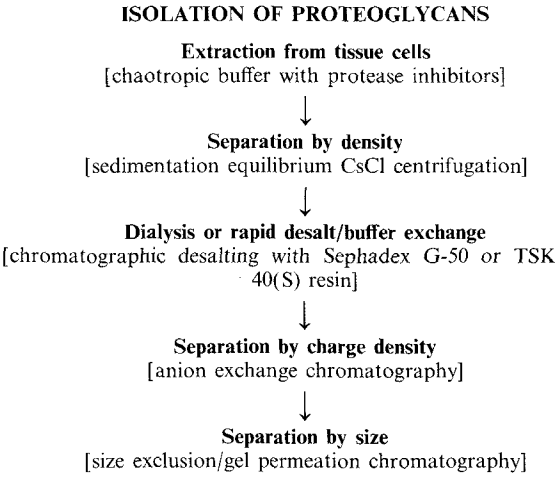
[a]Abbreviations: disacch., disaccharides; GlcA, glucuronic acid; GalNAc, N-acetyl galactosamine; IdoA, iduronic acid; GlcNAc, N-acetylglucosamine; GlcNSO_4, N-sulfated glucosamine.

PG isolation and Yanagishita et al.[160] discuss chromatographic methods for the isolation and analysis of PGs synthesized in vitro. The focus of this review will be on methods utilized in the last three years, especially those isolation methods that confer a high degree of selectivity and rapidity (refined sedimentation equilibrium techniques, affinity chromatography and adaptations of various forms of high performance liquid chromatography).

Proteoglycan source and extraction conditions

The source of the PG often determines the initial approach in purification. Tissue and organs can usually be obtained in large enough quantities to enable classical chemical purification. This involves tissue solubilization or extraction usually in the presence of protease inhibitors. The generalized procedure for PG purification requires extraction of the PG from the appropriate source (fig.). The standard extraction procedure developed by Sadjera and Hascall[123] for cartilage PGs involves a chaotropic buffer with 4 M guanidine HCl in 50 mM sodium acetate pH 6.0. This effectively solubilizes connective tissue matrix and basement membrane PGs. Protease inhibitors that are commonly used include benzamidine (serine proteases, 1–5 mM), N-ethylmaleimide (thiol proteases, 1–10 mM), 6-aminocaproic acid (plasmin, ~0.1 M), phenylmethylsulfonyl fluoride (PMSF, serine proteases, 0.1–1 mM), EDTA (metalloproteases, 0.1–1 mM), pepstatin (acid proteases, ~1 µg/ml), and leupeptin (thiol proteases and trypsin-like serine proteases, 0.5–1.0 µg/ml).

By varying the conditions of successive extractions one can obtain pools enriched with a given PG. Brand et al.[14] found when they extracted bovine articular cartilage with increasing concentrations of guanidine HCl a partitioning of small PGs to low concentrations of guanidine HCl, while large PGs required high guanidine HCl levels for efficient solubilization. Human articular cartilage can be sequentially extracted with phosphate buffered saline, 7 M urea and 4 M guanidine HCl to obtain pools differentially enriched in specific PGs[148]. The cell associated pool of PGs made by murine erythroleukemia cells can be separated into urea-soluble versus urea-insoluble HSPGs[103]. It is common to use different successive extraction procedures to infer gross localization of a given PG. For example, axonally transported PGs in goldfish were successively extracted with hypotonic buffer, 1 M NaCl, Triton X-100, or 1 M NaCl + Triton X-100 to attempt to ascribe a cytoplasmic, membrane-associated or membrane-bound location[32]. Since the inclusion of 0.5 M EDTA in extraction buffer dissolves hydroxyapatite mineral[141],



Figure

workers have distinguished PGs that are matrix associated (4 M guanidine HCl soluble) from those that are mineral-associated (4 M guanidine HCl insoluble/4 M guanidine HCl + 0.5 M EDTA soluble) in fetal porcine calvarial bone[45] and in MC 3T3 osteoblast-like cultures[139].

Plasma membrane-associated forms of PGs are usually distinguished by their extractability with high salt (an ionic interaction) and membrane-bound PGs by their release with detergent or a specific enzyme that is known to act on membrane-bound components. Human lung fibroblast CSPGs that have a membrane association have been defined in this manner[28]. From initial studies by Kramer and Tobey[69] on trypsin-release of HS through Höök's characterization of the mode of cell surface-association of HSPG[65], it has become routine to release membrane-associated HSPGs with salt, heparin, mannose 6-phosphate, or inositol hexaphosphate[21,23,55], of membrane-bound HSPGs with phosphatidylinositol specific phospholipase C[17,21,55,88,157–159] or with trypsin where susceptibility reflects core protein intercalated into the membrane[157–159].

Both nonionic and ionic detergents can be added to facilitate PG solubilization. Squid cranial cartilage CSPGs are effectively solubilized by 2% sodium dodecylsulfate[149], while 2% deoxycholate has been used for rat brain HSPGs[116] and for rat erythrocyte and liver membrane PGs[62]. A few workers have taken advantage of the temperature-sensitive phase transition that the detergent Triton X-114 can undergo (reviewed in ref. 109). Detergents of the Triton X series ([poly-oxyethylene]$_n$ p-t-octyl phenol) form micelles in aqueous solutions above their critical micelle concentrations at 0 °C. When the temperature is raised, micellular aggregates precipitate out of solution. The temperature depends on the number of hydrophilic oxyethylene moieties in the detergent. For Triton X-100, the number is 9–10 and the cloud point is 64 °C, while for Triton X-144, the number is 7–8 and the cloud point is at 20 °C. By increasing the temperature and causing a phase transition in a solution of proteins (and PGs), hydrophilic proteins remain in the solution phase while lipophilic components (integral membrane proteins) partition into the detergent gel phase. A DSPG from cultured rat glomerular epithelial cells was effectively solubilized in this way[110].

Precipitation is one of the most common methods in protein purification. Both proteins and PGs can be precipitated through perturbation of the solvent-protein interactions by the addition of ammonium sulfate, ethanol, polyethylene glycol, and acids such as trichloroacetic acid (TCA). Wight and co-workers, typically precipitate PGs from a buffer containing urea and high salt by adding 50 µg/ml CS as carrier, four volumes of 1.3% potassium acetate in 95% ethanol and chilling for 1 h at −20 °C[63]. The difference in solubility in ethanol of iduronate rich versus glucuronate rich DSPGs enabled their fractionation from human keloid scar tissue[43]. TCA precipitation of 4 M guanidine HCl + Triton X-100 extracts of rat HSPG yields HSPG in the suspension and not the pellet[77]. Similarly, rat liver membrane PGs extracted with detergent remain in solution after acid precipitation[62] and a TCA insoluble PG is the precursor to TCA soluble PG in bovine retina[92]. Because of their negative charge density, PGs may be somewhat selectively precipitated by cationic detergents. For example McBain and Mueller[86] took Zwettergent 3–12 extracts of cultured cells, treated them with cetylpyridinium chloride and then phenol extracted the pellet (to remove unglycosylated proteins) to yield lipophilic PGs of high density.

Assays

Ultraviolet absorbance

In any isolation procedure, it is necessary to have an assay to follow the yield of the component of interest at each step of the purification. A general assay for protein purification is to monitor peptide amide bond by ultraviolet absorbance at <210 nm or the presence of aromatic ring containing amino acids (tryptophan, phenylalanine, histidine and tyrosine) at 280 nm. Absorbance is limited not only by the amount of protein present and abundance of aromatic amino acid residues but also by the buffer systems employed. Extraction and chromatographic buffers containing UV absorbing components will interfere with absorbance monitoring (table 2). With buffers containing guanidine-HCl or urea, it is possible to remove UV-absorbing contaminates by using ultrapure grades and passing solutions over activated charcoal. Formamide, which can be substituted for urea, does absorb UV to a significant degree at 280 nm, so absorbance must be monitored at 290 nm[38]. Detergents that do not have a high UV absorbance at 280 nm can be substituted for those that do (table 2). Substitution of different detergents must necessarily take into account the differences in critical micelle concentration between detergents. Compatible buffers for monitoring PG purification by UV absorbance at 206–214 nm have recently been described[135].

Uronic acid and hexosamine assays

For PGs, more specific assays typically take advantage of the glycosaminoglycan portion of the intact PG. For HS, CS and DS-PGs containing uronic acid, the colorimetric carbazole-sulfuric acid assay as modified by Muir and coworkers[11] can be applied. This is a degradative assay so aliquots from extractions, and/or chromatographic separations are analyzed. The method can detect 4–400 µM D-glucurono-6,3-lactone (standard) and involves the reaction of unstable acid hydrolyzed

Table 2. Properties of buffer components

Reagents	UV concentration limits (permissible concentration)[a] 280 nm	205 nm		Comments
Buffers			pKa	
Acetate	0.1 M	10 mM	4.76	
Phosphate	1.0 M	50 mM	2.12, 7.21, 12.32	
Tris[b]	0.5 M	40 mM	8.10	
Detergents			cmc[c]	
Brij 35	1%	1%	0.02%	
CHAPS	10%	0.1%	0.40%	
Deoxycholate	0.3%	0.1%	0.17%	Precipitates at pH < 8
Genapol X-100[d]	> 10%	5%	0.01%	Developed for UV monitoring.
Octylglucoside	10%		0.73%	Can be removed by dialysis.
Triton X-100	0.02%	0.01%	0.02%	Precipitates at acidic pH.
Triton X-100(R)	> 10%	2%	0.02%	Reduction reduces UV absorption.
Tween 20	0.3%	0.1%	0.001%	
Miscellaneous				
EDTA	30 mM	0.2 mM		
KCl	100 mM	50 mM		High concentrations interfere with SDS PAGE.
NaCl	> 1 M	0.6 M		
Sucrose	2 M	0.5 M		
Urea	> 1 M	< 0.1 M		Ultrapure and deionized.

Data compiled from refs 100 and 134.
[a]Final concentration which does not produce an absorbance of 0.5 over water.
[b]Tris, hydroxymethyl aminomethane; Brij 35 (polyoxyethyleneglycol dodecyl ether); CHAPS (3-[(3-chloramidopropyl)-dimethylammonio]-1-propanesulfonate); Genapol X-100 (polyoxyethylene (10) isotridecyl ether); Triton X-100 (octylphenoxy polyethoxy ethanol); Triton X-100(R) carbon double bonds have been reduced to single bonds; Tween 20 (polyoxyethylene sorbitan monolaurate); EDTA ethylenediamine tetraacetic acid.
[c]Critical micelle concentrations determined for detergent/H_2O solutions at 25 °C.
[d]Available from Calbiochem.

dehydrated derivatives of hexuronic acids with carbazole. Neutral sugars will produce about 10% interference when present at similar molar concentrations and heparin and HS produce anomalously high absorbance values at 525 nm. Alternatively, and more laboriously, the hexosamine content of acid hydrolized samples can be assayed based on the Elson-Morgan reaction[33].

Dye-binding assays

The high degree of charge conferred on PGs by their sulfate and uronic acid content enables dye-binding assays using electrostatic interaction between dye and glycosaminoglycans. Stoichiometric assays such as those that use Alcian Blue[44,153] or 1,9-dimethylmethylene blue[52] involve precipitation of the dye-glycosaminoglycan complex and absorbance measurements after solubilization to quantitate dye levels. Assays that take advantage of metachromatic shifts upon binding of GAG to dye can use Alcian Blue[44], 1,9-dimethylmethylene blue[36,121] or the more sensitive Safranin O[72] to follow PGs.

Radiolabeling

When the system of interest can be modeled by cell, tissue or explant organ culture, radioactive precursors can be used to tag the PGs. [^{35}S]sulfate is, in many systems, incorporated almost exclusively into the sulfate moieties on glycosaminoglycan chains. [^{3}H]glucosamine ([^{3}H]glcN) is also often used as a metabolic precursor. Once inside the cell, [^{3}H]glcN rapidly interconverts to UDP-[^{3}H]glcNAc and UDP-[^{3}H]galNAc which are high energy intermediates involved in glycosminoglycan chain synthesis. General procedures for radiolabeling with [^{35}S]sulfate and [^{3}H]glcN have been recently reviewed[8,160]. [^{35}S]sulfate is used in the 25–100 μCi/ml range while [^{3}H]glcN at 5–100 μCi/ml is employed. One of the first steps in radiolabeling cultures is to determine the kinetics of label incorporation into PGs. For PG isolation, steady-state labeling is employed and by determining a time course of continuous radiolabel incorporation, one can determine the length of time sufficient for a steady-state to be reached. With radiolabeled metabolic precursors and suitable culture systems, PGs from different cell types can be compared. For example, Piepkorn et al.[107] studied fibroblasts, human keratinocytes and bovine aorta endothelial cell PGs under similar culture conditions. Metabolic labeling and comparison of PGs from organ cultures of human cornea with cultures of corneal fibroblasts provides information on how well a tissue culture system mimics an organ system that is closer to the state *in vivo*[48]. With [^{35}S]sulfate, the temporal sequence of PG sulfation, storage and secretion has been defined in exocrine cells[12].

As might be expected, the concentration of cold sulfate in the labeling medium will affect the specific activity of the [^{35}S]sulfate, while the levels of glucose in the medium will alter the specific activity of the [^{3}H]glcN pool. A facile method for monitoring alterations in the specific activities of metabolic precursor pools and determining the specific activity of [^{35}S]sulfate relative to [^{3}H]hexosamine has been recently described[161]. This method takes advantage of the fact that, given the repetitive structure of a glycosaminoglycan such as CS, disaccharides generated by enzymatic digestion (such as [ΔGlyA-GalNAc(4- or 6-SO_4)], where ΔGlyA represents the unsaturated uronic acid residue created in glycosidic bond cleavage) have a 1:1 mole ratio of sulfate to hexosamine. Thus, the specific activity of the sulfate in the disaccharide can be used to determine the specific activity of the hexosamine. By isolating and quantitating the ^{35}S and ^{3}H associated with a specific disaccharide, one can determine if the relative specific activities of the metabolic precursor pools are changing.

Radiolabeled precursors that target the protein (labeled amino acids) and other specific portions of the PG have also been employed. For example, [^{35}S]methionine has been used to track PG core proteins from smooth muscle cells of pig aorta[10], and a heterodimeric core protein of a large CSPG from human skin fibroblasts[16]. The incorporation of [^{3}H]inositol into PGs can be used as a marker for HSPGs that possess a phosphatidylinositol moiety[157]. Similarly, [^{3}H]myristic acid was used to label a HSPG from human colon carcinoma cells that possessed a covalently attached fatty acid component[54]. One of the biggest caveats for the use of radiolabeling is that one must be able to say precisely whether a radiolabeled product was generated biosynthetically by direct incorporation into the molecule or whether a radiolabeled product was the result of recycling of labeled break down products. This is done by isolating and determining the structure of the radiolabeled component and whether such a structure could arise from anabolic or catabolic recycling[54, 157].

Physical methods of proteoglycan isolation

Centrifugation

Perhaps nowhere are the unique physico-chemical properties conferred upon PGs by their composite structure more evident than isolation by equilibrium density gradient centrifugation. The technique of sedimentation equilibrium in a CsCl density gradient was developed by Meselson, Stahl and Vinograd[90]. The separations of macromolecules by ultracentrifugation in self-forming density gradients (of CsCl or $CsSO_4$) have been readily applied to bacteriophages, large macromolecules such as DNA (single stranded density ~ 0.018 g/cm^3, double stranded density ~ 1.7 g/cm^3), RNA, ribosomes, lipoproteins (density < 1.1 g/cm^3), proteins (density ~ 1.3 g/cm^3), and PGs (density $\sim 1.3-1.8$ g/cm^3). The actual mechanism of sedimentation equilibrium separation is very complex; but the purposes of this review, a simplified view can be taken. When a low molecular weight, high density solution (such as CsCl) is centrifuged, the salt redistributes until an equilibrium is reached and a concentration gradient is formed (less dense at the top of the tube and more dense at the bottom). Macromolecules will also sediment until they form a band at a position in the concentration gradient at which the density of the macromolecule equals the density of the solution. In general, components with a high buoyant density migrate rapidly through the gradient toward the bottom of the tube, while components with lower buoyant densities such as lipid or protein stay toward the top of the tube. Large PGs of a highly polyanionic nature behave similarly to DNA and possess a high buoyant density. The adaptation of sedimentation equilibrium in density gradients to the field of PGs was pioneered by Sajdera and Hascall[123]. By using either associative (0.5 M guanidine HCl) or dissociative (4 M guanidine HCl) buffers, PGs can be isolated as aggregates (with hyaluronan and link protein) or as monomers. The method is commonly used today as one of the initial steps in PG isolation. The approach of Fransson and coworkers in purifying PGs follows from density gradient centrifugation to gel and ion-exchange chromatography (for example, ref. 128). As a preparative method to separate large PGs from small PGs and other cellular products, density gradient centrifugation is a usual first step (table 3). However, the method is less useful for lower buoyant density PGs, i.e. those with lower glycosaminoglycan/protein ratios.

Refined sedimentation equilibrium methodology has been developed by Howell, Müller, Pita and Manicourt[49, 79–82, 96, 97]. Their work has shown that the method of extraction affects the size distribution of PG aggregates. PG aggregates from dissociative extraction have smaller molecular size than those recovered by non-dissociative methods[79]. The non-dissociative extraction procedure involves micropuncture aspiration of cartilage and treatment with highly purified collagenase and protease inhibitors. Their revised zonal centrifugation technique for preparative ultracentrifugation avoids plateau dilution and simplifies calculation of centrifugal properties[80, 96]. Using their system of analysis, a bimodal aggregate distribution was seen for both normal and model osteoarthritic cartilage PGs, whereas only one PG-aggregate peak was seen using conventional sedimentation equilibrium techniques[79–81, 97]. Two distinct families of PG aggregates were separated and shown to differ in terms of the presence or absence of link protein and the structure of the CS chains on the aggregating CSPG[81, 82, 97]. These advances in sample preparation and zonal centrifugation analysis have the

Table 3. Density gradient centrifugation in proteoglycan isolation

Dense salt	Isolation of	Reference
CsCl	Neoplastic and non-neoplastic human breast issue PGs	1
	Human B lymphoblastoid cell line CSPG	19
	Bovine intervertebral disc KSPG	29
	Human lumbar annulus fibrosus PGs	53
	Squid skin CSPG	60
	Mandible and femur PGs	67
	Human vascular endothelial cell PGs	76
	Rat liver HSPG	77
	Bovine articular disc large CSPG	78
	PG inhibitor of neutrite promoting activity of laminin	94
	Adult rabbit skeletal muscle PGs	106
	Rabbit articular chondrocyte CS/KSPGs and DSPGs	108
	Human tracheal cartilage PGs	117
	Copper deficient rat aorta PGs	121
	Arterial wall CSPGs from different animals	122
	Rabbit articular chondrocyte CSPGs	126
	Bovine articular disc small DSPGs	131
	Bovine arterial large CSPG	133
	Squid cranial cartilage CSPGs	149
	Chick embryo vitreous humor type IX collagen	155
	Cultured mesangial cell large CSPG	162
$(Cs)_2SO_4$	Experimental canine osteoarthritis PGs	81
$Cs(CF_3CO_2)$	Bovine articular cartilage	86

potential to maintain the in vivo integrity of PG-aggregate structure and to preserve the hydrodynamic stability of very slowly diffusing macromolecules.

Another advance in sedimentation methodology involves the application of sedimentation field flow fractionation to PG and PG-aggregate analysis[3, 64]. Although a specialized rotor and ultracentrifuge apparatus is required for this method of analysis, it is a promising one for it yields high resolution separation of a wide size range of macromolecules, from $< 10^6$ to 10^{13} molecular weight[64]. The basis of the technique is that a channel exists in the rotor through which a mobile phase is continuously flowing. Under force fields generated by centrifugation there is a differential rate of flow between fluid near the wall of the rotor (slow) and fluid in the center of the channel (fast). Macromolecules that are more dense than the mobile phase will migrate toward the outer wall while smaller components are caught up by the faster flowing streams in the center of the channel. With an appropriate sampling system, small macromolecules will elute first with the rapidly flowing solvent, while larger macromolecules will elute later. Another advantage of this method is that molecular weights can be determined during the separation and molecular weight standards are not required[64]. A bimodal PG-aggregate distribution was also observed during the analysis of cartilage PGs[3], consistent with the observations of Müller et al.[96].

Electrophoresis

Electrophoresis provides a simple method to identify and monitor PGs during purification and to determine their homogeneity. Electrophoretic separation of macromolecules involves the migration in an electrical field of charged macromolecules through a porous gel. Porosity of the gel is a function of the concentration of agarose or percentage of acrylamide and cross linker present. Hence, macromolecules that are highly polyanionic will migrate toward the cathode during electrophoresis. A composite acrylamide-agarose (1.2% acrylamide, 3% cross linker and 0.6% agarose) slab gel electrophoresis system was initially developed by McDevitt and Muir[87] to separate cartilage PGs. PGs separated in this fashion can be visualized by staining the gel with 0.2% toluidine blue in 0.1% acetic acid (alcian blue stains agarose and is thus not suitable). Radiolabeled PGs in composite gels can be visualized by fluorography[25]. More recently, the method has been adapted for rapid use with mini-slab gels (requiring only 75 min at 35 mA current to electrophorese). This system uses a composite agarose-acrylamide gel of 1% agarose, 1.2% acrylamide with piperazine diacrylamide in place of bisacrylamide as cross linker[146]. The electrophoresis buffer is 0.04 M Tris-acetate, pH 6.6, containing 0.02 M sodium acetate and 1.0 mM sodium sulfate.

The addition of SDS (sodium dodecyl sulfate) to electrophoretic buffers causes denaturation of macromolecules, the coating of their surface with the negatively charged detergent that is proportional to the size of the macromolecule. Macromolecules that do not possess an intrinsic negative charge can thus be fractionated on a basis of size in SDS-PAGE (polyacrylamide gel electrophoresis). Gradient SDS-PAGE systems for PG analysis have also been used. As with

protein analysis, the most common gradients are in the range from 4 to 30% T (where % T is the weight percentage acrylamide + cross linker, in g per 100 ml) acrylamide with a 3 or 4 to 20% gradient frequently used[144, 145]. A method using a 3–15% acrylamide gradient combined with a 0.04–0.06% bisacrylamide gradient has been employed to resolve several PGs from human bone cells grown in culture from each other, namely, a M_r 600,000 CSPG from a M_r 400,000 HSPG, a M_r 270,000 biglycan and a M_r 135,000 decorin[9, 38]. An SDS-PAGE system with a 1.32–10% T gradient with 4% agarose to stabilize the top of the gel has enabled human articular PGs of $< M_r\ 4 \times 10^6$ to penetrate the gel and separate[147]. Radiolabeled PGs separated by SDS-PAGE can be visualized by fluorography[9], while unlabeled PGs can be stained with a 0.2% toluidine blue in 0.1% acetic acid, 0.2% alcian blue in 3% acetic acid with 0.05 M $MgCl_2$[150] or Stains-all[147].

Chromatography

PGs differ in their charge, size, shape, hydrophobicity and specific functional groups. PGs are chromatographically fractionated by charge density (anion exchange chromatography) size and shape (size exclusion chromatography), surface hydrophobicity (hydrophobic interaction chromatography), and by the distribution of specific amino acids or carbohydrate residues at their surface (ligand-affinity chromatography). For all chromatographic modes but size exclusion, separation of compounds occurs through differential adsorption of solutes at the surface of the chromatographic resin. The basis of partition (adsorption) chromatography is the different polymers have differences in distribution between two phases. These two phases are the stationary phase (the chromatographic resin) and the mobile phase (eluant). Partitioning between the two phases is regulated at the molecular level by electrostatic interactions, dispersion forces, π-bonding, hydrogen bonding, charge-transfer interactions, and ligand coordination.

Anion exchange

Because of the high negative charge density contributed to PGs by their glycosaminoglycan chains, isolation based upon anion exchange chromatography is almost always employed. Ion exchange chromatography differs from other types of liquid chromatography in that the stationary phase carries covalently attached ionizable functional groups. Separation of proteoglycans by ion exchange involves PG binding to the fixed stationary phase charges and elution (displacement) by a new counterion (salt) that possesses a greater affinity for the fixed charges than the PG. In anion exchange chromatography, the most common resin employed is a diethylaminoethyl resin (DEAE, a weak base that possesses a net positive charge when ionized) and a NaCl salt gradient is used to elute the bound PGs. Specific resins and the type of PGs isolated are summarized in table 4.

For PG isolation, the most common anion exchange buffer contains 7 M urea (or 40–50% formamide), 0.05 M sodium acetate (or Tris HCl), pH 6–7, 0.5% detergent (Triton X-100 or CHAPS), with gradient elution using NaCl. Anion exchange resin can also be used to concentrate PGs by bulk volume loading and batch elution/extraction in high salt buffers[160]. A large volume of solution containing PGs is adsorbed onto a small bed volume of resin which is then extracted with a small volume of 4 M guanidine HCl.

Much attention has been recently focused on the use of HPLC anion exchange columns to bring all the advantages of HPLC to PG isolation[13, 38, 135]. A comparision of four HPLC anion-exchange columns in terms of yields of $^{35}SO_4$-labeled human bone cell PGs was done using the same buffer system, flow rate and gradient[38]. The yield of radiolabeled PGs off of the column was 30–40% for Mono Q, 85% for ToyoPearl TSK-GEL HW 650(S) DEAE, 60% for TSK DEAE 5PW, and 96% for Nucleogen 4000-10 DEAE in a formamide-KH_2PO_4 buffer system. The pore size of the resins in these columns are given as 4000 and 1000 Å for Nucleogen 4000-10 and TSK DEAE 5PW, respectively. The TSK-GEL Toyopearl HW 650(S) and Mono Q resins are based on non-porous supports whose protein exclusion limits are given as $M_r\ 5 \times 10^6$ and 10×10^6, respectively. Blake and McLean[13] reported high yields on a ProPac PA1 column in a urea based system for PGs from cultured corneal endothelial cells.

Size exclusion/gel permeation chromatography

Isolation and separation of PGs by gel permeation or size exclusion chromatography (SEC) is, at the simplest level, a function of the hydrodynamic volume of the intact PG. The chromatographic support (gel, matrix) contains pores of specific size ranges and the PG, depending on its size, is either excluded from the pores or will equilibrate between the internal pore volume and the external volume of liquid between the beads. PGs are fractionated by the degree to which they can penetrate the pores. Large PGs will be excluded from the internal volume and exit the column first, while smaller PGs will spend more time traversing the pores of the stationary phase and hence be delayed in elution.

The paradigm for SEC is a globular protein and the theoretical description typically employed is based upon an assumption of a hydrodynamic radius (volume) for a spherical protein. SEC can also provide an estimation of the weight average (M_r) of a component. By determining the volume required to elute macromolecules of interest (V_e), the elution volume of a totally excluded polymer (V_0), and a completely included polymer (V_i, determined usually by the elution of a small metabolite

Table 4. Anion-exchange resins frequently employed in proteoglycan isolation

Conventional resin[a]	Isolation of	Reference
Dowex AG 1 × 2	Rat hepatocyte HSPG	4
DEAE Tris-Acryl	Rat embryo and membrane betaglycan	2
DEAE-Sephacel	Bovine cementum mineralized matrix PGs	6
	Bovine periodontal ligament PGs	34
	Bovine articular disc PGs	59
	Rat liver HSPG	77
	Rat prechondrogenic limb bud PGs	84
	Mesangial cell PGs	162
DEAE-Cellulose	Human keloid scar tissue DSPGs	43
	Calf anterior lens capsule/lens epithelial HSPGs	93
DEAE-Sepharose	Arterial wall CSPGs from various species	122
	Human glomerular basement membrane HSPGs	144
Fast Flow Resins		
Q-Sepharose	Murine monocyte leukemic (M1) PGs	89
	Human cornea explant PGs	91
	Acidic FGF high affinity receptor HSPG	124
	Rat parathyroid cell line PGs	140
Mono Q	Rat liver HSPG	77
	Murine monocyte leukemic (M1) PGs	88
	Glomerular complement regulatory factor DSPG	110
	Human aorta PGs	136
	Rat parathyroid cell line	156
HPLC resins		
TSK DEAE 5PW	Cultured human bone cell PGs	38
	Cultured human skeletal muscle PGs	47
ProPac PA1	Cultured corneal endothelial cell HS and DSPGs	13
TSK-GEL HE 650(S) DEAE	Cultured human bone cell PGs	38
Nucleogen 4000-10 & 4000-7 DEAE	Human bone cell PGs	38, 39

[a]Resin sources: Dowex, Bio Rad; Tris-Acryl, IBF Technologies; Sepharose and Mono Q, Pharmacia; DEAE Cellulose, Whatman; ProcPac PA1, Dionex; TSK DEAE 5PW and TSK GEL HW, Toyosoda; Nucleogen, Machery Nagel.

such as glucose or by radiolabeled H_2O), one can calibrate the column, deriving a correlation between elution volume and M_r. By determining the partition coefficient for each polymer of known M_r by the formula $K_d = (V_e - V_0)/(V_i - V_0)$ and constructing a graphical plot of log M_r versus K_{av} one can interpolate the M_r of an unknown form where its elution volume lies on the resulting linear line.

The paradigm for a PG is microheterogeneity in terms of carbohydrate structure and hence a molecular weight distribution is what is measured. Aberrant elution of a PG from sieving resins can arise from its non-spherical nature. Instead of using protein standards, a column can be calibrated with PGs of known M_r. Caplan and co-workers derived calibration constants for chick limb bud chondrocyte PGs on Sepharose CL-2B equilibrated in 4 M guanidine HCl by using light scattering to determine M_r and hydrodynamic radius values[102]. The charged nature of a PG can give rise to electrostatic exclusions from sieving resins[115]. Electrostatic interactions can be overcome by increasing the ionic concentration of the eluant to >0.05 M salt. As long as the M_r determined by SEC is used for qualitative comparisons between PGs, then such deviations can be ignored. For the purpose of isolation, SEC in chaotropic eluants is invariably either the first or second chromatographic step and for analysis of purity, SEC is the last step. The most common resins employed are summarized in table 5. SEC is also utilized in place of dialysis to remove salts and/or unincorporated radiolabel[160]. Gel sieving resins such as Sephadex G-50 or ToyoPearl TSK-GEL HW 40(S) can be employed to desalt tissue/cell extracts rapidly and as such usually follow PG extraction as the next step to prepare the sample for anion exchange chromatography[38, 160].

The most common buffer systems employed are a) 4 M guanidine HCl and b) 0.05 M sodium acetate buffer pH 6.0, containing 8 M urea, 0.3 M NaCl, and 0.5% Triton X-100. A recent investigation of buffer composition and nonspecific adsorption to size exclusion resin has shown that the extent of nonspecific adsorption needs to be determined for each buffer system employed[50]. Their study using bovine nasal septum cartilage PGs determined that nonspecific adsorption a) is pH dependent with decreasing adsorption occurring at pH 8.0 and above; b) increases by guanidine HCl concentrations up to 0.5 M and decreases with higher guanidine HCl levels; and c) decreases with addition of detergents (CHAPS and SDS). A high yield buffer such as 0.05 M Tris-HCl, pH 8.0, containing 4 M guanidine HCl and 0.5% CHAPS possessed negligible adsorption while a buffer containing 0.05 M sodium acetate, pH 5.8, 4 M guanidine HCl and 0.5% CHAPS gave rise to a high degree (>40%) of nonspecific adsorption. It

Table 5. Gel sieving/permeation chromatography resins and applications

LC resins[a]	Isolation of	Reference
Sepharose CL-2B	Rabbit articular chondrocyte PGs	126
CL-4B	Polymorphonuclear leukocyte culture PGs	7
	Bovine periodontal ligament PG	34
	Human glomerular epithelial cell culture PGs	66
	Kurloff cell intracellular PGs	74
	BALB/c 3T3 fibroblast PGs	107
	Human aorta PGs	136
CL-6B	Human glomerular epithelial cell culture PGs	66
	Bovine skin DSPG	99
	Human glomerular epithelial cell HSPGs, CSPGs	142
	Neuro-2a neuroblastoma cell CSPG, HSPG	151
Bio-Gel A-5m	Cultured mesangial cell PGs	162
Sephacryl S-200	Human glomerular basement membrane HSPGs	144
Sephacryl S-400	Fibrotic schistosomal granuloma cell line PGs	132
Sephacryl S-1000	Calf cartilage explant monomer and aggregates	122
HPLC resins		
Superose 6	Human bone cell PGs	9
	3T3 L1 adipocyte PGs	20
	Bovine articular disc PGs	59
	UMR-106-01 rat osteoblast-like cell line PGs	88
	Murine monocyte leukemic (M1) PGs	89
	Human cornea explant PGs	91
	3T3 L1 adipocyte PGs	95
	Glomerular complement regulatory factor DSPG	110
	Rat parathyroid cell line PGs	140
Bio-Gel TSK-50/60, ToyoSoda G6000 (PW)	Cartilage PGs	119
TSK G 4000 SW	Bovine tracheal cartilage PGs	135
LiChrosphere Si500, Si1000, and Si4000	Cartilage PGs	130
TSK-GEL HW 55(S)	Rat hepatocyte cell line PGs	37
TSK-GEL HW 40(S) and 75(F)	Human bone cell PGs	38

[a]Resin sources: Sepharose Sephacryl and Superose, Pharmacia; Bio-Gel, Bio Rad; Bio-Gel TSK, TSK G 4000 and TSK-GEL HW, Toyosoda; LiChrosphere, E. Merck.

should be noted that above pH 8.0, some proteoglycan degradation may occur. For determination of molecular weight distributions, a 0.1 M KH_2PO_4 buffer pH 6.0, containing 6 M guanidine HCl (±0.5% detergent) yields consistent M_r values[85].

Finally, SEC under denaturing and reducing conditions can be used to assess the 'homogeneity' of an isolated PG. Funderburg and Conrad[41,42] describe a protein that co-purified with a KSPG by anion exchange chromatography, ethanol precipitation and by immunoaffinity chromatography that was only separated by SEC in a dissociative buffer. The PG TAP1 (PG-1000) upon reduction and dissociation was resolved into multiple components: a beta component with CS chains and M_r 18,000, 21,000, and 39,000 proteins[24,56]. The purity of a PG can be determined by monitoring absorbance at 210 and 280 nm where coincident and symmetrical peak profiles at the two wavelengths reflect purity[135].

Hydrophobic interaction chromatography

Hydrophobicity is a property of repulsion between a nonpolar component and a polar environment – it is the structure of water that creates hydrophobic interactions[61]. Separations based on surface hydrophobicity are accomplished by either hydrophobic interaction or reverse-phase chromatography. When organic solvents are used to elute bound components, the technique is called reverse-phase chromatography (RPC), while elution effected with descending salt gradients or increasing nonionic detergent gradients are called hydrophobic interaction chromatography (HIC)[114]. In general, HIC resins contain a much lower ligand-density than RPC resins. In HIC, the stationary phase consists of short chain octyl or phenyl groups. It should be stressed that hydrophobic interactions are neither attractive forces nor a binding of hydrophobic groups to each other. Hydrophobic interactions are 'forced' on nonpolar moieties by the structure of water. By changing the structure of water (adding salts, organic solvents, detergent), hydrophobic interactions are altered. In general, increasing ionic strength increases hydrophobic interactions, while detergents or chaotropic salts that disrupt the structure of water decrease the strength of hydrophobic interactions. Hence, elution from an HIC column can be done by a) an increasing detergent gradient, b) a descending salt gradient or c) an increasing gradient of chaotropic salts.

Neural retinal cell-associated PGs can be fractionated on phenyl-Sepharose[18]. Arterial wall CSPGs have been separated by octyl-Sepharose chromatography[111, 112]. Rosenberg and coworkers typically use octyl-Sepharose to separate the two small DSPGs biglycan and decorin from each other[26]. Biglycan, decorin and fibromodulin from human skin and sclera fibroblasts have also been isolated using octyl Sepharose[152]. Membrane-associated PGs bind avidly to octyl-Sepharose allowing the isolation of HSPGs from rat liver[15], a rat parathyroid cell line[140], and KSPG from explants of human cornea[91].

Binding to HIC resin is purported to reflect the membrane association of PGs via hydrophobic patches of the core protein. This is not necessarily the case as freely soluble secreted PGs (such as decorin and biglycan) can bind to octyl-Sepharose[26]. The interpretation of HIC binding as mimicking an affinity for lipids in vivo or in vitro is tenuous. Nagasawa and coworkers investigated glycosaminglycan binding to HIC resins and demonstrated that distinct species of HS could be separated on phenyl-Sepharose by ammonium sulfate descending gradients into fractions that differed in N-acetyl and sulfate groups[98]. Subsequent work determined that fractionation was occurring through the differential contributions of the position of the sulfate and conformation of the carboxyl groups in glycosaminoglycans to their ability to precipitate in ammonium sulfate[143]. Glycosaminoglycans were retained in hydrophobic interaction gels by an interfacial precipitation at high salt and were released into the mobile phase as the salt concentration decreased.

Hydroxyapatite chromatography

Adsorption and elution of macromolecules from hydroxyapatite occurs by a complex mechanism that is distinct from that involved in ion exchange chromatography. Recent work elaborating the mechanisms involved in hydroxyapatite binding have shown that: adsorption and elution are not simple reversals of a single process (as in ion exchange). Adsorption to hydroxyapatite is influenced differently by amino and carboxyl groups; and elution from hydroxyapatite by different salts follows different mechanisms[46]. The surface of a hydroxyapatite crystal contains both positive (calcium) and negative (phosphate) groups. For chromatography, most columns are equilibrated by extensive washing with phosphate buffer at pH 6.8. Under these conditions, the net charge of the resin's surface is negative because of partial screening of the positive calcium charges by the solvent's phosphate ions. Amino groups facilitate binding by nonspecific electrostatic interactions. Carboxyl groups are repelled electrostatically from the negative phosphate charge of the column and bind specifically by complexing to calcium sites on the column. PGs can thus interact via their protein portion and through the charged glycosaminglycan chains. An increasing gradient of phosphate is sufficient to elute PGs from hydroxyapatite columns.

Human aorta PGs were fractionated into four distinct peaks on a Bio-Gel HTP hydroxyapatite column using a 0.01 M sodium phosphate buffer, pH 6.8 with a linear gradient to 0.7 M sodium phosphate, pH 7.2[135]. The same resin was used to isolate fetal porcine calvarial bone PGs into three fractions employing a 0.01 M Tris HCl buffer, pH 7.4, containing 0.01 M sodium phosphate and 7 M urea and a 0.01–0.5 M phosphate gradient[45]. Kojima et al.[68] also used the Bio-Gel HTP resin in the purification of rat microvascular endothelial cell PGs. PGs were eluted by gradient from 0.01 to 0.12 M sodium phosphate, pH 7.0. Bio-Gel HTP (Bio Rad) hydroxyapatite has a small particle size that restricts flow rate depending on the dimensions of the column. HA-Ultragel (IBF Biotechnologies, Inc.) consists of beads of hydroxyapatite-agarose that have a large exclusion limit (5×10^6 Da for globular proteins), pH stability between 4 and 14, fast flow rate capacity and resistance to denaturing agents such as 8 M urea or 6 M guanidine HCl.

Ligand affinity chromatography

While certain macromolecules may have similar charge, size and hydrophobicity, they are readily distinguished by function. Affinity chromatography takes advantage of the ability of macromolecules to specifically bind one or more molecules (ligands) with high affinity. The covalent attachment of such a molecule (ligand) to a chromatographic resin is the basis of affinity chromatography. Once a macromolecule is bound to the modified resin, it may be eluted specifically by competition with free ligand or nonspecifically by salt gradients or by changing the pH.

Because HSPGs have the capacity to bind to a large number of proteins, they are the type of PG most frequently isolated by affinity chromatography. Proteins that have been used to affinity purify HSPGs and the systems they were applied to include: antithrombin III (rat endothelial cells[68]), TGF-β and wheat germ lectin (rat embryo neutrophages[2]), lipoprotein lipase (rat brain[116]), type IV collagen (rat hepatocyte monolayers[4]), laminin and fibronectin (neonatal rat Schwann cells[22, 23]), thrombospondin (mouse mammary epithelial cell surface PGs[138]), and transferrin (cultured liver capillary endothelial cells[105]). Both polyclonal and monoclonal antibodies have been used for immunoaffinity purification of HSPGs, with lymphocyte CD44 being purified with Hermes-1 monoclonal antibody[58] and mouse mammary syndecan with monoclonal antibody 281.2 affinity chromatography[57]. Large CSPGs have been affinity purified using low-density-lipoprotein (LDL) in a bovine arterial wall cell system[133] and immunoaffinity purified using antibodies against CS/KS PGs from brain[113], and using monoclonal antibody L5

in adult mouse brain and cultured astrocytes[137]. Dye-ligand affinity chromatography enabled the isolation of a CSPG from rat neonatal superior colliculus[129]. Funderburg and Conrad[42] employed a monoclonal antibody to immuno-purify a KSPG from bovine cornea. High performance chromatographic resins that have the appropriate matrix chemistry for covalent ligand attachment are readily available from such supplies as Pharmacia, Toyosoda, Pierce and PerSeptive Biosystems.

Conclusions

The researcher interested in the isolation of PGs has, at her or his disposal, many techniques. The most generalized approach is one where PGs are extracted, desalted, subjected to anion exchange chromatography followed by gel permeation chromatography. Certain specialized techniques such as sedimentation equilibrium centrifugation, hydrophobic interaction chromatography, and ligand affinity chromatography can be employed when the researcher knows a priori certain physical characteristics of the PG(s). Recent advances in the chemistry of chromatographic supports have enabled the application of high performance liquid chromatography to PG isolation. The development of functional assays for PGs does not appear to be too far away. Lee and Lander[73] described a quantitative system similar to a gel shift mobility assay for analyzing PG/glycosaminoglycan interactions with specific proteins. Levine and coworkers[75] have employed crossed immuno-electrophoresis to characterize PG and platelet factor 4 interactions. The adaptation of techniques (such as gel shift mobility and crossed immuno-electrophoresis) as biologically functional assays to follow PG purification are a logical next step.

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