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AQUEOUS EXCLUSION CHROMATOGRAPHY

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Introduction

The exclusion chromatography of water-soluble polymers has been a field of intense activity in recent years. Although polysaccharide gels like Agarose and Sephadex have long been used for the size separation of proteins ("gel filtration"), only the advent of new hydrophilic column packings could lead to the widespread application of aqueous GPC to synthetic polymers. These new substrates are produced with the mechanical strength and the uniformity and control of both particle- and pore-size distributions requisite to the packing of high-efficiency columns. Such columns offer good separation of species of different molecular weight (MW) along with minimal band-spreading, and so can provide, in principle, detailed and accurate MW distribution data coupled with short analysis times. MWD accuracy and assay speed - relatively unimportant in most biochemical research laboratories - are paramount considerations in the industrial laboratory involved with synthetic water-soluble polymers.

The exploitation of new opportunities arising from this breakthrough in column technology has been accompanied by a growing awareness of problems unique to aqueous polymer solutions.

Like water-soluble polymers, aqueous GPC substrates ("gels") owe their solvation to strongly polar (ionic or non-ionic) functional groups. Electrostatic or hydrogen-bonding forces between such moieties on polymer and gel, respectively, can lead to adsorption or anomalous elution of the former. That these attractive forces persist in strongly solvating media is due at least in part to the cooperative nature of polymer adsorption.

A second set of problems arises from the ionic character of many water-soluble polymers and aqueous GPC packings. Either of these may possess an electrostatic domain, the range and intensity of which is a function of both linear or surface charge density and the charge type and concentration of small ions. The resulting electrostatic interactions lead to a variety of effects, sometimes difficult to resolve from classical "steric" behavior. Simplistic invocations of "ion-exclusion" or "ion-exchange" may conceal the complexities of these interactions. Lastly, polymer-substrate affinity through hydrophobic interactions is clearly a phenomenon unique to aqueous systems.

This report will attempt to present recent developments in the field of aqueous GPC from several perspectives. Aspects of column technology include a description of commercially available organic and inorganic substrates, along with comments on their comparative virtues. Some information on the nature of these packings is proprietary to the instrument companies but there is much that may be presented. The applications of aqueous GPC to polymeric solutes is a second theme. Much of the literature in both of these areas prior to 1979 is cited in a comprehensive review by Barth[1]; hence, the focus here will be on the more recent reports in this intensely active field. Lastly, an attempt will be made to offer a critical review of mechanistic studies that explore separation phenomena unique to aqueous GPC, including adsorptive behavior. It is appropriate to note that two comprehensive texts on exclusion chromatography have recently appeared. One[2] deals especially well with efficiency, resolution and column packing technology, but rather less with

specific electrostatic and adsorptive effects central to aqueous separations. The other offers the most complete description available of polysaccharide gels and their properties[3] but, reporting on the field only until 1977, fails to present recent major developments in theory and technique.

I. Column Technology

Before ca. 1970, only two types of aqueous GPC packings were available: "soft" gels, such as dextran or polyacrylamide gels, and porous glass or silica. While the latter substrates offer advantages in resolution and assay time vis-a-vis the relatively inefficient compressible gels, they also tend to adsorb proteins, cationic polymers, and some synthetic nonionic polymers. Great progress has been made in the last decade in solving these problems of efficiency, mechanical strength and adsorption, both with polymeric and siliceous packings. First, these substrates are now prepared, with a wide range of pore sizes, as spherical, narrow size distribution particles with diameters in the 5-50 μm range. These uniform microspheres may be slurry-packed at high linear flow rates and the resulting columns offer excellent resolution due to dense packing of porous beads; they also exhibit very little band-spreading because irregular packing effects, such as channeling and eddy diffusion, are minimized. The mechanical strength of porous silica and modern organic GPC gels permit operation at moderate or high flow rates. An obvious consequence of high efficiency coupled with rapid flow rate is short assay times, typically three to ten times less than with earlier generation columns. Lastly, the development of techniques for modifying the surface of porous silica through versatile silane coupling reactions has made possible the use of inorganic substrates for proteins and nonionic polymers, without adsorption.

Table I lists both conventional and state-of-the-art packed columns (and packings) now available commercially. It would seem useful to compare the performance of these products, at least in

TABLE I. COMMERCIALLY AVAILABLE PACKINGS FOR AQUEOUS GPC.

PRINCIPLE TRADENAME (SUPPLIER) *COMMERCIAL PSEUDONYMS	CHEMICAL TYPE PARTICLE SIZE	FLOW LIMITS: LINEAR FLOW RATE[a], PRESSURE DROP[b]	TYPICAL EFFICIENCY (PLATES FT^{-1})[c]	UPPER MW LIMIT, (CALIBRATOR)	TYPICAL ASSAY TIME[d]
I. Polymeric, Rigid					
TSK PW (Toyo Soda) *Spherogel PW (Altex) *Bio-Gel Tsk (Bio-Rad)	Cross-linked hydroxylated polyether 11 - 15 μm	160 cm hr^{-1} 200 p.s.i.	3500	3×10^7 (dextran)[e]	30 min.
Shodex Ionpak (Showa Denko) *Shodex Aqueous (Perkin Elmer)	Sulfonated Cross-linked polystyrene 10 μm	170 cm hr^{-1} 500 p.s.i.	5000	5×10^6 (dextran)[e]	15 min.
#Spheron (LaChema) (Koch-Light) (Knauer KG)	Cross-linked poly(2-hydroxyethylmethacrylate) 20 - 40 μm	100 cm hr^{-1} 500 p.s.i.	800	1×10^7 (dextran)[e]	60 min.
II. Polymeric, Compressible					
#Toyopearl (Toyo Soda) *Fractogel (E. Merck)	Cross-linked hydroxylated polyether 30 - 60 μm	25 cm hr^{-1}	800	1×10^7 (dextran)[e]	150 min.
#Sephadex (Pharmacia)	Cross-linked dextran 40 - 120 μm(dry)	10 cm hr^{-1} ———	200	2×10^5 (dextran)	———
#Sephacryl (Pharmacia)	Sephadex, sequentially reacted with allylchloride and methylene bisacrylamide.	20 cm hr^{-1} 14 p.s.i.	500	1×10^8 (4000Å) (dextran)[e]	90 min.
#Ultrogel AcA (LKB)	Agarose, post-polymerized with acrylamide 25 - 55 μm(dry)	25 cm hr^{-1} 2 p.s.i.	500	2×10^6 (protein)	100 min.
#Bio-Gel A (Bio-Rad)	Agarose 40-80 μm; 80-150μm; 150-300 μm (dry)	10 cm hr^{-1} 2 p.s.i.	200	1×10^8 ?	250 min.

TABLE I (CONTINUED)

III. Siliceous, underivatized					
#LiChrospher (E. Merck)	Porous silica 10 µm	1800 cm hr^{-1} 1000 p.s.i.	7000	5 x 10^6 (dextran)[e]	10 min.
DuPont SEC Zorbax SE (9-11 um) Zorbax PSM (5-7 um)	Porous silica 5 - 11 µm	4000 cm hr^{-1} ———	2500	5 x 10^6 (dextran)[e]	10 min.
#Spherosil (Rhone-Progil)	Porous silica 40 - 110 µm	—— ———	300	——	100 min.
#CPG (Electronucleonics)	Porous glass 55, 100, 150 µm (all ± 30%)	300 cm hr^{-1} ———	250 700[f]	3000 Å	60 min.
IV. Siliceous, derivatized					
#LiChrosorb Diol (E. Merck)	1,2-dihydroxy-propyl silica, 5 µm	400 cm hr^{-1} 2000 p.s.i.	5000	2 x 10^6 (dextran)[e]	10 min.
SynChropak (Synchrom)	" " , 10 µm	" "	3500	1 x 10^7 (dextran)[e]	" "
Aquapore (Brownlee)	" " "	"	"	"	" "
Aquachrom (Chromatix)	" " "	"	"	"	" "
TSK SW (Toyo Soda)	Derivatized silica treatment unknown 8 - 12 µm	300 cm hr^{-1} 800 p.s.i.	5000	6 x 10^5 (dextran)	30 min.
µBondagel (Waters)	" " ("polyether phase")	500 cm hr^{-1} 1200 p.s.i.	3500	1 x 10^7 (dextran)[e]	——
#GlycophaseG (Pierce) *Glyceryl CPG (Electro-nucleonics)	1,2-dihydroxy-propyl glass 55, 100 (±20) µm	300 cm hr^{-1} ———	200	———	50 min.

(a) Limitation on linear flow rate as imposed by loss of efficiency, with or without gel compression.
(b) Pressure drop corresponding to flow rate in (a), or maximum encountered in normal operation (30 cm column).
(c) Obtained with low MW nonionic solute.
(d) Typical values found in published literature.
(e) Extrapolated from data on lower MW dextrans, or inferred from results with larger particles, e.g. viruses.
(f) Column packed with sieved glass.
Substrate sold as bulk packing; optimal data from literature.

regard to resolution and speed, but there are several difficulties confronting such comparisons. For substrates sold in bulk, reported column resolutions reflect in part the experimenters' packing techniques. Variations in column dimensions - from 8 to 4 mm I.D. for most commercial columns and both greater and less for laboratory packed columns - mean that normalization of efficiencies on the basis of column length may be incomplete. Finally, good resolution demands that the sample MW lie in the optimal resolving range of the column.

Several workers have compared the efficiency of commercial columns using specific solute pairs. The resolution factor employed to do this usually resembles that presented by Bly[4]:

$$R_{1,2} = \left(\frac{V_2 - V_1}{\log M_2 - \log M_1}\right)\left(\frac{1}{4\bar{\sigma}}\right) \qquad (1)$$

where $R_{1,2}$ is a measure of the extent of separation of species 1 and 2, V is the retention volume and $\bar{\sigma}$ the mean variance of the peaks (very nearly equal to one-fourth the mean base width). Since V_2-V_1 increases with column length L while σ increases as $L^{\frac{1}{2}}$, $R_{1,2}$ increases with $L^{\frac{1}{2}}$. In order to normalize for the effect of column length[5] we may write:

$$R^*_{1,2} = b\left(\frac{1}{L^{\frac{1}{2}}}\right)\left(\frac{1}{4\bar{\sigma}}\right) \qquad (2)$$

where b is the negative of the reciprocal slope of the calibration curve, equal to the first term in eq (1). Introducing the familiar expression for the solutes' plate number (e.g. plates ft^{-1}), $\bar{n} = \bar{N}/L = \bar{V}_{1,2}/\bar{\sigma}L$, (i.e. $\sigma_1 \cong \sigma_2$), we find :

$$R^*_{1,2} = \frac{\bar{n}^{\frac{1}{2}}\, b}{4\,\bar{V}_{1,2}} \qquad (3)$$

This expression is essentially identical to that of Pfannkoch *et al.*[6] who write (their eqs (7) and (14)):

$$R_{sp} = V_i N^{\frac{1}{2}}/4mV_e$$

where m is the slope of the calibration curve. V_i, the pore volume, appears in the numerator since m is defined as $(d\log M/dK_D)$

$= V_i(d\log M/dV_e)$. It would seem that this expression might incorrectly suggest an effect of V_i on R_{sp} beyond its contribution to the calibration slope.

Since b and $\bar{V}_{1,2}$ increase linearly with total column volume, the effect of increased column diameter is embodied in $\bar{n}$ which, as $\bar{V}_{1,2}/\bar{\sigma}$, will increase with the cross-sectional area (as long as uniform packing is feasible), although at the expense of assay time. If, however, the flow rate may be increased accordingly without a proportional increase in σ, a wider diameter column will provide more resolution than a narrow one in the same assay time, as has been empirically noted elsewhere[7-9]. The rate of increase of σ with flow velocity has been analyzed in detail in terms of the solute diffusion coefficient and particle and pore sizes[10].

Table I provides only values of n obtained with low MW solutes; it is recognized that $\bar{n}$ for macromolecular solutes will typically be smaller since their lower diffusion coefficients lead to values of σ that are both larger and more sensitive to linear flow velocity. This consideration aside, it is suggested that comparison of n for different columns will provide a good indication of their relative resolving powers; as seen from eq (3), differences in substrate porosity or column diameter will tend to have compensating effects on b and $\bar{V}_{1,2}$, as long as the MW of the polymer in question falls within the effective resolving range of the column.

The "typical assay times" tabulated reflect a variety of factors which presumably lead most workers to the corresponding "typical" conditions of total column length and column volume, and flow rate. The first two parameters are determined by the efficiency of the columns and the required span of MW resolution, in light of the separation desired. Flow rate is limited by loss of efficiency due to diffusion-controlled dispersion, and, for "soft" gels, by bed compression.

Some descriptive remarks may supplement the data of Table I. TSK PW gels have been used successfully in the author's laboratory

for polycations[11] and appear to be uniquely suited to this group of polymers. Other applications include polyacrylamide, polyvinylalcohol and polyvinylpyrrolidone[12], polyacrylic acid, heparin and chitosan[13]; and most frequently, proteins[14]. The manufacturer further recommends the use of TSK PW columns vs. TSK SW columns for MW $<10^3$ or MW $>10^5$, in which ranges the latter provide little resolution. Toyopearl gels, apparently based on identical polymer chemistry as TSK PW gels, provide the same MW resolution ranges and presumably may be applied to the same solutes. With particle sizes three or four times greater, the Toyopearl gels are supplied in bulk to be packed by the user[15a-c,16]. Although far less efficient than TSK PW columns, columns packed with Toyopearl gel compare favorably in resolution and mechanical stability to polysaccharide gels[17].

The characteristic feature of Shodex Ionpak columns is the intense negative surface charge which acts to repel and hence exclude polyanions, and indeed may confer a mixed-mode aspect on all separations. In fact, monosaccharides may be separated, clearly via some non-exclusion effect[18]. These columns are primarily recommended for the analysis of nonionic oligomers[19].

Despite some interesting properties, Spheron[20] gels are apparently not marketed in the U.S. P1000 20-40 μm beads were slurry packed in methanol to yield 4 mm I.D. columns with good efficiency (at least 700 plates ft^{-1} at 500 cm hr^{-1}) and long-term stability[21]; 8 mm I.D. columns packed with aqueous suspensions of the same gel exhibited 1300 plates ft^{-1} at an optimal flow rate of 40 cm hr^{-1}, corresponding to 100 min assay time[20]. Commercially packed (aqueous slurry) 8 mm columns gave about 300 plates ft^{-1} at 200 cm hr^{-1} although only resolution data for dextrans were presented[22]. As will be discussed later, much of the chromatographic behavior of Spheron involves adsorption and partition.

The other commercially available polymeric packings are the compressible polysaccharide gels: Sephadex, Biogel A (similar to Sepharose), Sephacryl and Ultrogel. Sephadex (dextran) and

Sepharose (agarose) gels are too soft for high efficiency packing and application. To enhance gel rigidity, Sephadex gels have been reacted sequentially with allylchloride and N,N'-methylenebisacrylamide; similarly, Sepharose beads have been permeated with acrylamide and then post-polymerized. In the resultant products - Sephacryl and Ultrogel AcA, respectively - the synthetic monomers are thought to form a secondary network that supports and strengthens the polysaccharide fibrils. Indeed, these substrates can be packed with higher efficiency and used at higher flow rates than their untreated bases.

Porous glass, prepared by selective dissolution of biphasic glass, has been used for aqueous GPC for nearly as long as cross-linked polysaccharide gels. Resolution, primarily limited by lack of uniformity in particle size and shape, may be somewhat improved by sieving. Thus, conventionally dry-packed 100-250 μm GPC glass yields 10 min I.D. columns with ca. 250 plates ft^{-1}, while 130-170 μm sieved glass gave columns with 700 plates ft^{-1} (at 10 cm hr^{-1})[23]. N, for 10^5MW dextran, declined only by a factor of three as flow-rate increased ten-fold[23], making it possible to operate several meters of columns at high flow rate with good efficiency. Smaller particle size CPG presumably provides yet higher efficiency[24]. A more serious defect of porous glass is its adsorptive behavior with regard to biopolymers and most synthetic nonionic polymers. Coating CPG with polyethyleneoxide (PEO) prevents the adsorption of viruses and some proteins, but these coatings are impermanent and irreproducible.

We noted that reduction in particle size and dispersity greatly enhanced packing efficiency for polymeric gels; similarly improved silica particles yield the same benefit. Thus, slurry packing of 10 μm Lichrospher in IPA into 5 mm I.D. columns, gave 10,000 plates ft^{-1} at 450 cm hr^{-1} flow velocity[25]. For DuPont PSM silica particles, plate counts ranging from 3000-8000 ft^{-1} were obtained with slurry packed 6-8 μm particles[8]. Since polymer band-spreading increased only one-third as fast as solvent

velocity, flow rates of 150 ml hr^{-1} could be employed to give 20 min assays with good resolution.

While offering greatly improved chromatographic efficiency relative to porous glass, small particle-size silica exhibits more severe adsorption properties and dissolution behavior. Thus, unmodified silica columns may be used for dextrans and anionic polymers[25], but most proteins and nonionic synthetic polymers are fully retained. Polymers with Bronsted base residues - e.g. polyvinylpyrrolidone, polyethyleneoxide, polyvinylpyridine - show particularly strong adsorption to glass and silica. Dissolution of silica is expected to increase for small particles[26] and seems especially pronounced for DuPont SEC[21] in contrast to LiChrospher[25], although one must note the use of pH 5 acetate buffer in the latter case, possibly a less basic medium than the unbuffered water used with the SEC columns. (It is not clear how the current "Zorbax SE" or "Zorbax PSM" differ from earlier "SEC" and "PSM" products.) It is also of interest to note that 100 μm silical (Spherosil XOB 075) and ca. 50 μm silica (Porasil C) did not exhibit such instability[21].

In order to minimize these difficulties, a major effort has been made to develop methods for the permanent (covalent) bonding of nonionic and hydrophilic groups to porous glass and silica. Fortunately, trichloro- and trialkoxy-organosilanes provide a facile and versatile approach to the synthesis of such bonded phases. By far the most popular reagent is glycidoxy-propyltrimethylsilane, and the resultant "glycol" group (-Si-$(CH_2)_3OCH_2CHOHCH_2OH$) is the surface functionality of all the derivatized glasses and silicas of Table I except for μBondagel ("polyether" bonded phase) and TSK SW (surface treatment unknown). The packing efficiencies of these materials are very similar to those of underivatized substrates, e.g. 5 μm LiChrospher Diol can be slurry packed to yield ca 5000 plates ft^{-1} (6 mm I.D.) or 10,000 plates ft^{-1} (25 mm I.D.)[7]. (The wider column provided the better resolution in a given assay time, when operated at 280 cm hr^{-1}, i.e. 21 ml min^{-1}.)

Differences in performance among these surface-treated silicas probably correspond to variations in the extent of surface coverage. For example, PEO was only partially retarded on a glycol-phase SI-100 silica[27] but was 75% adsorbed on a similarly derivatized SI-500[9], which also partially adsorbed polyvinylalcohol (PVA) and totally retained polyvinylpyrrolidone (PVP). Again, SynChropak GPC 100 and LiChrosorb Diol show marked differences in Lysozyme retention[6] despite having identical substrates and surface coatings. On the other hand, the adsorption properties of μBondagel and TSK SW appear to bear out their different surface treatments. The former exhibits adsorption of proteins[1]; this effect may be attributed to the hydrophobic nature of the surface which is also attested to by its relatively intense affinity for phenylethanol[6]. In contrast, TSK SW columns show minimal "salting-out chromatography" of lysozyme[6] and little other evidence of anomalous protein adsorption; and are reportedly suitable for PEO, PVA and PVP, although not for polyacrylamide[28].

II. Applications

A common feature of all aqueous GPC columns is the absence of specific interactions with dextran. Since this neutral polysaccharide is furthermore available as well characterized fractions (Pharmacia), its use in aqueous GPC studies is ubiquitous and deserves no special note per se. Anionic polysaccharides also seem to chromatograph easily, e.g. heparin on TSK SW[28], LiChrospher[25], Sepharose CLB[29], or Sephadex[30]; gum arabic on TSK SW[28]; and carboxymethylcellulose on LiChrospher[25] or Synchropak[31]. More complex acidic polysaccharides such as chondroitin sulfate and hyaluronic acid have been analyzed with Spheron 1000[32], Sepharose[29], and TSK SW[28]. All available data suggest that synthetic polyanions, particularly acrylates, may be chromatographed readily on all aqueous GPC supports.

The behavior of many neutral synthetic polymers presents a contrast to that of dextran inasmuch as polyethyleneoxide (PEO), polyvinylpyrrolidone (PVP), and commonly, polyvinylalcohol (PVA)

all adsorb on untreated glass or silica. "Glycol" bonded phases offer at best a partial reduction of this effect for the first two polymers[9]; on the other hand, TSK SW modified silica is reported to be applicable to all three[28]. Interestingly, PAM is not readily characterized on TSK SW[28] even though it may be eluted from porous glass or silica. No such restrictions seem to apply to polymeric gels: for example, TSK PW literature is illustrated with specific applications to PEO, PVP, PVA, and PAM[13].

The principal motivation in the commercialization of surface treated glass and silica has been the analysis of proteins, and the literature on these packings abounds with data on their resolution, capacity and retention behaviour for globular proteins. The last property may be complicated by the superposition of the electrostatic and hydrophobic forces that take place between protein and substrate, and experimental efforts to resolve among these effects are described by Pfannkoch et al[6]. Polymeric gels also have been used with some success for protein GPC[15a,33,34] (see also manufacturers' literature for Ultrogel, BioGel, and Sephadex, etc. products); in this case, electrostatic interactions are expected to be negligible, while hydrophobic factors can become more dominant.

The successful exclusion chromatography of viruses and high MW proteins and protein multimers poses some difficulties related to their size, in addition to the adsorption problems noted above. First, the packing pore size must well exceed the solutes' dimensions to produce adequate resolution; secondly, ultrahigh MW standards are needed to calibrate the column. Using plant viruses and the very high MW hemoglobin-like protein chlorocruorin (all presumably characterized by ultracentrifugation) Himmel and Squire found the resolution limit of a G5000 PW column to be 1.4×10^6[35], although tobacco mosaic viruses, MW 4×10^7, was separated, possibly via "hydrodynamic chromatography"[36]. With a G6000 PW column, Okazaki et al separated blood serum lipoproteins up to MW 2×10^7[37], although they did not specify the

absolute method used to measure these MW values. Using PEO-coated CPG glass with pore sizes up to 3100 Å, Haller et al resolved the components of a viral solution into a primary 500 Å species and aggregates ranging in diameter up to 2800 Å[38].

Large pore siliceous packings have also proven useful in the characterization of polymer latex particles in colloidal aqueous suspensions. Polystyrene and poly(methylmethacrylate) lattices were first fractionated on 500 - 50,000 Å porous silicas[39]; later, porous glasses were used for similar separations.[40] Coll and co-workers pointed out the value of surfactants and supporting electrolyte to stabilize latex particles and reduce ionic repulsive forces between latex and packing[41]. A uniform dependence of the retention factor on particle diameter (determined by SEM) could be established for chemically different lattices[42]. More recent studies focussed on the separation of 90-360 nm diameter polystyrene spheres on CPG with pore sizes ranging from 500 Å - 10,000 Å[43a-c]. Special attention was paid to the influence of ionic strength on the calibration curve; the interpretation of detector response, i.e. turbidity, in terms of particle size and concentration; and mathematical procedures for the deconvolution of the chromatogram to yield true particle size distributions.

It is difficult to obtain suitable synthetic standards for aqueous GPC in the very high MW range. Very narrow MWD sulfonated polystyrene (Pressure Chemical Co.) samples are available from MW $2x10^3$ to $1.2x10^6$. Dextran T fractions (Pharmacia) encompass the range $10^4 < \bar{M}_w < 2x10^6$. Recently, Toyo Soda has supplied narrow MWD PEO standards, MW 10^4 - 10^6, which supplement the lower MW narrow-distribution materials available from Dow Chemical Co. or Union Carbide Corp. The resulting calibration plots can be extended into the range of MW$>10^6$, if data for viral or synthetic particles can be corrected for differences in molecular configurations.

Basic or cationic polymers have, in general, not been amenable to analysis with siliceous supports, although Chitosan, a glucosamine-rich polysaccharide, was chromatographed on Glyco-

phase G[44]. Porous glass or silica with a quaternized bonded phase was used for poly(2-vinylpyridine) and quaternized poly(4-vinylpyridine), although no evaluation of elution data with regard to MW was made for the latter[24]. Uncharged polymeric gels may be more appropriate for cationic polymers; PW columns seem particularly well suited for strong polycations[45,46].

While GPC has been used to study polymerization reactions in organic media, analogous applications to aqueous solutions are rare. The polymerization of silicic acid was examined using Sephadex chromatography[47]. Studies of noncovalent multimerization with aqueous GPC are appearing with increasing frequency. For example, exclusion chromatography on polysaccharide gels has been used to investigate the size of micelles formed from n-alkyl polyoxyethylene ethers[48] and mixtures of Triton X-100 with phosphatidylcholines[49], while CPG was used to separate casein micelles[50]. Gel chromatography has also become a fundamental tool for studying reversible aggregation of proteins[51,52], on the basis of the pioneering work of Winzor and Scheraga[53] and Ackers[54].

Novel applications of aqueous GPC continue to emerge. The use of exclusion chromatography to measure polymer-ligand interactions[55] has been recently extended to complexes of serum albumin with warfarin[56] and heparin with methylene blue[30]. In a somewhat related fashion, aqueous GPC has been used to study the preferential solvation of amylose in DMSO-H_2O[57]. The chromatography of biopolymer-surfactant complexes may be important for difficultly soluble proteins; given the multiplicity of possible electrostatic and solvophobic interactions among protein, detergent, and substrate, the resultant chromatographic behavior and its response to variables such as concentration and ionic strength may be quite complicated[58,59].

III. Separation Mechanisms

The dependence of elution volume on the dimensions of polymer molecules and the geometry of substrate pores has been the

subject of considerable theoretical and experimental study. All of these treatments are based on the supposition that specific interactions between solutes and substrates are absent. However, such interactions - electrostatic, hydrogen-bonding, or solvophobic - can influence or even dominate the separation, if the substrate and solvent are not chosen judiciously for the polymer in question. Hence, it is appropriate to first consider "anomalous" retention effects before addressing the "ideal" case.

A. Adsorption and Partition

A useful distinction may be made between non-ideal retention based on strong, specific interactions such as the formation of hydrogen bonds or ion-pairs, and that resulting from non-specific van der Waals and solvophobic forces. Interactions of the first class involve pairing of complementary sites on polymer and substrate, e.g. Lewis acid and base, or anion and cation. These "adsorptive" phenomena are likely to be cooperative, increasing with MW, and may be irreversible. Solvophobic partitioning, on the other hand, should exhibit equilibria readily shifted by solvent or temperature change, and may decrease for higher MW solutes for which the available gel surface is less[60]. Clearly, adsorptive phenomena are typical of siliceous packings, and partitioning more characteristic of polymeric substrates.

Solvophobic partitioning has been studied extensively for polysaccharide gels; indeed, apolar groups are coupled to such packings to provide very selective separations via "hydrophobic chromatography"[61]. Unsubstituted polysaccharide gels also exhibit unique affinities for certain low MW solutes. That Sephadex can bind aliphatic alcohols[62], surfactants[63], bile salts[64] and tetraalkylammonium salts[65] in a manner that increases with temperature and ionic strength and decreases in the presence of organic co-solvent, is compelling evidence for the hydrophobic nature of this interaction. There is less agreement about the gel site of binding. Marsden, for example, proposed that one face of the anhydroglucose residue could

provide a hydrophobic surface[66], while others find the epichlorohydrin-derived crosslinks more reasonable loci[67], a proposal consistent with the observation of greater partitioning with the smaller pore-size, more densely crosslinked gels. On the other hand, Janado et al[63] use the same observation to support a model in which "bound" water, relatively more abundant in the "concentrated", less porous gels, provides an environment entropically more favorable for hydrophobic solutes than bulk water. This postulate gains credence from their finding that concentrated dextran (but not glucose) solutions solubilize apolar solutes, but no quantitative comparisons are made between these solubilities and the excess GPC retentions.

Aromatic compounds exhibit particularly intense affinity for Sephadex, generally attributed to hydrogen bonding between gel hydroxyls and aromatic pi electrons, an explanation entirely consistent with measured substituent effects[68]. The binding of phenols is the subject of some controversy which focusses on the role of solute hydroxyl hydrogen-bonding to gel ether groups[69]. To complicate matters further, the elution of phenols exhibits a pH dependence that reflects both phenolate ion formation and also the dissociation of the gel. Sephadex contains typically about .005 meq gm^{-1} COOH[70] whose presence leads to progressive perturbation of the elution of ionic solutes from pH 2 to 10; above this pH, intense effects of polysaccharide hydroxyl ionization become evident[65].

Given this multiplicity of non-ideal retention modes for Sephadex gels, their utility in macromolecular size separation might seem fortuitous. In effect, aromatic and strongly apolar groups are usually internalized within the polymer's spatial domain, and so not exposed to the gel surface. Weak electrostatic forces due to acidic gel groups are readily screened out by the mobile phase electrolyte. Nevertheless, the influence of specific interactions may become apparent upon careful comparison of different polymer types. For example, polyethyleneoxide fractions elute from Sephadex much earlier than do dextrans of the same

molecular size, but explanations of this effect do not agree[71, 72]. In general, it may be observed that the understanding of sorption on polysaccharide gels lags far behind the practical application of this phenomenon to separations of numerous low MW solutes[73].

Other polymeric packings also exhibit sorptive behavior, although none have been studied as much as Sephadex. Using Spheron as a GC packing, Hradil compared the sorption of pentane and ethanol[74] and was able to conclude that non-specific apolar interactions - largely attributable to the ethylenedimethacrylate comonomer - were predominant, with hydrogen bonding playing a secondary role. Partitioning on this gel in aqueous GPC operation could be used to separate uracil derivatives[75]; while elution increased with alkyl substituent size, K_D varied inversely with temperature and was insensitive to ionic strength, thus suggesting a mixed mode of sorption rather than pure hydrophobic partitioning. On the other hand, hydrophobic effects appear to dominate the retention of proteins, inasmuch as K_D for chymotrypsinogen doubles with a 10°C temperature increase, and increases ten-fold as salt concentration is changed from 0.6 M to 2.0 M[76]. It may be that the functional group orientations necessary to develop cooperative hydrogen bonds between protein and gel are not consistent with the conformation of the native protein, and thus nonspecific hydrophobic attractions predominate.

Rather little is known about the sorptive properties of TSK PW gels. Protein adsorption occurs at very high ionic strength (e.g. 50% ammonium sulfate) an effect ascribed to hydrogen bonding[77]. Phenolic compounds are totally bound from aqueous solvent, while aliphatic alcohols exhibit K_D values ranging from 1.0 to 2.4; these effects are reduced but not eliminated in 50% methanol[78]. Since the composition of this packing is only partly disclosed, satisfactory interpretation of these results is difficult.

As noted above, siliceous packings exhibit tenacious adsorption of polymers with cationic or proton-accepting

residues, attributable to the properties of surface silanol groups. These effects typically involve cooperative MW-dependent adsorption of polymer chains, e.g. CPG elutes in the total column volume pyrrolidone and N,N-dimethylacetamide, but totally retains poly(N-vinylpyrrolidone) and poly(N-vinylacetamide)[78]. Such adsorption properties are yet more pronounced with porous silica. The adsorptive interactions of polymeric and low MW, ionic and nonionic solutes, with the surface of silica are lucidly discussed by Iler[79].

While the introduction of bonded phases is intended to eliminate the adsorptive properties of the silanol group, it is incorrect to assume that total derivatization is possible. Steric considerations suggest that about ¼ of the available silanol groups can react with trialkylchlorosilane[80] and that "only methanol is small enough" to fully combine with all silanol sites[79]. Thus, while dry silica carries a surface concentration of SiOH of 8-13 μmoles m^{-2}[80], the best coverage attained from a series of triethoxysilanes was 5 μmoles m^{-2}[27] (and only half that for a "diol" or "glycol" phase). CPG packings with the same concentration of silanol groups (i.e. 8 per 100 $Å^2$)[81] behave at best in a similar manner. The recovery of basic proteins, often used as a probe for the extent of derivatization, is in fact a poor measure of residual silanol groups: for example, the adsorption of Cytochrome C on glycophase G could be reduced from 80% to 8%, but the concentration of bonded phase never exceeded 2 μmole m^{-2}[82] (about 1 molecule per 100 $Å^2$ or at most 20% of reactive sites).

The influence of such residual sites may, fortunately, not be proportionate to their concentration, since they can be shielded by the bonded phase. Thus, the effect of anionic groups on Glyceryl-CPG can usually be nullified if the ionic strength is above 0.1, especially at pH less than 4[83]. Similarly, TSK SW, Synchropak, LiChrosorb Diol, and μBondagel all display anomalous retention of the basic amino acid arginine at pH 7 (isoelectric pH 10.8), which is virtually eliminated at ionic strengths above

0.1[6]. The use of salt to screen out such electrostatic effects is not without some hazards, since hydrophobic retention increases with ionic strength. The K_D of phenylethanol, intended as measure of hydrophobic partitioning[6], increases slowly with ionic strengths above I = 0.2, the extent of partitioning increasing in the order SynChropak < TSK SW < LiChrosorb Diol < μBondagel. (It should be noted that the possibility of pi electron hydrogen bonding with phenylethanol was neglected.) Presumably as a consequence of these opposing effects of salt on electrostatic and hydrophobic adsorption, all of these substrates display a minimum in the adsorption of Lysozyme at $0.3 < I < 0.6$[6]. As pointed out in the last reference, the adsorption of proteins is difficult to predict from other data, For example, SynChropak, with relatively low values of K_D for arginine and phenylethanol, shows strong retention of lysozyme[6]. One problem could be that the selection of suitable low MW hydrophobic probes may not be obvious; thus, 5-cyclobutyluracil, which strongly partitions onto Spheron, is only slightly retained on a glycerol bonded phase porous glass[76] in 0.7 M $(NH_4)_2SO_4$, an observation that suggests that the retention of phenylethanol[6] may overestimate the role of hydrophobic bonding on such "diol phase" siliceous packings.

The most comprehensive program for the preparation of hydrophilic bonded phases was described by Engelhardt and Mathes who first[27] prepared SI-100 silica with the functional groups: (1) Y-NH-CO-CH_3, (2) Y-NH-CO-CF_3, (3) Y-NH-SO_2-CH_3, (4) Y-NH-CO-CH_2-NH-CO-CH_3, (5) Y-O-CH_2-CHOH-CH_2OH, and (6) Y-$(CH_2)_{14}$-CH_3, where Y = -Si-$(CH_2)_3$-. With water as the solvent, only the "amide" (1), and "glyinamide" (4) bonded phases eluted polyethyleneoxides without adsorption, and only the former eluted proteins before the total column volume. The superior performance of the "amide" phase relative to the similarly hydrophilic "glycol" phase (5) may be attributed in part to its better surface coverage, i.e. 4.0 vs. 2.2 μmole m^{-2}. Later, the same authors compared 14 bonded phases[84] with regard to adsorption of nonionic synthetic polymers, and found that amine surfaces, e.g. -Si-$(CH_2)_3NH(CH_2)_2NH_2$,

gave excellent, non-adsorptive separations of polyethyleneoxides (plate count ca. 5000 ft^{-1} for ethylene glycol) in water, although buffer was recommended to maintain a local pH below that leading to silica dissolution. The more stable "amide" phase, $-Si-(CH_2)_3NHCOCH_3$, was satisfactory for dextrans and proteins in pure water. Adsorption of poly(vinylpyrrolidone) in water to all phases was attributed to hydrophobic factors, but the suppression of this effect on "amide" phase at pH 8, with 0.5 ionic strength Li_2SO_4 and 10% ethylene glycol, could be also ascribed to the disruption of polymer-substrate hydrogen bonds, particularly given the tendency of Li^+ to complex with polymeric amides[85].

B. Size Exclusion Theories

The goal of theoretical treatments for GPC, in its most basic form, has been to predict retention volume from the known configurational properties of the macromolecule in conjunction with measurements of the porosity of the substrate. Further refinements must deal with the consequences of the distributions of both molecular weights and pore sizes, the influence of flow velocity on separation, and effects of solute concentration, inter alia. After a decade during which models that conflicted with reality were slowly discarded, the equilibrium theory of Casassa[86], also developed from a different direction by Giddings[87], achieved broad acceptance. Although rigorous confirmation of the details of this treatment are elusive due to the difficulty of attaining accurate and unambiguous data on substrate pore dimensions[88], the general result - that the radius of gyration is the dimensional parameter that determines the GPC distribution coefficient for random coil polymers - is entirely in accord with the "Universal Calibration" parameter $[\eta]M$[89]. The last quantity is of considerable intuitive appeal, as it can be identified with the hydrodynamic volume of an equivalent sphere[90].

It might appear, at first blush, that extensions of these treatments to aqueous exclusion chromatography should confront no

opposition. Three effects make the situation more complex than for GPC in organic media. First, the macromolecular dimensions for polyelectrolytes, a major class of water-soluble polymers, are very sensitive to ionic strength. This property, and the related dependence of molecular size on polymer concentration in low-salt solutions, are well-understood phenomena[91]. Secondly, the substrate surface also may exhibit ionic character, as discussed above, and so interact electrostatically with a charged polymer. Finally, the diversity of macromolecular conformations in aqueous systems, approximating for example impenetratable spheres, rigid rods, and random coils, for globular proteins, helical polypeptides and neutral synthetic polymers, respectively, provides a challenge and a valid test for models of the separation process, if anomalous sorption effects can be obviated.

Conflicting data concerning the universality of $[\eta]M$ as a calibration parameter have been characteristic of the literature on GPC at large. For aqueous exclusion chromatography, as for the field in general, deviations from universal calibration behavior often turn out to be related to non-steric effects, such as polymer aggregation, adsorption and partition, or other polymer-substrate interactions. For example, while the universal calibration plots of sodium polystyrene sulfonate (NaPSS) and dextran on CPG failed to coincide at high MW in 0.1 M Na_2SO_4[92], these polymers, as well as polyacrylic acid, exhibited coincidental plots in the presence of 50% methanol[93]. It is likely that this solvent reduced interactions that led to the late elution of high MW dextran from CPG. Failure to observe universal calibration of NaPSS and dextran and on Sephadex CL-6B in 0.1 - 0.9 M NaOH[94] may well be a consequence of the ionization of the second polymer and the gel at this high pH, and resultant variations in electrostatic effects.

Electrostatic factors, as expected, play a significant role for charged polymers on charged substrates, at low ionic strength[25,31,95]. This effect may be viewed as a diminution in available pore volume as the extension of the electrical double

layer of the substrate reduces the domain in which a polyion of like charge will permeate. Electrostatic repulsion also provides the basis for "ion exclusion chromatography", in which ionic and non-ionic solutes are separated from each other on ion-exchange resins; hence this term has been applied to the diminished retention of polyions from ionizable packings. No quantitative treatment of this phenomenon has been attempted to date.

Semi-empirical separation relations, other than the classical universal calibration approach, have been offered in the context of aqueous GPC. Haller found with dextrans on CPG that plots of log $(1-K_D)$ vs log M were linear[96] and related the limiting value of $M_{K_D \to 0}$ to the pore diameter. This treatment was further refined by Basedow et al[23], and extended to the chromatography of viral aggregates[38]. Hester and Mitchell[97] replotted the data of Spatorico and Beyer and found a linear dependence of ln K_D on $([\eta]M)^{1/3}$, a relation that follows reasonably from the theory of Casassa and co-workers[86] (not cited by Hester and Mitchell). More recently, Squire and co-workers[98, 99] attempted to extend an earlier treatment for protein gel filtration[100] to random coil parameters. They essentially used protein calibration data to obtain "equivalent sphere" hydrodynamic radii for polyethyleneoxide samples, but achieved only fair agreement between these values and configurational dimensions from other methods. This issue is most clearly confronted by Casassa and Tagami[86a] who conclude that only results for branched chains can provide a decisive basis for choosing between hydrodynamic radii and other random-coil dimensional measures as calibration parameters.

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