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Separation of Macromolecules in Gels: Permeation Chromatography and Electrophoresis

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The principal methods for separating macromolecules in gels, permeation chromatography and electrophoresis, are reviewed. The emphasis of the review is put on the steric exclusion between the macromolecules to separate and the gel, and on the migration mechanisms. In gel permeation chromatography, a heterogeneous assembly of gel particles leads to a mechanism of steric exclusion in which the largest molecules migrate the fastest. In gel electrophoresis, the steric exclusion occurs in a volume homogeneously filled with gel, and the reverse, i.e. the smallest molecules migrating fastest, generally occurs. These general rules offer exceptions, however, and several mechanisms of migration used for practical applications are described in the review. The present experimental state-of-the-art is also discussed in some detail, and the different aspects and limitations of the two techniques are discussed (gel media, instruments, detection and analysis). The conclusion points to the probable convergence of these two complementary techniques in the near future.

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List of Abbreviations and Symbols

GPC	Gel Permeation Chromatography
GE	Gel Electrophoresis
GFC	Gel Filtration Chromatography
HPC	High Performance Chromatography
SEC	Size Exclusion Chromatography
V_e	Elution volume
K_a	Adsorption constant
S	Specific surface area
V_o	Volume of interstititial solvent
V_{gel}	Gel Volume
V_{mat}	Matrix Volume
V_p	Pores volume
K_{gpc}	Partition coefficient
K_{excl}	Exclusion coefficient
N	Chain size
Z_{pore}	Partition function of a chain in a pore
Z_{free}	Partition function of a chain in free solvent
k	Boltzmann constant
ΔF	Excess free energy
T	Absolute temperature
d_p	Particle diameter
w	Peak width
M	Molecular weight
η	Viscosity
V_h	Hydrodynamic volume
N_p	Number of theoretical plates
HETP	Height Equivalent of a Theoretical Plate
w	Peak width
F(V)	Experimental chromatogram
W(V)	Theoretical chromatogram
G(V)	Instrumental function
ISF	Instrumental Spreading Function

OD	Optical Density
ID	Internal Diameter
HPLC	High Performance Liquid Chromatography
UV	Ultra Violet
LALLS	Low-Angle Laser Light Scattering
MALLS	Multi-Angle Laser Light Scattering
THF	Tetrahydrofuran
ΔP	Pressure drop
r_c	Capillary radius
l_c	Capillary length
Q	Solvent flow rate
M_n	Number-average molecular weight
M_w	Weight-average molecular weight
M_v	Viscosity-average molecular weight
M_z	z-average molecular weight
I_p	Polydispersity index
Da	Dalton
DNA	DeoxyriboNucleic Acid
bp, kbp	Base-pair, kilobase-pair
T%	Total acrylamide content
C%	Bisacrylamide/total acrylamide content
μ	Electrophoretic mobility
μ_o	Electrophoretic mobility in free solvent
μ_1	Limiting mobility
f	fractional volume
s, 1, n	Surface, line and number density of the matrix
L, S, V	Mean length, excluded area and excluded volume of a particle
K_r	Retardation coefficient
R, r	Particle radius, fiber radius
R_s	Polymer Stokes radius
a	Average pore diameter
ζ	Friction coeffcient
t_{rep}	Reptation time
t_A	Equilibration time of a blob
q	Blob charge
E	Electric field
$\varepsilon = \frac{Eqa}{kT}$	Reduced electric field
$\dot{S}$	Curvilinear velocity
F	Effective force
t_{dis}	Disengagement time
ρ	Reduced end-to-end projection
$Q(\theta, \varepsilon)$	Distribution function of blob orientations
N*	Critical chain size for mobility saturation
t_{or}	Orientation time of a chain
τ	Pulse time

1 Introduction

Macromolecules are generally difficult to purify and to characterize. In particular, the crystalization processes widely used for small molecules are often inapplicable to the separation and/or the characterization of macromolecules with a large molecular weight. As an alternative, very sophisticated and efficient separation methods using the migration properties of macromolecules in gels were developed.

The typical scale of the microscopic structure of gels can be varied in a large range, typically from a few Å to several μm. Because of this rather unique property, gels are very extensively used in industry and research for the separation of molecules, macromolecules and microparticles. The simplest of such applications is gel filtration. The gel there acts as a membrane in which only particles smaller than its average pore size can penetrate, leading to the separation of a population of particles or molecules in two "families". The natural weakness of swollen gels, however, generally make them less suitable than solid organic or inorganic membranes for this purpose (indeed, the distinction between gels and membranes is often rather arbitrary, since the latter are often prepared by a sol-gel process, and may be partly "reswollen" by vector fluids). In this review, we focus on more specific transport properties in gels, which have provided separation technology in the last few decades with most spectacular and powerful tools, i.e. Gel Permeation Chromatography (GPC) and Gel Electrophoresis (GE). These techniques are very important tools for the characterization of polymers and biomolecules, and they are also extensively used on a preparative scale. Gel filtration, gel electrophoresis and GPC present numerous common features. They use an external field (hydrodynamic for filtration and GPC, electric for electrophoresis) to "push" solutes (particles or molecules) in the gel, which in turn hinders motion by restricting the space available to them. In spite of this common "excluded volume" origin, the migration mechanisms in gels may be rather subtle and very size-or-shape-specific. Describing these mechanisms, their use and their limits, is the aim of the following sections. There is a very strong interconnection of research and applications in this field, and it is probably fair to say that our understanding of transport in gels would be much poorer, had not progress been constantly promoted by technological needs and questions. Therefore, we also strongly refer to methodological aspects in the following presentation. A brief history of GPC is given in Sect. 2.1, and the different mechanisms of solute retention and transport are described in Sect. 2.2. The applications and methodology are discussed in Sect. 2.3. Electrophoretic transport is considered in Sect. 3. A brief history, and a description of the most extensively used gels are given in Sect. 3.1. The mechanisms of separation in electrophoresis are recalled and discussed in Sect. 3.2. Finally, we describe in Sect. 3.3 recent methodological and fundamental progress in electrophoresis, based on the introduction of pulsed fields

and spectroscopic techniques of investigation. A general conclusion is given in Sect. 4.

2 Gel Permeation Chromatography (GPC)

2.1 History of GPC

The fractionation of macromolecules according to molecular size by liquid chromatography has its origin in the University of Uppsala in 1959. Using cross-linked dextran gels swollen in aqueous media, J. Porath and P. Flodin [1] obtained the size-separation of various water-soluble polymers, introducing the term of Gel Filtration Chromatography (GFC) [2] and the Sephadex family [3] still widely used today by biochemists. These "soft gel" packings only operate under low pressure which involves very long analysis times.

Some years later, J.C. Moore [4] in the Dow Chemical Company, achieved the separation of organo-soluble polymers using packings based on cross-linked polystyrene gels, immediately commercialized by Waters Associates under the name of Styragel [5]. These semi-rigid packings, partially swollen with organic solvents, could operate under moderate pressure, leading to a shorter analysis time. A new instrument, the Waters GPC 100 liquid chromatograph was simultaneously introduced, giving birth to Gel Permeation Chromatography (GPC) which came rapidly into extensive use for polymer analysis in industry as well as in the university laboratories.

The first totally rigid porous packing was introduced in 1966 by De Vries et al. [6–7] in Pechiney-St-Gobain. It consisted of porous silica gel commercialized under the name of Spherosil or Porasil. These packings were fully compatible with both aqueous and organic solvents and very pressure-resistant, but their use was restricted by the strong interactions between the silica surface and a number of solutes, leading to abnormal retention in GPC experiments.

In 1974, the development of new packings consisting of small porous particles with a typical diameter of around 10 μm instead of 50–100 μm initiated technological improvement. High Performance (HP) chromatography was born. High pressure technology and the reduction of column volumes, as a consequence of the smaller particle diameter and of the high efficiency of the columns, decreased analysis times from a few hours to some ten minutes. The first small particles introduced commercially for HPGPC were μ-Styragel by Waters Associates [5, 8] with the same chemical structure as Styragel but a diameter of 10 μm. Other similar packings [9, 10] were introduced at once. At the same time, Kirkland [11, 12] and Unger [13, 14] described porous silica micro-beads capable of withstanding high pressure and compatible with the majority of solvents. In order to prevent interactions with solutes, other

packings were developed, involving silica surface modifications by organic grafting (μ-Bondagel [5], TSK-Gel SW [15], Lichrosphere-diol [16], etc.) or by organic coating [17] as SynChropak Catsec [18].

The most recent developments led to the commercialization of smaller cross-linked polystyrene beads with a diameter of around 7 μm and a very narrow particle size distribution, providing a very high column efficiency: ultra-Styragel [5], TSK-gel [15], Shodex-A gel [19], PL-gel [20]. At the same time, manufacturers have developed new families of porous hydrophylic gels for aqueous GPC with the same characteristics as organic GPC packings. They are fully compatible with aqueous solvents and withstand relatively high pressure (TSK-Gel PW [15], Shodex OH-pak and Ion-pak [19], PL-aquagel [20], . . .).

Modern high performance Size Exclusion Chromatography (HPSEC) or simply Size Exclusion Chromatography (SEC) is now the most commonly used term for GPC when using small porous particles, since, as well as for Gel Filtration Chromatography (GFC), the size exclusion by the gel is the dominant fractionation mechanism. As we shall see, however, several other mechanisms (often unwanted) may be responsible of chromatographic retention of macromolecules on a cross-linked gel.

2.2 *Solute Retention in GPC*

2.2.1 The Chromatographic System

Gel permeation chromatography is a particular liquid chromatography process in which the stationary phase is a porous cross-linked gel carefully packed in a chromatographic column and swollen by a mobile phase. For chromatographic reasons, the gel generally consists of spherical beads with the smallest possible diameter and a narrow distribution in size. For retention mechanism reasons, the size of the pores should have the same order of magnitude as that of the macromolecules to be separated in solution. Therefore, it is generally not possible to use gel beads with a diameter much smaller than 10 μm, in contrast to modern conventional Liquid Chromatography (HPLC).

A GPC column is represented in Fig. 1. The total column volume V_{col} can be considered as the addition of two volumes: the dead volume V_o corresponding to the interstitial solvent and the gel volume V_{gel}. V_{gel} is in turn divided into the gel matrix volume V_{mat} and the porous volume V_p filled up with the solvent:

$$V_{col} = V_o + V_{mat} + V_p \tag{1}$$

When considering GPC solute retention, only the total solvent volume V_m in the column must be taken into account: if K_{gpc} is the partition coefficient between the mobile phase V_o and the stationary phase V_p, the macromolecule elution volume V_e can be written:

$$V_e = V_o + K_{gpc}V_p \tag{2}$$

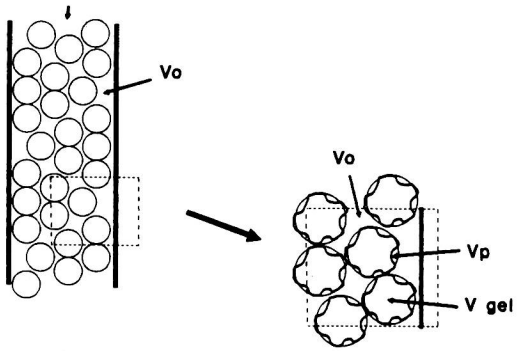

Fig. 1. Representation of the GPC column; V_o is the dead volume, V_p is the porous volume and V_{gel} is the gel volume

where K_{gpc} varies from 0 for excluded macromolecules (no access to the stationary phase) to 1 for macromolecules in total permeation (total access to the stationary phase). As we shall see later, K_{gpc} depends on the size of macromolecules, which means that the biggest macromolecules, excluded from the gel, elute first at V_o, then intermediate ones, the smallest ones being eluted last at $V_o + V_p$.

In fact, in order to cover the whole range of molecular weights of usual polymers, packings should have pore sizes from a few Angstroms to a few thousands of Angstroms. For technical reasons, it is only possible to synthesize packings with a limited range of pore sizes, and the GPC column is generally an assembly of several columns in series, packed with several gels of different porosities (Fig. 2). Another possibility is to use only one column packed with a mixture of several different gels with various porosities (mixed beds).

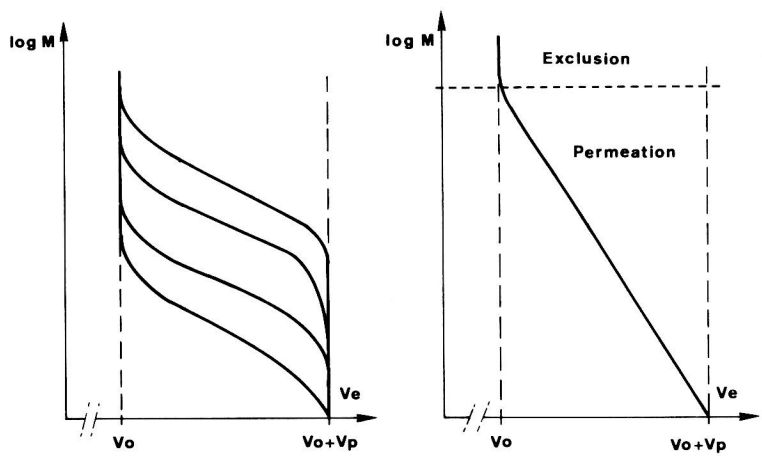

Fig. 2. Influence of pore size packing on the response in size of a GPC column: *on the left*, responses corresponding to various pore sizes are represented. The response corresponding to a combination of these pore sizes is represented *on the right*

2.2.2 Retention Mechanisms

Many attempts have been made to explain polymer fractionation in GPC (see the reviews of Audebert [22] and Hagnauer [23]), and the size exclusion mechanism is now widely accepted and demonstrated [24–28]. In actual experiments, however, several additional mechanisms based on interactions between the solute and the stationary phase may occur in the column, such as adsorption and liquid-liquid partition.

Adsorption Mechanism. Adsorption mechanism mainly occurs with mineral porous gel particles well-known for their surface activity. This is the classical mechanism of solid-liquid adsorption chromatography. The elution volume takes the form:

$$V_e = V_o + K_a S \tag{3}$$

where K_a is a constant representing adsorption forces and S the specific surface area. In the first approximation, the adsorption energy per molecule linearly increases with the number of adsorption sites it occupies on the surface. This generally leads to a retention time which exponentially increases with molecular weight, and it can produce an irreversible adsorption of the polymer onto the stationary phase. This phenomenon is extremely undesirable in classical GPC experiments and the trend is to modify the surface by grafting or coating organic compounds onto silanol groups, mainly responsible of these effects, to prevent adsorption when using those kinds of particles as the GPC stationary phase [15–18].

Liquid-Gel Partition Mechanism. Liquid-gel partition mechanism is the classical liquid chromatography process where the mobile phase is the solvent and the stationary phase is the swollen gel. In modern GPC where gels are highly cross-linked, only very small molecules are capable to enter the gel matrix, but with "soft gels" or "semi-rigid gels", the characteristic dimensions of gels are greater and macromolecules can penetrate the gel. In this case, the elution volume can be written according to the partition equilibrium:

$$V_e = V_m + K_{par} V_{mat} \tag{4}$$

where V_m is the volume of mobile phase ($V_m = V_o + V_p$), V_{mat} is the volume of the gel matrix and K_{par} is the partition coefficient. This equation represents the equilibrium theory of conventional liquid chromatography. The partition coefficient K_{par} depends on thermodynamic interactions between solutes and the swollen gel but also on the solutes molecular weight.

Figure 3 represents the liquid-liquid partition equilibrium. When the solute enters the pore volume V_p (by a size exclusion mechanism which will be discussed later), it has access to the gel matrix swollen by the solvent and a thermodynamic equilibrium may occur between these two phases. This equilibrium depends mainly on the relative solute affinity for the gel. Therefore, the elution volume will depend on the solute size, since the gel volume accessible to the solute is controlled by steric exclusion, but also on interactions between the

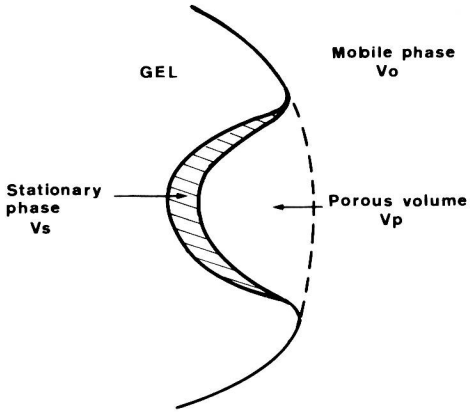

Fig. 3. Liquid-liquid partition equilibrium: V_o is the volume of the mobile phase, V_p is the porous volume and V_s is the volume of the stationary phase

solute and the gel (the latter depending on chemical affinity and on molecular weight).

This phenomenon mainly occurs in the low molecular weight range but it is not reproducible from one macromolecule to the other. As shown later, the GPC column has to be calibrated in molecular weight for general use with many different polymers, and the liquid partition mechanism appears as a disturbing phenomenon which should be minimized by a suitable selection of stationary and mobile phases. A general rule is to run GPC experiments with a thermodynamically good solvent of the polymer as mobile phase, the stronger the affinity of the solute for the solvent, the lower the affinity for the gel, and conversely. When using a poor solvent of the polymer, abnormal retentions may occur leading to a wrong interpretation of GPC chromatograms.

Size Exclusion Mechanism. Steric exclusion (or size exclusion) is the main process of polymer fractionation by GPC. This mechanism is based on a thermodynamic equilibrium between two phases: the interstitial solvent in the dead volume V_o and the solvent filling the porous volume V_p. If K_{gpc} is the partition coefficient, the elution volume V_e of a macromolecule is defined by:

$$V_e = V_o + K_{gpc} V_p \tag{5}$$

The evolution of the partition coefficient as a function of chain size and topology on the one hand, and of pore size distributions on the other, has a purely entropic origin, and it is well predicted by the theory of Casassa [29–35]: The partition function for a chain of a given size N trapped in a pore with volume V_p and inpenetrable walls, Z_{pore}, can be obtained by an enumeration of all possible random paths of N steps which start in the pore volume and never cross the wall (e.g. such as the full line and unlike the dotted line in Fig. 4).

The partition function of the same chain in an equivalent element of the dead volume V_o, Z_{free}, is given by the same enumeration without the condition of not crossing a wall: It is larger than the Z_{pore} by an amount representing all conformations such as the dotted line in Fig. 4. Therefore, entering the pore

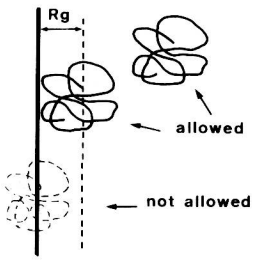

Fig. 4. Representation of different conformations of a randomly coiled polymer near a wall. *Solid line*: authorized conformation; *Dashed line*: forbidden conformation

implies for the chain an excess free energy of order

$$\Delta F = -kT \log(Z_{pore}/Z_{free})$$

By application of the Boltzmann equipartition law, this leads to a depletion of chains in the porous volume by a factor.

$$K_{excl} = \exp(-\Delta F/kT) = Z_{pore}/Z_{free}, \quad 0 < K_{excl} < 1 \tag{6}$$

An equivalent although mathematically less straightforward way of considering size exclusion is to say that the steric repulsion of the wall creates in its vicinity a depletion layer of order R_g (Fig. 5), so that only a fraction $V'_p = K_{excl}\, V_p$ of the pore volume is really available to the chain in the pore (Fig. 6).

The topology in actual gels is more complicated, but gel strands are uncrossable to macromolecular solutes, and they play the role of the "walls" pictured above. The K_{excl} coefficient depends on the macromolecule size and varies from 0 for a very high molecular weight polymer unable to penetrate the gel to 1 for a very small macromolecule capable of entering the total gel volume. Consequently, the elution volume of a macromolecule through a GPC column takes the following form:

$$V_e = V_o + K_{excl} V_p \tag{7}$$

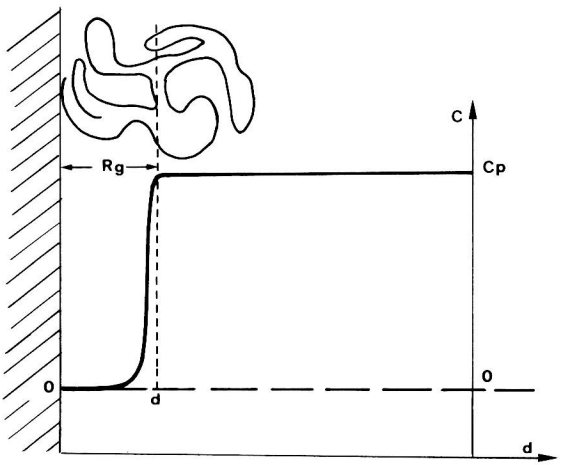

Fig. 5. Concentration profile of a randomly coiled macromolecule near a wall. R_g is the radius of gyration, C_p is the polymer concentration and d is the limiting distance for exclusion

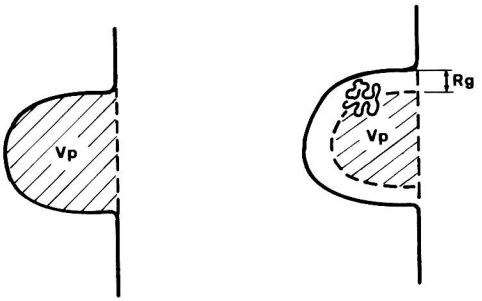

Fig. 6. Representation of the size exclusion of a macromolecule by the wall inside a pore

Accordingly, for a pure GPC experiment only controlled by the size exclusion mechanism, we have $K_{gpc} = K_{excl}$.

It is interesting to remark that, in this mechanism, gels with a monodisperse distribution of pore sizes are capable of performing the fractionation of molecules over a certain range of molecular weights (around two decades for random-coil polymers, around the half for rod-like polymers [21]). In practice, these ranges are greater since GPC packings generally have a substantial pore-size distribution which increases their fractionation capacity (but decreases the maximum resolving power). In general, as this distribution is not very wide for synthesis reasons, it is usual to mix together several gels of different porosities or to assemble several columns with different fractionation ranges in series in order to form a "custom-made column" appropriate for a specific problem. Another interesting approach is the bimodal distribution [36] used by Du Pont [37] with their porous silica microspheres (PSM). These packings have only two kinds of pores of different sizes. Nevertheless, they are characterized by a good linearity of the calibration curve over a wide range of molecular weights.

Another particularity of GPC is that the partition coefficient K_{gpc} only varies from 0 to 1, which means that all the solutes are eluted between two limits V_o and $V_o + V_p$ (volume V_p being experimentally of the same order of magnitude as volume V_o). This restricted volume of fractionation contrasts with other chromatographic methods. Figure 7 schematically represents the variations of elution volume in a GPC column with a single pore size distribution as a function of polymer size.

The mechanism of size exclusion in GPC is only controlled by entropy considerations, contrary to all other mechanisms encountered in chromatography, including the previously described adsorption and liquid-partition mechanisms based upon enthalpy differences. This unique feature makes the generality of size exclusion chromatography. To preserve this generality, however, the polymer analyst must be very careful with adsorption and liquid-liquid partition, since they are very difficult to detect yet nevertheless strongly disturb the GPC experiment. In particular, the stationary and mobile phases must be chosen according to solute properties in order to minimize such effects.