Polymer Bulletin 33, 339–346 (1994)

Polymer Bulletin

Determination of complexation equilibria of macromolecules with small molecules by means of size exclusion chromatography

C.-H. Fischer[1], D. Berek[2,*], and T. Macko[2]

[1]Hahn-Meitner-Institute, D-14109 Berlin, Germany
[2]Polymer Institute of the Slovak Academy of Sciences, SK-842 36 Bratislava, Slovakia

Summary

A new size exclusion chromatographic procedure for determination of complexation of macromolecules with low molar mass substances (such as preferential solvation or drug-protein-binding) was proposed. The method is based on the assessment of system peaks formed as result of differences between eluent composition and bulk solvent composition due to the complexation within the initial sample solution. The composition of the eluent, which contains the same components as the sample solvent, is adjusted in such a way that the system peak disappears. Under these circumstances the bulk solvent composition equals to the eluent composition and the former can be easily calculated. This procedure was tested by measuring the preferential solvation in a model system polystyrene plus toluene plus methanol and by comparing the result with the data obtained with two other chromatographic methods.

Introduction

Complexations of macromolecules with small molecules play an important role in many areas including living organisms. Numerous methods were developed for assessment of complexations of this type and the choice often depends on the stability of complexes formed.

The most difficult task presents the quantitative determination of parameters of complexations where does exist a dynamic equilibrium between complexes and their non complexed constituents. Typically, it is the temporary "binding" of various low molecular ligands to dissolved macromolecules and the preferential solvation of macromolecules in two-or multicomponent solvents. In both cases small molecules compete to occupy the appropriate sites on polymer chains and the difference between both processes is more or less formal only. Customarily, we do speak about the ligand binding in the case when the small molecules preferentially interacting with macromolecules are present at relatively low concentration, usually less than a few percent. On the other hand, the concentrations of components of mixed solvents that preferentially solvate macromolecules are as rule much higher.

Chromatographic methods present a powerful tool for studying complexation phenomena (1). They include frontal analysis, equilibrated column scanning and, the most important elution methods. In the following we shall discuss the particular elution methods more in detail.

*Corresponding author

Description of Methods

a) Direct Analysis Method

In 1962 Hummel and Dreyer (2) proposed an original method for determination of the drug binding to proteins. The method is based on the size exclusion chromatographic (SEC) - gel permeation chromatographic separation of the both noncomplexed protein molecules and complexes formed by proteins and drug molecules on the one hand from the free drug on the other hand. The experiments are done in the differential arrangement i.e. the solution of the drug in water is used as SEC eluent and the protein dissolved in eluent is injected into an appropriate SEC column. The protein consumes drug from the solvent for complexation. The protein molecules and the complexes of protein with drug are larger than the drug molecules and leave SEC column earlier than the bulk solvent which initially surrounded the dissolved protein molecules and from which drug was extracted by the protein. Using a detector which monitors the concentration of the drug, one observes two peaks on the resulting chromatogram: A positive peak at low retention volume which belongs to protein and protein/drug complexes and in which the concentration of drug exceeds that one in the eluent as well as a negative peak ("trough") which is caused by the deficiency of drug in the bulk solvent. The chromatographic peaks produced just by the local change of eluent composition are often called system peaks and we shall adopt this term in the present paper, as well. The amount of drug consumed by protein molecules can be calculated from the size of system peak after appropriate calibration i.e. after consecutive injections of a series of drug solutions with known concentration under identical conditions as the samples studied except they do not contain proteins. We shall call this procedure the Direct Analysis Method (DAM).

A similar approach was independently applied to the determination of preferential solvation of synthetic macromolecules in two-component solvents (3). In this case the two-component solvent for polymer is used as SEC eluent. The non-specific or eluent-specific detector sees the system peak because of the local difference between the composition of bulk solvent and eluent i.e. the initial sample solvent. After appropriate calibration the bulk solvent composition is determined from the size of system peak and the extent of preferential solvation can be calculated.

The described approach was used for the study of numerous systems protein plus drug (1, 4-11) or polymer plus two-component solvent (12-19). In the SEC of stabilized inorganic sols negative system peaks were also observed (20). This may offer an opportunity for determination of the stabilizer amount which is bound to the surface of colloid particles.

Evidently, the DAM method described can be used for assessment of various kinds of complexation of macromolecules and nanoparticles with small molecules including both very weak solvates of macromolecules with solvent molecules and much more stable associates of macromolecules and particles with small ligand molecules.

After their separation from the bulk solvent the complexes are, however, surrounded by eluent i.e. by initial solvent. The complexation equilibrium is perturbed and further portions of small molecules may be additionally extracted into complexes. As result the calculated data on polymer complexation may be incorrect. The reestablishment of complexation equilibrium would be manifested by the appearance of distorted system peak with the "fronting" shape.

b) Zero System Peak Methods

Sébille et al (21) proposed an interesting approach to SEC measurement of drug-protein binding. They used a ternary system protein plus drug plus solvent as SEC eluent and injected just solvent (e.g. a buffer) and series of drug solutions with different concentrations. Two peaks were observed also in this case indicating deficiency of both protein and drug in the injected solution. At a certain concentration of drug in the injected solution the system peak of the drug disappeared. This was the situation when the concentration of drug in the injected solution equalled with the concentration of free drug within the saturated column. One can speak about a "zero system peak" method. Sébille et al called this vacancy chromatographic procedure "saturation method".
In the Sébille arrangement the system is near to an equilibrium, however, the polymer consumption is rather high and the SEC eluent is too viscous to get fast and highly efficient separation.
Two alternative approaches to chromatographic assessments of complexation of macromolecules with small molecules can also be called "zero system peak methods":

i. Bulk Solvent Adjustment Method (SAM)

The procedure is similar to DAM but the composition of bulk solvent in a series of injected polymer solutions is adjusted by small additions of ligand or that solvent component which was consumed by macromolecules. When the composition of bulk solvent approaches the eluent composition the size of corresponding system peak decreases and, eventually, system peak vanishes or even changes its sign. The composition of the initial bulk solvent is calculated from the amount of added solvent component or ligand which makes the system peak disappear (22,23) (bulk Solvent Adjustment Method - SAM). SAM represents a non-equilibrium approach: the delicate dynamic equilibrium between polymer complexes and bulk solvent in the injected solution is perturbed by the added bulk solvent component or ligand: The extent of complexation can be changed e.g. further solvent or ligand molecules can be consumed by macromolecules due to changed bulk solvent composition. In other words, when system peak vanishes we do have an equilibrium between polymer complexes in the injected solution and eluent, which may be different from the equilibrium between complexes and initial bulk solvent. In fact, the situation in the equilibrated SAM system corresponds to the situation at the column outlet in the case of the direct analysis method.

ii. Eluent Adjustment Method (EAM)

A series of solutions with different polymer concentrations in studied mixed solvent is successively injected into appropriate LC column flushed with eluents of different compositions. Eluent composition is changed in such a way that the system peak successively becomes smaller and eventually vanishes or changes its sign. The composition of bulk solvent is directly calculated from interpolated or extrapolated eluent composition at which system peak disappears. We shall designate this original procedure Eluent Adjustment Method (EAM). EAM is almost an equilibrium method since if we neglect the effects of dilution of sample solution during its passage along the column we can consider macromolecules permanently in contact with their initial bulk solvent.
In this work we tested the eluent adjustment method and compared it with the direct analysis method and with the bulk solvent adjustment method.

Preferential solvation of polystyrene molecules in mixed solvent toluene plus methanol was determined to evaluate the experimental feasibility and the reliability of data afforded by the above three methods.

Experimental

High performance SEC apparatus consisted of the Merck Hitachi Model L-6000 pumping system, *(Hitachi, Tokyo, Japan)*, electrically driven three-way six-port injection valve provided with a 10 μl loop *(Knauer, Berlin, Germany)*, 250x4 mm column packed with LiChrospher 60, 10 μm particle size, 6 nm pore diameter, spherical, bare silica gel *(Merck, Darmstadt, Germany)* and of the refractive index detector Model RI 71 *(Merck, Darmstadt, Germany)*.
Flow rate 1 $cm^3 min^{-1}$ was controlled with a flowmeter *(Phase Separations, Queensferry Clwyd, UK)*. Working pressure was about 12 MPa. Column and injection valve were thermostated in a custom made air box at temperature 25 + 1 °C. Data were collected and processed by the Chromstar data system *(Bruker, Bremen, Germany)*. System peak areas from at least two independent injections were considered.
Both methanol and toluene of analytical grade *(Merck, Darmstadt, Germany)* were used without further purification. Narrow polystyrene (PS) with molar mass 4000 $g.mol^{-1}$ was from Pressure Co., Pittsburgh, Pennsylvania, USA. All mixtures and solutions i.e. mixed solvent for polymer, polymer solutions, mixed eluents, as well as calibration mixtures of toluene and methanol were prepared on the weight basis.
Precautions were taken to minimalize preferential evaporation and moisture absorption effects. Polymer solutions were prepared either in the 5 cm^3 gas tight syringes *(Hamilton, Bonaduz, Switzerland)* - methods DAM and EAM - or in 50 cm^3 glass vials provided with septum and magnetic stirrer - SAM method.

Results and discussion

A series of polymer solutions with concentrations in the range from 2.65 to 10.85 mg per one gram of solvent was prepared by dissolving polystyrene in the mixed solvent toluene plus methanol containing 79.33 weight % of toluene. These solutions were successively injected into the column flushed with the eluent of the same composition. *(Direct analysis method.)*. The resulting system peaks increased with increasing polymer concentration. Examples of chromatograms are shown in Fig. 1.
The calibration dependence for the DAM method i.e. the dependence of system peak area on the composition of injected binary mixtures toluene plus methanol is shown in Fig. 2. The calibrating mixtures of toluene plus methanol were prepared in glass vials containing 20 cm^3 of eluent which were provided with rubber septum.
In the bulk solvent adjustment method, small amounts of toluene were successively added into a series of solutions of polystyrene in eluent containing from 1.74 to 6.00 mg of polymer per one gram of solvent. After each addition of toluene the solution was injected into column. Some chromatograms obtained are shown in Fig. 3. The negative system peak first decreased in size and later changed its sign. Small changes in the overall polymer concentration caused by toluene additions cannot be seen by the refractometric detector.
In the eluent adjustment method, polystyrene solutions in the starting eluent containing 79.33 wt. % of toluene were successively injected into the column flushed with the eluents containing 78.9; 79.1; 79.25; 79.33 and 79.5 wt. % of toluene, resp. The resulting chromatograms for one

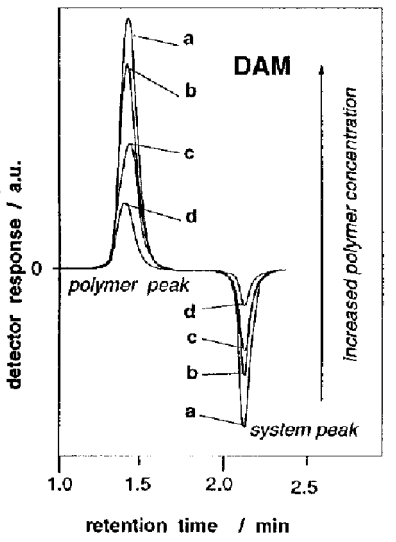

Fig. 1 Typical chromatograms obtained with the direct a analysis method (DAM). Polystyrene concentrations [mg/g solvent]: a) 10.85; b) 8.43; c) 5.50; d) 2.65. Solvent and eluent: 79.33 % toluene, 20.67 % methanol.

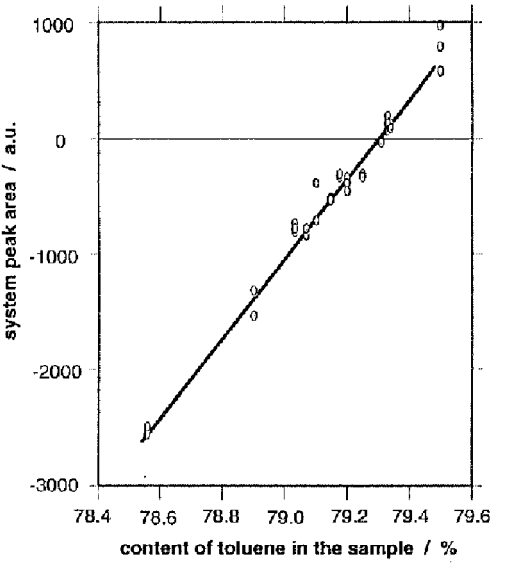

Fig. 2 Calibration dependence: system peak area vs. composition of mixed solvent toluene plus methanol injected. No polymer was present.

polystyrene solution in five eluents are shown in Fig. 4.
One can see that system peaks obtained by all three methods were rather symmetrical. This means that the effect of preferential solvation equilibrium reestablishment during passage of sample along the column was not important in the direct analysis method, at least for the present system where the extent of preferential solvation was not too high.
Commonly, the extent of preferential solvation of macromolecules in mixed solvents is expressed in terms of the coefficient of preferential solvation, λ . The coefficient λ gives the excess of one solvent component in the domain of polymer molecules in comparison with the starting solvent composition and it is conventionally expressed in millilitres of the solvent component preferentially solvating macromolecules per one gram of dry polymer.
We calculated λ parameters from the slopes of constructed dependences: composition of bulk solvent vs. injected polymer concentration shown in Fig. 5. The particular procedures DAM, SAM and EAM gave the λ values 0.43, 0.44 and 0.42, resp which represents an excellent agreement. For comparison, λ values for polystyrenes with similar molar masses measured by various methods in mixtures benzene plus methanol are given in Table I. They are well comparable with our data. This is somewhat surprising since polystyrene molecules used in this study were not excluded from the pores of column packings. In other words, macromolecules could displace molecules of solvent component(s) adsorbed on the large inner packing surface (3) and thus influence the system peak size and, consequently, the resulting λ values. Probably the displacement effects are negligible in the case of nonpolar macromolecules of polystyrene.

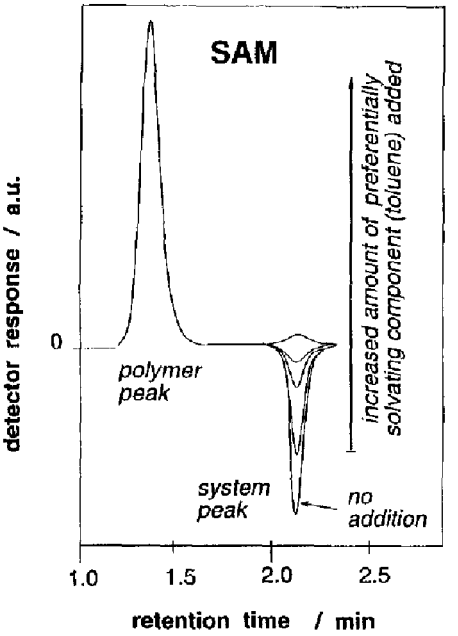

Fig. 3 Typical chromatograms obtained with the bulk solvent adjustment method (SAM). 6.0 mg polystyrene /g solvent. Additions of toluene in wt. %: a) 0.0458; b) 0.1853; c) 0.2349; d) 0.2852

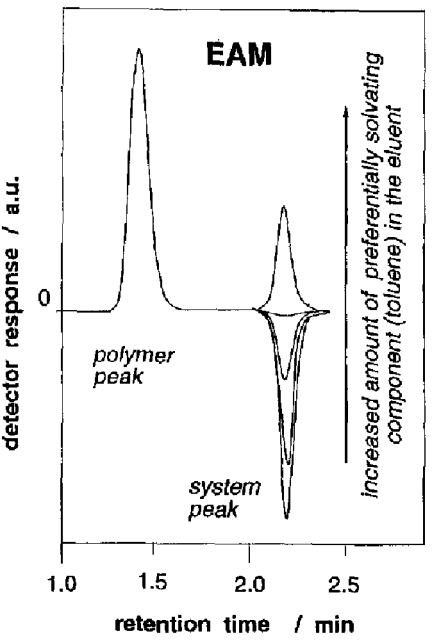

Fig. 4 Typical chromatograms obtained with the eluent adjustment method (EAM). 5.5 mg polystyrene/g solvent (79.33 % toluene, 20.67 % methanol). Percentage of toluene in the eluent: 78.9; 79.1; 79.25; 79.33; 79.5.

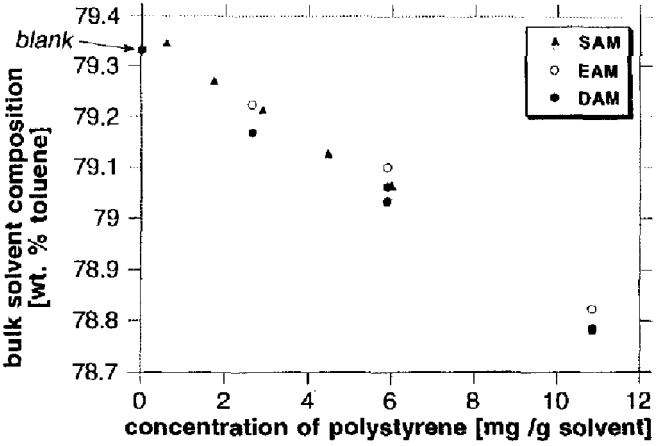

Fig. 5 Bulk solvent composition determined by the three different chromatographic methods as function of polymer concentration. Coefficients of preferential solvation λ are derived from the slopes of particular straight lines.

Table I The λ values for low molecular mass polystyrenes measured by various methods in mixtures of benzene plus methanol (a) or toluene plus methanol (b) of similar composition

Molar mass of PS [g.mol^{-1}]	*Composition of mixed solvent (volume fraction of methanol before mixing)*	λ [ml.g^{-1}]	*Method*	*Literature*
4 500	0.160 (a)	0.42	SEC-DAM	14
	0.100 (a)	0.15	light	
4 500	0.222 (a)	0.56	scattering	24
	0.255 (a)	0.69		
	0.190 (a)	0.41	viscosity	
4 800	0.225 (a)	0.56	& light	
	0.255 (a)	0.69	scattering	25
6 500	0.200 (a)	0.45	dialysis	
	0.300 (a)	0.80		26
	0.223 (b)	0.43	SEC-DAM	
4 000	0.223 (b)	0.44	SEC-SAM	this
	0.223 (b)	0.42	SEC-EAM	paper

Conclusions

Values of coefficients of preferential solvation λ determined by the direct analysis method, the bulk solvent adjustment method, and the eluent adjustment method agree very well. They are also comparable to the λ values found for polystyrene-benzene-methanol system of similar composition by means of conventional methods - light scattering, dialysis and viscometry. We can conclude that all three SEC methods are equivalent and the perturbations of complexation equilibrium do not play any important role - at least for low polymer concentrations, for relatively high overall concentration of the preferentially solvating solvent component, and for the systems where the extent of complexation (solvation) is not extremely high. Similarly, the perturbation of sorption equilibrium in the system bare silica gel plus toluene plus methanol by the non excluded polystyrene macromolecules is not evident.

A set of dilute polymer solutions of different concentrations is needed for all three methods. The direct analysis method is most convenient from the experimental point of view. Theoretically, it needs only one injection per sample solution. The bulk solvent adjustment method is experimentally rather demanding, especially if one has to cope with the problem of preferential evaporation of one solvent component. In order to relatively decrease the effect of preferential evaporation, larger volumes of polymer solutions must be treated. This increases polymer consumption. Eluent adjustment method is experimentally feasible when using an appropriate HPLC equipment including a solid, non swelling column packing. Since it is "an almost equilibrium method" it will give precise results also in the systems exhibiting high extent of complexation which might be important especially in some biological systems. This method does not need any calibration.

References

1 Wood G C, Cooper P F (1970) Chromatogr Rev 12:88
2 Hummel J P, Dreyer W J (1962) Biochim Biophys Acta 63:530
3 Berek D, Bleha T, Pevná Z (1976) J Polym Sci Polym Lett Ed 12:323
4 Colmon R E (1972) Anal Biochem 46:358
5 Andreu J M (1985) Methods in enzymology 117:346
6 Šoltés L, Brée F, Sébille B, Tillement J P, Ďurišová M, Trnovec T (1985) Biochem Pharmacol 34:4331
7 Sébille B, Thuaud N, Piquion J, Beher N (1987) J Chromatogr 409:61
8 Motorin Yu A (1987) J Chromatogr 404:254
9 Zaton A, Martinez A, de Gandaries J M (1987) J Liq Chromatogr 10:899
10 Oravcová J, Mlynárik V, Bystrický S, Šoltés L, Szalay P, Boháčik Ľ, Trnovec T (1991) Chirality 3:412
11 Šoltés L, Sébille B (1994) J Liq Chromatogr 17: in press
12 Chaufer B, Lesec J, Quivoron C (1977) C R Acad Sci Paris C 284:881 and 2:633
13 Campos A, Borque L, Figueruelo J E (1977) J Chromatogr 140:219
14 Soria V, Campos A, Figueruelo J E (1978) Ann Quím 74:1026
15 Katime I, Campos A, Rivera J M T (1979) Eur Polym J 15:291
16 Strazielle C (1979) Makromol Chem 180:1375
17 Basedow A M, Ebert K M, Emmert J (1979) Makromol Chem 180:1359
18 Straub P G, Brant D A (1980) Biopolymers 19:639
19 Cesteros C, Katime I, Strazielle C (1983) Makromol Chem Rapid Commun 4:193
20 Fischer Ch-H, Henglein A (1983) J Phys Chem 93:5578
21 Sébille B, Thuaud N, Tillement J P (1979) J Chromatogr 180:103
22 Ratica J, MSc Thesis, Polymer Institute of the Slovak Academy of Sciences (1983)
23 Kristol L, Grushka E (1989) 13th Int Symp Column Liquid Chromatography, Stockholm Th-L-008
24 Dondos A, Benoit H (1970) Makromol Chem 133:119
25 Dondos A, Benoit H (1977) J Polym Sci Polym Lett Ed 15:137
26 Hert M, Strazielle C (1976) J Polym Mater 4:195

Accepted June 12, 1994 St