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Simulation of size exclusion chromatography for characterization of supramolecular complex: a theoretical study

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Abstract

A simple chromatographic model was used to simulate the retention behavior of supramolecular complex in size exclusion chromatography (SEC). Two fundamental model complexes, directional complementary couple (AB) and self-complementary unit (AA), were employed to investigate the influence of various parameters in the SEC characterization/confirmation of supramolecular association. Peak tailing, peak fronting, peak splitting and retention time shifting were observed under different conditions. It was found that the chromatographic peak shape and retention time were strongly affected by the association constant, sample concentration, as well as the addition of a reactant in the mobile phase. Furthermore, using the same model, the chromatographic process of Hummel–Dreyer procedure was simulated, and the results indicated that the procedure can be a good method for the determination of association constants for AB type complexes.

Keywords: Simulation; Supramolecular complex; Retention behavior; Association constant; Size exclusion chromatography

1. Introduction

Supramolecular chemistry is a rapidly developing new branch of chemistry which is defined as the chemistry of molecular association and complex held together by non-covalent interactions. The most important feature in the supramolecular system is that components are linked together reversibly by intermolecular forces, not by covalent bonds [1–4]. True supramolecular compounds are reversible aggregates that can break and recombine on experimental time scales. Regardless of many impressive progresses in the synthesis of supramolecular compounds, their characterization still remains a task which is not easy to handle [2].

For over 40 years, size exclusion chromatography (SEC) has been used as an essential tool for the estimation of molecular weights of various samples. The technique separates molecules on the basis of their hydrodynamic volume or size, with the biggest elute first. Stable covalently linked macromolecular samples are routinely characterized by SEC [5–7]. In the analysis of non-covalent complexes, on the other hand, the applicability of SEC is strongly dependent upon the stability of the sample. For complexes which are stable under the chromatographic conditions, analysis can be carried out by standard SEC. Successful examples include the analysis of some stable metal complexes and protein complexes [8,9]. However, for complexes which are less stable, normal SEC procedures are not adequate. These complexes will dissociate during the chromatographic process, and thus shifting the retention time and altering the measured molecular weights. This type of chromatographic retention behavior could significantly hinder the direct confirmation of non-covalent assembly and its molecular weight determination. Unfortunately, on these aspects, the influence of complex dissociation still has not been fully investigated yet.

Despite of the lack of fundamental investigations, interesting applications of SEC in the characterization and/or confirmation of supramolecular assembly have already been published [10–12]. In these applications, SEC provided strong evidence for the formation of reversible assembly. It was also demonstrated that a stable complex gave a sharp and well-defined chromatographic peak, while a less stable one gave a broadened and tailing peak which was supposed to be due to the complex dissociation on the SEC column. No efforts were made in these articles to further correlate

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the SEC retention behavior with the stability of the complex and with the related chromatographic parameters.

In addition to molecular weight measurement, SEC has also been used for the determination of association constants. Actually, the change of retention time and peak shape of non-covalent complex has been used to study the interactions of dissociable aggregates, such as protein–protein, antigen–antibody and protein–drug interactions [13–16]. For the association constant determination, one of the most successful methods is the Hummel–Dreyer procedure that has been employed for quantitating the binding of a small ligand molecule (X) to a macromolecule (M) [17]. In this procedure, a solution of X is used as the mobile phase. M dissolved in the same solution is applied to the column. From the peak and trough (negative peak) areas, the concentrations of MX and M in equilibration with X can be calculated, and thus the association constant. An implicit assumption of the procedure is that the macromolecule and macromolecule–ligand complex should migrate at the same speed [18]. However, this requirement normally cannot be fulfilled with many different types of supramolecular complexes, and there are debates on whether the method can still be applied under these circumstances [19,20]. To the best of our knowledge, no consensus has yet been reached. Therefore, in order to make full use of the procedure, further theoretical studies are needed.

The aim of this contribution is to improve the understanding of what kind of parameters and how these parameters could affect the chromatographic behavior of supramolecular compounds in SEC. The SEC processes of two model complexes, AA and AB, were simulated. The effects of various parameters, including the association constant, the sample concentration, and the addition of a reactant in the mobile phase, were investigated to see how the dissociation of a complex could influence the chromatographic retention time and peak shape. Finally, the possibility of using the Hummel–Dreyer SEC procedure for the determination of association constants of AB complex was discussed.

2. Theory

Retention in SEC is normally described by the distribution function, K_{SEC}, which is expressed by:

$$V_E = V_0 + K_{SEC} V_p \tag{1}$$

where V_E is the elution volume of the solute, V_0 the column interstitial volume (mainly the liquid volume between particles) and V_p the pore volume of the column packing [5]. K_{SEC} is constrained to values between 0 and 1, representing the extremes of complete exclusion and permeability of the pore volume by the solute. However, the K_{SEC} value is not directly related with the ratio of time spent by the solute in and out the stationary phase (inside and outside the pore of the column packing), and is inconvenient to be used in the simulation. In the following sections, therefore, K_{SEC} is transformed into k, which is equivalent to the capacity factor in chromatography and is expressed directly as the ratio of time spent by the solute in and out the stationary phase:

$$k = \frac{V_E - V_0}{V_0} = K_{SEC}\frac{V_p}{V_0} \tag{2}$$

When a compound is introduced onto the SEC column, it will migrate at a speed depending on its hydrodynamic volume. A Craig-type apparatus can be used to simulate the SEC process [21]. In this work, it will be assumed that the volumes of the stationary phase cells and the mobile phase cells are equal.

For a supramolecular complex sample, it might dissociate into its reactants during its migration along the SEC column. Therefore, instead of observing only one complex peak, there might be several peaks corresponding to the complex and its reactants. Retention time of the sample components will not only depend on their hydrodynamic volumes, but also on the association constant and presence of the other components. In our study, it is assumed that equilibration can quickly be established for the complexes. Hence, for AB type, we have:

$$A + B \leftrightarrow AB \tag{3}$$

$$K_{AB} = \frac{[AB]}{[A][B]} \tag{4}$$

The k-value of each individual component can be expressed by

$$k_A = \frac{[A]_{stationary}}{[A]_{mobile}} \tag{5}$$

$$k_B = \frac{[B]_{stationary}}{[B]_{mobile}} \tag{6}$$

$$k_{AB} = \frac{[AB]_{stationary}}{[AB]_{mobile}} \tag{7}$$

In the Craig distribution model, the sample is introduced in the first cell, and after equilibration the mobile phase is then moved into the second cell, fresh mobile phase is added to the first cell, and the process repeated. With the continuous shifting of the mobile phase against the stationary phase, sample components are moved along the column and separated chromatographically. For the supramolecular system, the mass conservation for equilibration (3) should be satisfied at any point in the development of the chromatogram:

$$\Delta A = \Delta B = -\Delta AB \tag{8}$$

More specifically, for any given ith cell after a certain number of jth shifts, we have:

$$\Delta A_i = [A]_{stationary,i,j} + [A]_{mobile,i,j} - \{[A]_{stationary,i,j-1} + [A]_{mobile,i-1,j-1}\} \tag{9}$$

$$\Delta B_i = [B]_{stationary,i,j} + [B]_{mobile,i,j} - \{[B]_{stationary,i,j-1} + [B]_{mobile,i-1,j-1}\} \tag{10}$$

$$\Delta AB_i = [AB]_{\text{stationary},i,j} + [AB]_{\text{mobile},i,j} - \{[AB]_{\text{stationary},i,j-1} + [AB]_{\text{mobile},i-1,j-1}\} \quad (11)$$

where $[\,]_{\text{stationary},i,j}$ and $[\,]_{\text{mobile},i,j}$ represent, respectively, the concentrations in the stationary phase and mobile phase in the *i*th cell after *j*th shift, and $[\,]_{\text{stationary},i,j-1}$ and $[\,]_{\text{mobile},i-1,j-1}$ represent, respectively, the concentration in stationary phase of *i*th cell and the concentration in the mobile phase of the $(i-1)$th cell after $(j-1)$th shift.

For any given chromatographic cell after a new shift, there are six unknown concentration values, i.e. the concentrations of A, B and AB in the mobile phase and stationary phase, respectively. Combining Eqs. (4)–(11) yields six independent equations for any cell, and thus the unknowns can be calculated.

Similarly for AA, we have:

$$2A \leftrightarrow AA \quad (12)$$

$$K_{AA} = \frac{[AA]}{[A]^2} \quad (13)$$

$$k_A = \frac{[A]_{\text{stationary}}}{[A]_{\text{mobile}}} \quad (14)$$

$$k_{AA} = \frac{[AA]_{\text{stationary}}}{[AA]_{\text{mobile}}} \quad (15)$$

$$\tfrac{1}{2}\Delta A = -\Delta AA \quad (16)$$

$$\Delta A_i = [A]_{\text{stationary},i,j} + [A]_{\text{mobile},i,j} - \{[A]_{\text{stationary},i,j-1} + [A]_{\text{mobile},i-1,j-1}\} \quad (17)$$

$$\Delta AA_i = [AA]_{\text{stationary},i,j} + [AA]_{\text{mobile},i,j} - \{[AA]_{\text{stationary},i,j-1} + [AA]_{\text{mobile},i-1,j-1}\} \quad (18)$$

By combining these equations, the component concentrations in any cell after a given number of shifts can also be calculated (four unknown concentrations with four independent equations).

In developing the equations given earlier, it is assumed that the equilibration of complex association/dissociation can quickly be established. Such an assumption can only be true for some certain occasions. If this is not the case, then the supramolecular complex will not be dissociated sufficiently to reach its local equilibrium concentration before moving into the next cell, which indicates that less complex will be dissociated. In an utmost case where the kinetics is extremely slow, no considerable association/dissociation would occur in the chromatographic time scale. The complex and its reactant(s) would move like independent compounds, and sharp and symmetric peaks could be expected at retention times according to their corresponding hydrodynamic volumes. Obviously, a real complex system is between the two ends of extremely fast and slow kinetics. For a given complex, the model simulation based on quick equilibration can thus provide useful information for the prediction of its chromatographic retention behavior. Furthermore, by using these model equations, the effects of complex association/dissociation can be studied theoretically. Another assumption in these equations is that separation is based on ideal SEC mechanism. This might be contentious especially when the molecules are not associated together. Fortunately, for many supramolecular systems, the assumption should be reasonably true because the complexes are usually designed based on specific non-covalent interactions [1–4]. Even when the molecules are not associated together, their interactions with the mobile phase or with the stationary phase might still be negligible if suitable chromatographic conditions are employed.

3. Simulation

Simulations were based on the Craig-type apparatus [21] and carried out with a program written in C language. Chromatograms were developed by shifting the mobile phase against the stationary phase. At the beginning, the sample was introduced into the first cell of the mobile phase. Then, a shift was made and the contents of the two opposing cells were equilibrated. The concentrations of components in the corresponding cells were calculated according to the equations derived in Section 2. The process was continued until the required number of shifts had been performed.

The Craig-type apparatus employs a simple chromatographic model that does not depend on a specific retention mechanism. In order to obtain a chromatogram that is developed against time, a pseudo-detector was attached to the end of the column and its response to different components was recorded with the shifting of mobile phase. The SEC column was arbitrarily supposed to have 5000 stationary phase cells and the mobile phase was shifted at a speed of 40 cells/s. Chromatographic peaks were then plotted according to the variation of mole concentration of the individual components in the detector.

4. Results and discussion

In SEC analysis of supramolecular complexes, a very important issue that must be considered is their possible dissociation. This is in clear contrast with the traditional SEC application for compounds that are formed via covalent bonds and stable enough under chromatographic conditions. For a complex, the equilibrium between association and dissociation is a dynamic process which is controlled by many parameters. When the complex is applied to SEC separation, the situation becomes even more sophisticated because of the repeated distribution/separation of the components between the stationary and the mobile phase along the column. Therefore, it can be envisaged that the SEC results will depend not only on the complex properties (mainly the association constant and concentration), but also on the chromatographic parameters, such as the column type and length,

the mobile phase composition, etc. In the following sections, SEC processes of two model complex systems, AB and AA, were simulated to study the effects of various parameters.

4.1. Effects of association constant

The association constant (K) is determined by the strength of supramolecular binding. It describes the relationship of equilibrium concentrations of the reactants and the complex. A high K-value reflects a strong tendency for association. Using the simulation program, an important advantage is that the movement of every individual sample component can be studied. Figs. 1 and 2 illustrate how the association constant affects retention time and peak shape of the individual components in AB and AA types of complexes, respectively. For complex AB, equal amount of A and B were mixed together prior to SEC. In order to produce a frame of reference, corresponding compounds (A_0, B_0, AB_0 and AA_0) that are supposed to be stable enough and will not associate/dissociate during the SEC separation, are also shown in the figures for comparison. Obviously, retention of the supposed stable compounds only depends on their corresponding hydrodynamic volumes. Simulations were carried out at many different combinations of retention factors and association constants, and the chromatograms shown in Figs. 1 and 2 are some typical results.

4.1.1. AB type complex

It can be seen clearly from Fig. 1 that, for the AB type complex, the chromatographic behavior of the system is strongly influenced by the association constant. At low K-values, no complex peak but only two single peaks corresponding to pure A and B were observed. This is because at such low K-values, significant amount of complex was broken down into A and B. With the repeated chromatographic distribution, A and B were continuously separated and the separated components could not meet again to form AB in the column. The chromatographic process further shifted the complex equilibration to the direction of dissociation. Eventually, after sufficient number of chromatographic steps, no AB was left in the column.

With the increase of K-values, the complex peak gradually became visible and its retention time moved closer to that of AB_0. However, for K-values below $10^6\,M^{-1}$, no Gaussian peaks were observed for A, B and AB at the retention times according to their respective hydrodynamic volumes. Instead, peculiar peak shapes (fronting, tailing and split peaks) were developed and significant amount of complex was broken down into A and B. The overall retention times of A and B were shifted towards higher masses. Under the given conditions and for K over $10^4\,M^{-1}$, association should strongly be favored over dissociation in the initial complex system prior to SEC. The peculiar peak shapes and the breakdown of complex must be the combined results of the reversible supramolecular binding and the chromatographic process. Unlike the situation discussed

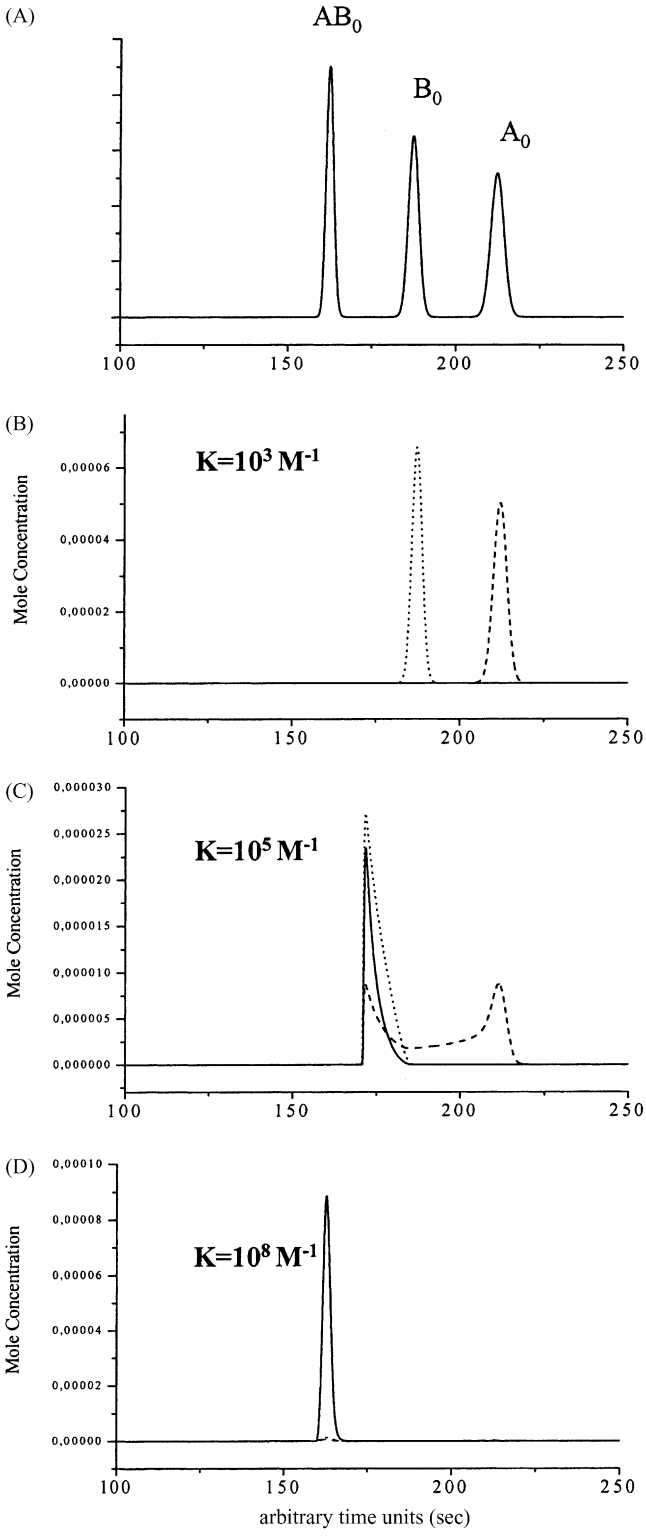

Fig. 1. Effects of association constant on the chromatographic behavior of AB complex. The chromatogram was simulated by plotting the variation of component concentration at the end of a column with 5000 stationary phase cells; the mobile phase shifted against the stationary phase at a speed of 40 times/s. $k_A = 0.7$, $k_B = 0.5$, $k_{AB} = 0.3$; initial concentrations: $[A]_{0,total} = [B]_{0,total} = [AB]_{0,total} = 10^{-2}\,M$. In the chromatogram, AB complex in solid line, A in dashed line, and B in dotted line.

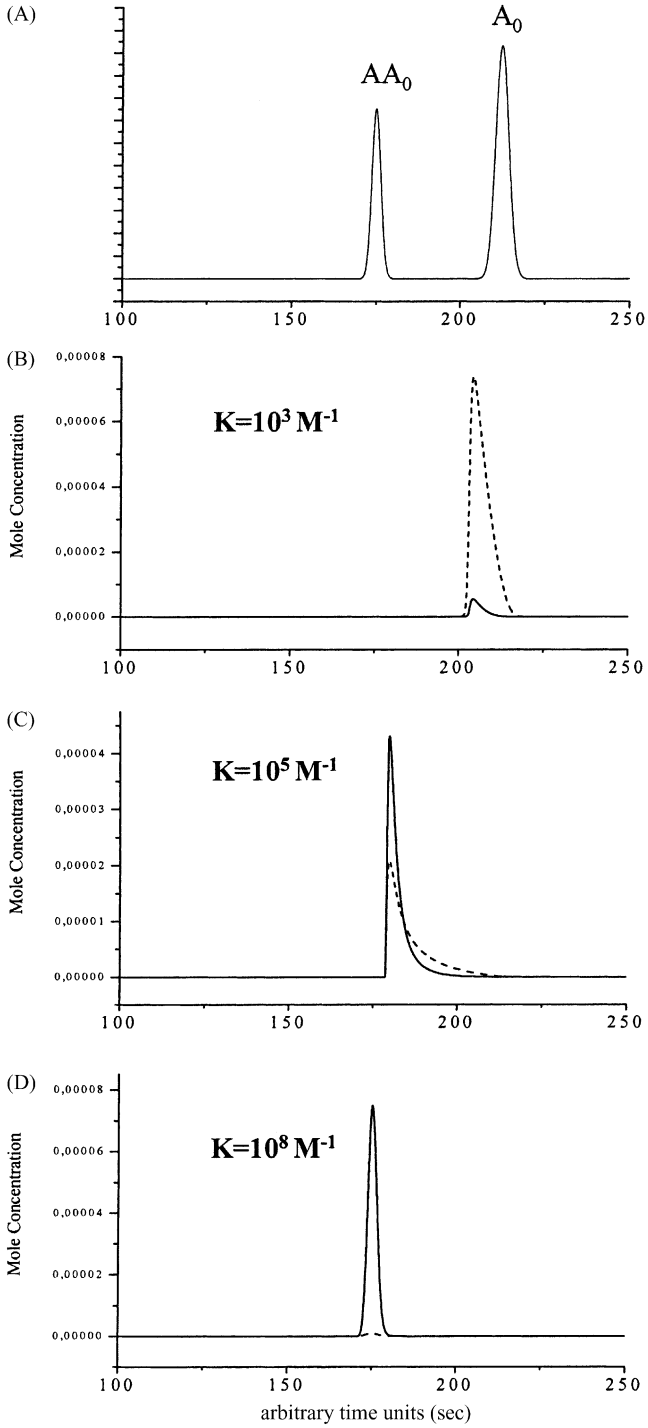

Fig. 2. Effects of association constant on the chromatographic behavior of AA complex. $k_A = 0.7$, $k_{AA} = 0.4$; initial concentrations: $[A]_{0,total}/2 = [AA]_{0,total} = 10^{-2}$ M. In the chromatogram, AA complex in solid line and A in dashed line. Other details as in Fig. 1.

above with very low *K*-values where the complex quickly broke down into A and B in the SEC column yielding two single symmetric peaks, here the complex survived the repeated chromatographic process. In this circumstance, one component could not move freely without the interference of other components. The retention of one component was not only controlled by its own hydrodynamic volume, but also by the presence of the other complex components.

When the *K*-values reached above 10^7 M^{-1}, a sharp peak of AB was observed, and its retention time was very close to that of AB_0. Moreover, no substantial amount of complex was broken down into A and B. At these conditions, the binding was probably so strong that dissociation was negligible. The complex moved along the SEC column like a stable covalently bonded compound. No considerable change in the chromatogram was observed by further increasing the *K*-values.

4.1.2. AA type complex

The binding of AA complex is self-complementary. Therefore, A and AA are always coexisting and in equilibration in the column. This is in clear contrast with the AB system where the separated A and B cannot meet again in the column to form AB.

Fig. 2 shows the effects of association constant on retention and peak shape in the AA system. It is interesting to see that the peak shapes of AA and A are very much similar with exactly the same retention time at their peak top. This is obviously because of the self-complementary binding. Therefore, instead of two peaks corresponding to AA and A, only one peak (sum of A and AA) could be recorded for this complex system in a real SEC chromatogram. Since it was assumed that A and AA was in quick equilibration, one might also expect that the retention time of the peak should simply be the average retention time of the sample components multiplied with their corresponding percentages at the initial conditions, However, the real case was much more complicated because equilibration was continuously shifting during the chromatographic process. At low *K*-values, an almost symmetric peak which contained mostly of A and a small amount of AA was observed. Its retention time was very close to that of A_0. With the increase of *K*, the retention time shifted towards AA_0, the percentage of AA increased, and the peaks became tailing. The reason for tailing is that the front of the peak (that contains more AA) moved faster than the back (that contains more A). When *K* reached above 10^7 M^{-1}, a symmetric peak that was very close to the AA_0 peak was the result. In this case, AA also moved like a stable covalently bonded compound.

From the discussions above, the effects of association constant can briefly be summarized as the following. At low *K*-values, only (or mainly) the reactant peak(s) can be observed, which means that SEC is not a suitable method for these complexes. With the increase of *K*, peculiar peaks (fronting, tailing and splitting) for the AB complex system and tailing peaks for the AA system will be developed, and retention time be shifted from the reactant(s) towards the complex. Although, in this case, SEC cannot give correct molecular weight values, the results (retention time and peak shape) can still be used as a strong evidence of supramolecular binding. When *K* reaches a certain value at a given condition, the complex will move like a stable covalently bonded

compound and a symmetric peak can be recorded. Under this circumstance, SEC can provide conclusive evidence for the supramolecular association and even be used for the determination of molecular weight. Although the conclusions derived here were based on two simple model complexes (AB and AA), some of them might also be applicable to the SEC of more complicated supramolecular samples.

4.2. Effects of concentration

The effects of concentration include two possible aspects. Firstly, supramolecular coupling is dependent upon sample concentration together with the association constant. A high concentration will obviously favor the formation of complex. Secondly, the hydrodynamic volume of a sample component might also be influenced by concentration. In this study, however, this effect is neglected. This is because the molecular weights of the model complexes are normally below 10^4 Da and the problem concerning the concentration dependence of hydrodynamic volume can be ignored [6]. Regarding the concentration, it should also be noted here that in our simulation the compounds were introduced only into the first mobile phase cell. However, in a practical SEC analysis (e.g. on a 75 mm × 300 mm column having more than 10,000 plates and total volume of $V_0 + V_p$ about 10 ml), for example, typically 20 μl sample volume was injected which means that the sample was introduced into about 20 plate cells. It could be argued, therefore, that the applicable concentration in our simulation should also be about 20 times higher than that normally applied in a real analysis.

It has been shown in Section 4.1 that for both model systems with K of 10^8 M^{-1}, the complexes move like stable compounds at a concentration of 0.01 M (see Figs. 1 and 2). By simply decreasing the sample concentration, relatively more complexes would be dissociated, and at concentrations below 10^{-7} M only the reactant peaks were left. Fig. 3 shows the simulated chromatograms of the complexes at a concentration of 10^{-5} M. By comparing Fig. 3 with Figs. 1C and 2C, respectively, it can be seen that the shapes of the corresponding chromatograms are exactly the same. It is notable that the products of K and concentration are identical in the corresponding figures. Similar results were also obtained at other conditions. From this, it appears that the association constant and concentration have closely related effects on the retention behavior with the chromatograms as a function of their product. Higher values of the product favor the equilibration toward complex association. As a result, retention volume in SEC at a given K-value will decrease with increased concentration until the complex moves like a stable compound. This effect is quite unique for the supramolecular complex. In SEC of covalently bonded macromolecules, in contrast, retention volume normally can only increase with increased concentration if no aggregation/complexation occurred [6]. The effects of concentration can, therefore, probably be used as a tool for the confirmation of supramolecular binding. In some certain cases where molecular weight is

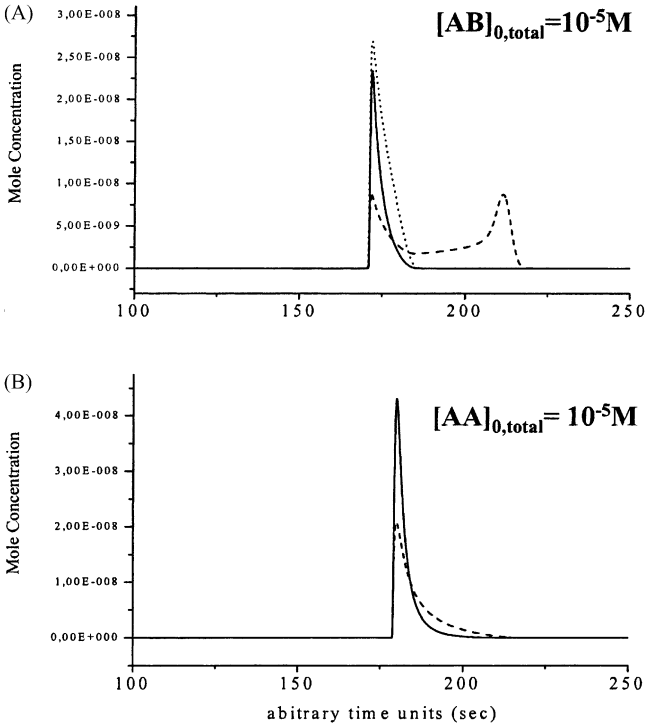

Fig. 3. Effects of concentration on the chromatographic behavior. (A) AB complex: $k_A = 0.7$, $k_B = 0.5$, $k_{AB} = 0.3$, $K = 10^8\,M^{-1}$; AB complex in solid line, A in dashed line, and B in dotted line. (B) AA complex: $k_A = 0.7$, $k_{AA} = 0.4$, $K = 10^8\,M^{-1}$; AA complex in solid line and A in dashed line. Other details as in Fig. 1.

to be determined, which can only be carried out when the complex moves like a stable compound, it is profitable to use the sample concentration as high as possible.

4.3. Effects of adding a reactant in the mobile phase

For the AB system, a very important issue is that A and B were continuously separated based on their difference in hydrodynamic volume. The separated A and B could not meet again in the column to form AB, resulting in the complex equilibrium to be shifted towards dissociation. This situation could be changed if a solution of one reactant (for instance A), instead of a pure organic solvent, was used as the mobile phase. In this way, B could always meet A in the column because component A was continuously delivered in the mobile phase.

Fig. 4 shows the effects on the chromatographic behavior of AB complex by the addition of A in the mobile phase. As illustrated earlier in Fig. 2 for K at 10^5 M^{-1}, without the addition of A, peculiar peaks were developed and the retention time for AB was considerably longer than that of AB_0. By raising the concentration of A in the mobile phase, it can clearly be seen in Fig. 4 that the peaks eventually became symmetric, and more importantly, the retention time of AB got closer to that of AB_0. At $[A]_m$ of 10^{-4} M, the AB peak was almost identical to that of AB_0. The results indicate that clearer information of complex association, particularly

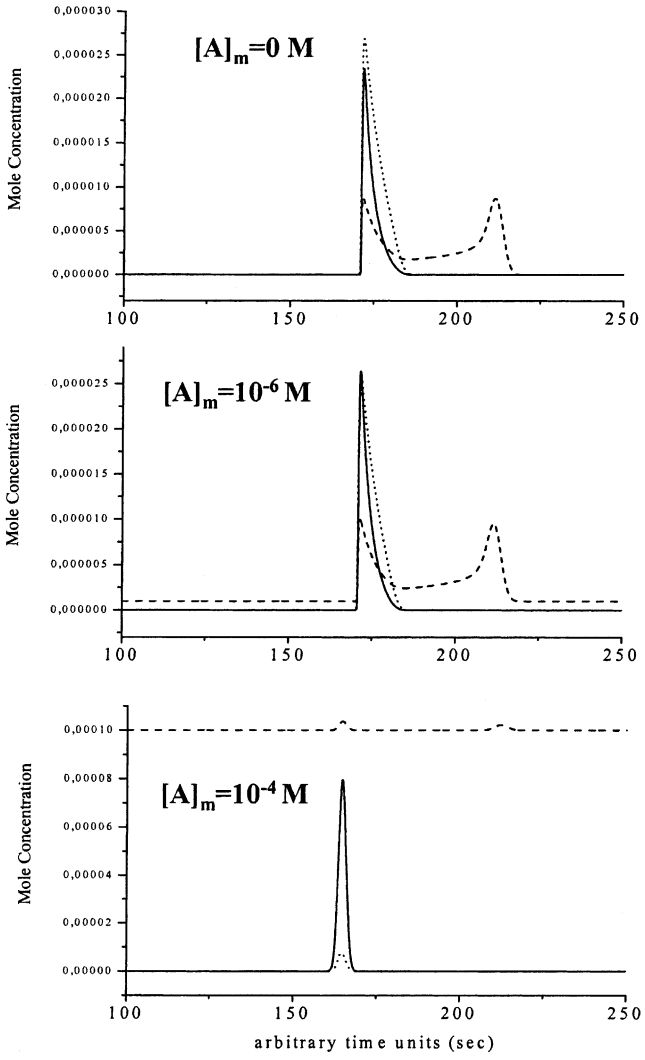

Fig. 4. Effects of adding reactant A in the mobile phase on the chromatographic behavior of AB complex. $k_A = 0.7$, $k_B = 0.5$; $k_{AB} = 0.3$, $K = 10^5\,M^{-1}$. In the chromatogram, AB complex in solid line, A in dashed line, and B in dotted line. Other details as in Fig. 1.

for complexes with moderate association constant, could be obtained by introducing suitable amount of reactant into the mobile phase.

4.4. Hummel–Dreyer method for the determination of association constant of AB complex

In the Hummel–Dreyer method [17], it is assumed that the macromolecule and the complex migrate at the same speed down the column. In literature, however, there are conflicting reports about the applicable conditions of this method. Some stressed that the assumption described earlier must be satisfied, while some others argued that the assumption is unnecessary and the only requirement is the baseline separation between the small ligand (with which the column is pre-equilibrated with a known concentration) and the complex [19]. In this section, simulations were carried out for AB complex with different retention behavior and association constants. In the following simulations, the chromatographic peak and trough were assumed to be baseline separated.

After being injected into SEC, B will remove A that was used to pre-equilibrate the column until the binding equilibrium is satisfied by the concentrations of AB and B at the baseline concentration of A ($[A]_m$). If now B and AB move at the same speed, equilibration at $[A]_m$ will be maintained along the rest of the column. This is exactly what was observed from our simulation (Fig. 5). Under this condition, the Hummel–Dreyer profile can be obtained and the association constant can be calculated by the following equation:

$$K = \frac{Q_{AB}}{[A]_m(Q_{B0} - Q_{AB})} \tag{19}$$

where $[A]_m$ is the baseline line concentration of A, and Q_{B0} the total amount of B applied. The only unknown value of Q_{AB} can be calculated from the chromatographic peak areas.

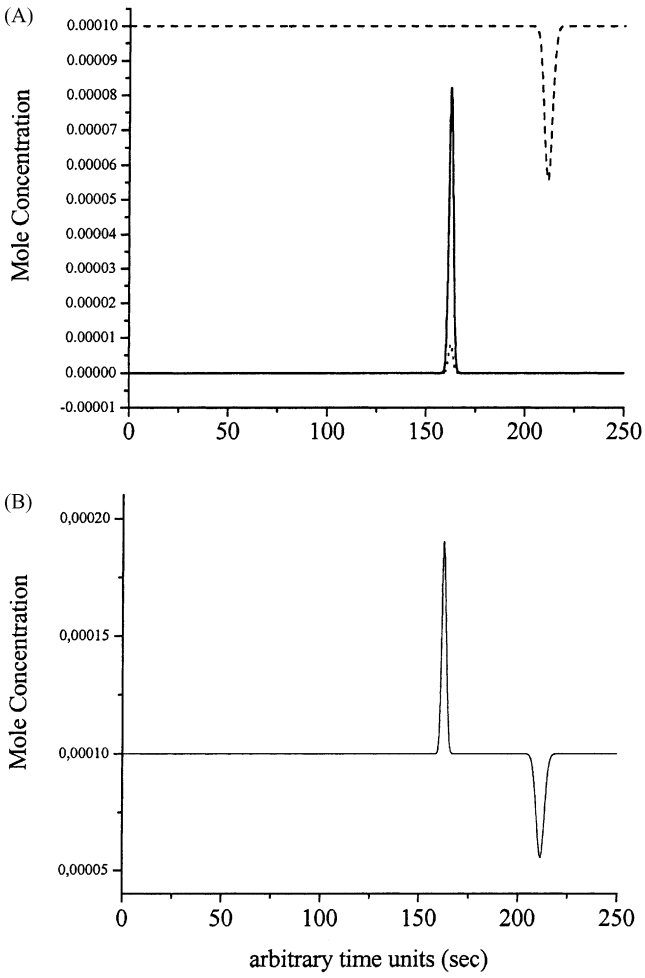

Fig. 5. A schematic representation of Hummel–Dreyer chromatographic profile at $k_B = k_{AB}$. $k_A = 0.7$, $k_B = k_{AB} = 0.3$, $K = 10^5\,M^{-1}$, $[A]_m = 10^{-4}$ M, $[B]_0 = 10^{-2}$ M. (A) Chromatograms of individual components: AB complex in solid line, A in dashed line, and B in dotted line. (B) Chromatogram of total component concentration. Other details as in Fig. 1.

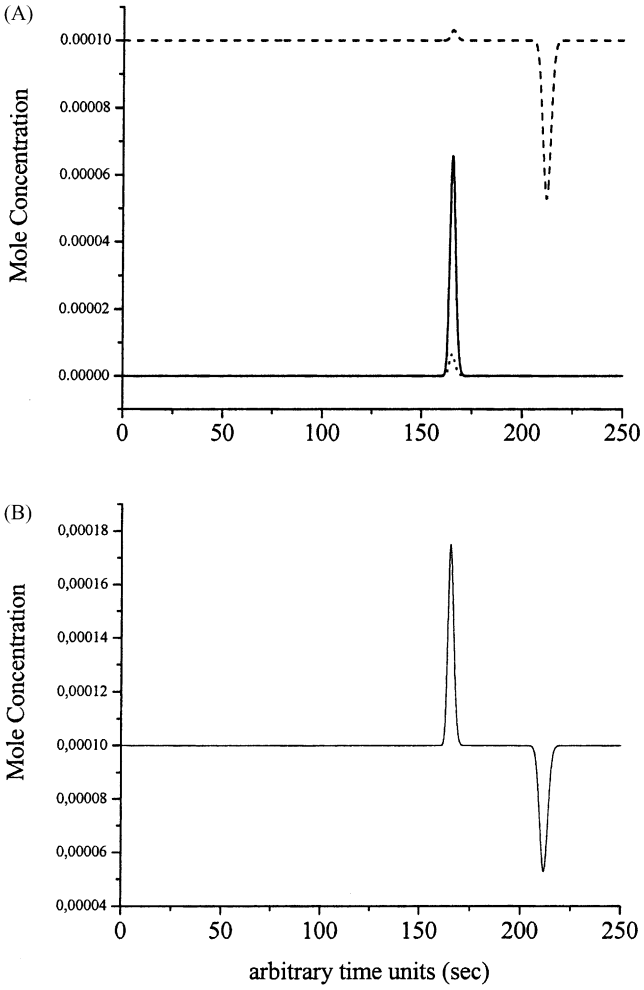

Fig. 6. A Schematic representation of chromatographic profile at $k_B \neq k_{AB}$. $k_A = 0.7$, $k_B = 0.5$, $k_{AB} = 0.3$, $K = 10^5\,M^{-1}$, $[A]_m = 10^{-4}\,M$, $[B]_0 = 10^{-2}\,M$. (A) Chromatograms of individual components: AB complex in solid line, A in dash line, and B in dot line. (B) Chromatogram of total component concentration. Other details as in Fig. 1.

For many complexes where A and B are of comparable size, however, the hydrodynamic volume of AB might be considerably larger than that of B ($k_{AB} < k_B$). In this case, B and AB tend to be separated from each other, and the equilibrium cannot be maintained stably at the baseline concentration of A. Although the Hummel–Dreyer profile can still be acquired (Fig. 6B), the continuous shifting of equilibration might have resulted in the concerns that Eq. (19) might not be able to provide accurate *K*-values. This is because: (1) no stable equilibrium was established at $[A]_m$; and (2) the right values of Q_B and Q_{AB} cannot be determined chromatographically due to the small peak of A co-eluting with AB and B (see Fig. 6A). If the same calculation procedure as in the Hummel–Dreyer method is applied, it is likely that the *K*-values will be somewhat overestimated. Table 1 lists some typical results for *K*-values calculated using the Hummel–Dreyer procedure supposing that equilibrium was at $[A]_m$. From this table, it can be seen that accurate *K*-values can be obtained with the Hummel–Dreyer procedure if $k_B = k_{AB}$. This requirement can more or less be satisfied when, for example, a small ligand is bond to a macromolecule. The accuracy deteriorates with the increase of the difference between k_B and k_{AB}. Fortunately, even when k_B is two times higher than k_{AB}, and A and B are of comparable hydrodynamic volumes, the calculated *K*-values are still in the right level although considerably overestimated. From the data listed in Table 1, it can be said that the Hummel–Dreyer method can reliably be used if the difference of hydrodynamic volume between B and AB is negligible ($k_B = k_{AB}$). In addition, the simulation data also supported the findings [19] that even if $k_B \neq k_{AB}$, the method might still be reasonably good as long as the chromatographic peak and trough could be baseline separated.

5. Conclusions

From our simulation study, the retention behavior of a supramolecular complex in size exclusion chromatography can be explained. The asymmetric peak shape and shifting retention time are the results of coordinated effects of the reversible complex association/dissociation and continuous chromatographic process. The association constant, sample concentration, and the addition of a reactant in the mobile phase are found to be important parameters controlling the chromatographic behavior of a supramolecular complex. Under conditions where the equilibration is favored towards dissociation, (almost) no complex peak and only (or mainly) reactant peaks can be observed. With the increase of association strength, asymmetric peaks can be developed and retention time be shifted toward complex mass, which can be used as an evidence of the presence

Table 1
Comparison of association constants between theoretical values and simulated experimental data of AB complex

Theoretical value	10^3	10^4	10^5	10^6	10^7
Simulated experimental value[a]					
$k_B = 0.3$ ($k_{AB} = 0.3$)	10^3	10^4	10^5	10^6	10^7
$k_B = 0.4$ ($k_{AB} = 0.3$)	1.4×10^3	1.5×10^4	1.4×10^5	1.5×10^6	1.4×10^7
$k_B = 0.5$ ($k_{AB} = 0.3$)	2.2×10^3	2.3×10^4	2.1×10^5	2.3×10^6	2.1×10^7
$k_B = 0.6$ ($k_{AB} = 0.3$)	4.6×10^3	4.8×10^4	4.7×10^5	4.7×10^6	4.1×10^7

[a] Data calculated supposing that equilibrium is established at the baseline concentration of A ($k_A = 0.7$).

of supramolecular binding. Symmetric complex peak and correct molecular weight information can be obtained for complex with strong association. The simulation results also indicate that the Hummel–Dreyer procedure can be a good method for the determination of association constants for AB type complexes as long as the chromatographic peak and trough can be baseline separated. However, the procedure tends to give somewhat overestimated values if the difference in distribution function between the complex and its reactant with the larger size is not negligible.

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