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Affinity capillary electrophoresis and density functional theory applied to binding constant determination and structure elucidation of hexaarylbenzene-based receptor complex with ammonium cation

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ABSTRACT

Affinity capillary electrophoresis (ACE) and quantum mechanical density functional theory (DFT) calculations have been employed for the investigation of noncovalent interactions between hexaarylbenzene-based receptor (**R**) and ammonium cation NH_4^+. Firstly, by means of ACE, the binding constant of the NH_4R^+ complex in methanol was estimated from the dependence of the effective electrophoretic mobility of the receptor **R** (in advance corrected by our earlier developed procedure to a reference temperature of 25 °C) on the concentration of ammonium ion in the background electrolyte using non-linear regression analysis. The logarithmic form of the apparent binding (stability) constant of $NH_4\mathbf{R}^+$ complex in the methanolic background electrolyte (25 mM Tris, 50 mM chloroacetate, pH_{MeOH} 7.8) was evaluated as $\log K_{NH_4R} = 4.03 \pm 0.15$. Secondly, the structural characteristics of $NH_4\mathbf{R}^+$ complex were determined by DFT calculations.

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1. Introduction

Noncovalent molecular interactions play an important role in many chemical and biological systems. The achievement and/or acceleration of chemical reactions based on the formation of intermediate reagent–catalyst complexes, signal transduction ensured by hormone–receptor binding, the regulation of enzyme activity by enzyme–substrate or enzyme-inhibitor binding, immunity based on antigen–antibody complexation and ion transport performed via ion–ionophore interactions are only a few examples of the processes accomplished by non-covalent, more or less specific (bio)molecular interactions. The effects of drugs can be also explained in terms of binding to receptors, enzymes or ion channels.

In current analytical chemistry, the interactions of analytes with various complexing agents (further called receptors) are widely employed to increase the selectivity of their separations by chromatographic and electromigration methods; e.g. the complexation of analytes with stereoselectors, such as cyclodextrins, crown ethers and macrocyclic glycopeptide antibiotics, is frequently used for a chiral analysis of biomolecules and other relevant compounds [1–3]. The strength of these noncovalent interactions is quantitatively estimated by the binding (stability, formation, association or dissociation) constant of the complexes between an analyte and receptor; hence, the determination of binding constants is one of the important steps in the investigation and characterization of molecular interactions.

There are several techniques available to measure the binding constants, such as UV–Vis absorption, fluorescence, nuclear magnetic resonance (NMR), mass spectrometry (MS), potentiometry, polarography, conductometry, chromatography and electrophoresis, see review [4] and references cited therein. Among these techniques, affinity capillary electrophoresis (ACE) [5–10] is now established as a sensitive analytical method, in which the migration patterns of interacting molecules and ions in the electrical field are recorded and used to identify and quantify their specific bindings and to estimate binding constants of the formed complexes [4,11–17]. Various ACE modes (classified according to the fact whether analyte interacts with free or immobilized receptor molecules before and/or during electrophoresis) are available that can be utilized for investigation of thermodynamically weak and strong bindings and kinetically fast and slow interactions both in homogeneous liquid phase and on the heterogeneous solid–liquid interface [5,7,10,18]. The advantages of ACE methods comprise low sample size requirement, relatively low analyte concentration, high separation efficiency, high resolving power and mostly short analysis times. Moreover, the analyte need not be perfectly pure (the

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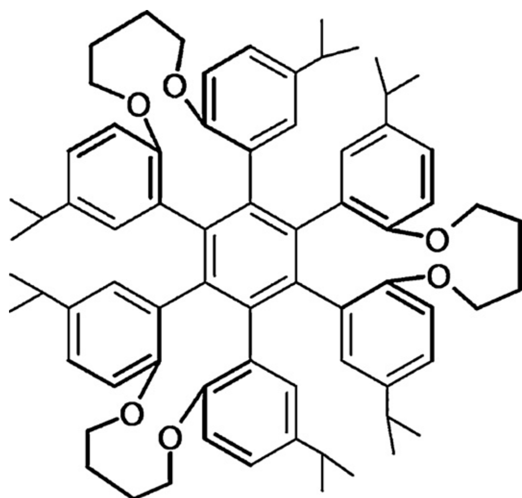

Fig. 1. The structure of the hexaarylbenzene-based receptor **R**.

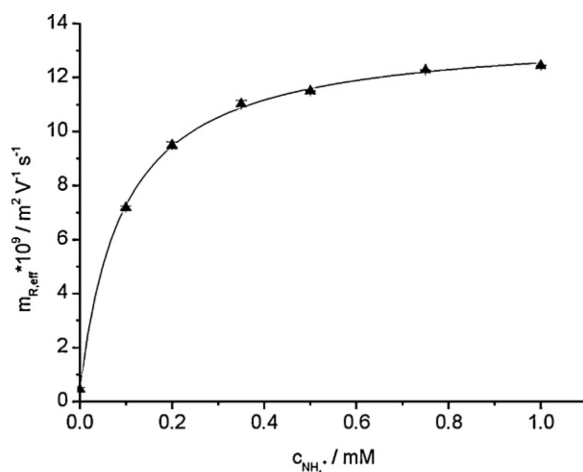

Fig. 3. The dependence of the effective mobility of hexaarylbenzene-based receptor **R**, $m_{R,eff}$, corrected to reference temperature, 25 °C, on ammonium ion concentration in the background electrolyte, $c_{NH_4^+}$.

admixtures can be separated during ACE experiment) and concentration of the analyte need not be exactly known since in the most frequently used ACE mode, mobility shift assay, the estimation of a binding constant, K_b, is not based on the determination of a free analyte concentration but on its effective mobility. ACE has proved to be a useful and sensitive tool for studying variety of (bio)molecular non-covalent interactions and for determining binding constants of the formed complexes in aqueous, nonaqueous or mixed hydroorganic media [7,15,19–22].

New chemical structures based on heaxaarylbenzene (HAB) derivatives have recently received a great attention because they can be potentially employed for the preparation of modern graphitic materials and for various applications in the emerging areas of molecular electronics and nanotechnology [23]. Recently, polyaromatic hexaarylbenzene-based receptor, **R** (see Fig. 1) was synthesized with the aim to be applied in practical sensors for various metal ions [24]. The structural details of the complex of this receptor **R** with potassium cation K^+ were characterized by NMR spectroscopy and X-ray crystallography [24] but these techniques could not provide estimation of the binding constant of this complex. In K**R**$^+$ complex, a single potassium cation synergistically interacts with the polar ethereal fence and with the central benzene ring via cation–π interactions [24]. It is known, that K^+ ion interactions with the ethereal oxygens play an important role also in its binding with 18-crown-6 ether. Moreover, it is reported in literature that in methanol, 18-crown-6-ether forms fairly strong complex with NH_4^+ cation (log K_b = 4.32) [25]. Hence, we have assumed that receptor **R** could form similar complex with ammonium cation and that the strength of this complex can be estimated by ACE. Thus, this is the first study dealing with investigation of noncovalent interactions between HAB-based receptor **R** and NH_4^+ cation. The first aim of the present study was to employ ACE for determination of the binding constant of NH_4**R**$^+$ complex. The apparent binding (stability) constant of NH_4**R**$^+$ complex in methanol was determined via non-linear regression analysis of the dependence of effective electrophoretic mobility of the receptor **R** (beforehand corrected to reference temperature 25 °C) on the concentration of ammonium ion (added in the form of chloride) in the background electrolyte (BGE). The second aim of this work was to describe the structural characteristics of the receptor **R** and its complex with NH_4^+ ion by means of quantum mechanical density functional theory (DFT) calculations.

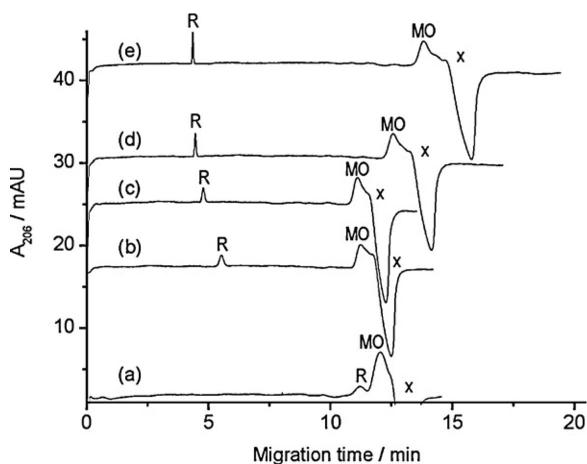

Fig. 2. The typical electropherograms of hexaarylbenzene-based receptor (**R**) in the background electrolyte (BGE) composed of 50 mM $ClCH_2COOH$, 25 mM Tris, pH_{MeOH} 7.8, containing various concentrations of NH_4Cl: (a) 0 mM, (b) 0.1 mM, (c) 0.2 mM, (d) 0.5 mM, and (e) 1.0 mM. Experimental conditions: total/effective capillary length 306/200 mm, id/od 50/375 μm; separation voltage + 12 kV, A_{206}, absorbance at 206 nm; MO, mesityl oxide (EOF marker); x; system peaks.

2. Materials and methods

2.1. Chemicals

All chemicals used were of analytical reagent-grade. Methanol was obtained from Penta (Chrudim, Czech Republic), ammonium chloride and sodium hydroxide from Lachema (Brno, Czech Republic), mesityl oxide (MO) and Tris from Merck (Hohenbrunn, Germany), and chloroacetic acid from Fluka (Buchs, Switzerland). The hexaarylbenzene-based receptor (**R**) was synthesized in the group of R. Rathore, for more details see Ref. [24].

2.2. Apparatus

For the ACE experiments, an adapted home-made CE apparatus [26] equipped with a UV photometric detector monitoring absorbance at 206 nm was used. The ACE separations were performed in the internally uncoated fused silica capillary with an outer polyimide coating, a total/effective length of 306/200 mm, an id/od of 50/375 μm. The separations were carried out at ambient temperature, 23–26 °C. A Clarity chromatography and electrophoresis station (DataApex, Prague, CR) was used for data

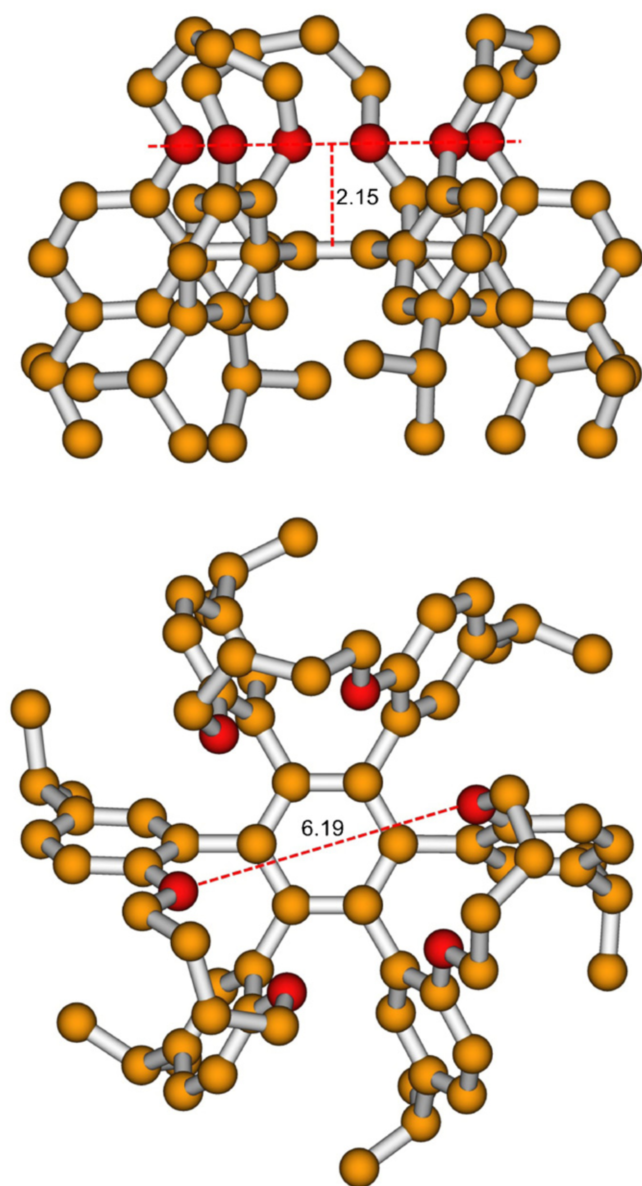

Fig. 4. Two projections of the DFT optimized structure of a free hexaarylbenzene-based receptor (**R**) (B3LYP/6-31G(d)) (hydrogen atoms omitted for clarity). The depth and the diameter of the receptor cavity are 2.15 and 6.19 Å, respectively.

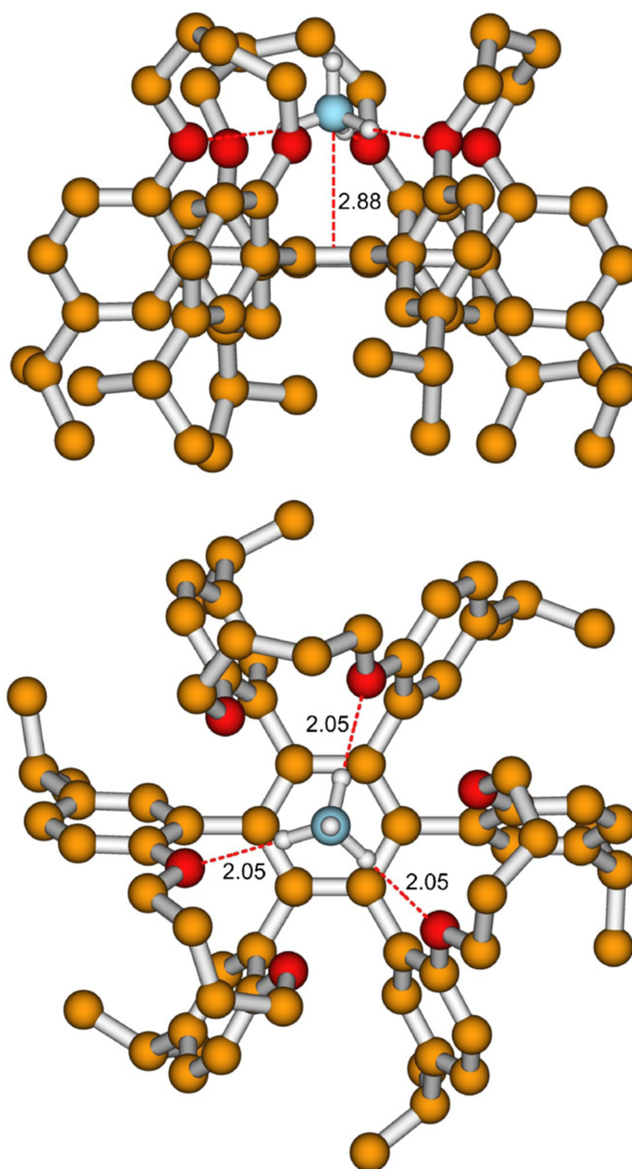

Fig. 5. Two projections of the DFT optimized structure A of the NH_4**R**$^+$ complex (B3LYP/6-31G(d)) (hydrogen atoms omitted for clarity except those of NH_4^+). The distance between the mean plane of the bottom benzene ring and the nitrogen atom of NH_4^+ in the NH_4**R**$^+$ complex is 2.88 Å. The depth and the diameter of the cavity in NH_4**R**$^+$ complex are 2.24 Å and 6.04 Å, respectively. Three hydrogen bonds that are formed between NH_4^+ and receptor **R** are each 2.05 Å long.

acquisition and evaluation, and the Origin 6.1 program (OriginLab Corp., Northampton, MA, USA) was employed for the non-linear regression analysis.

2.3. ACE conditions

The background electrolyte (BGE) consisted of Tris (25 mM) and chloroacetic acid (50 mM), with the addition of variable concentrations of ammonium chloride (0–1 mM), in methanol. The pH value of the BGE according to the conventional pH scale, pH_{MeOH}, described by Porras et al. [27], was equal to 7.8 (the pK_a value of chloroacetic acid in methanol at 25 °C is 7.8) [27]. Receptor **R** (20 μM in mixed solvent Cl_2CH_2/CH_3OH, 1:1, v/v) used as analyte, and mesityl oxide MO (2.5 mM in MeOH) employed as EOF marker, were consecutively introduced hydrodynamically into the capillary by pneumatically induced pressure (10 mbar) for 5 s each. The applied separation voltage was +12 kV (the anode at the injection end) and the electric current was in the range of 10–12 μA. Before the first use and between the series of analyses in different BGEs, the capillary was conditioned by subsequent rinsing with water (2 min), 0.1 M aqueous sodium hydroxide (10 min), water (2 min), methanol (10 min), and the BGE (20 min). Between runs in the same BGE, the capillary was rinsed with methanol (2 min), water (1 min), 0.1 M of NaOH (1 min), water (1 min), methanol (1 min), and BGE (4 min). All the rinsing was performed at a pressure of 1 bar.

3. Results and discussion

3.1. Selection of separation conditions

In ACE studies of host–guest inclusion complexes (which is the case of the studied NH_4**R**$^+$ complex), usually a guest, e.g. metal cations or organic bases or acids (central ions) are employed as analytes, and the host (further called receptor), e.g. non-charged

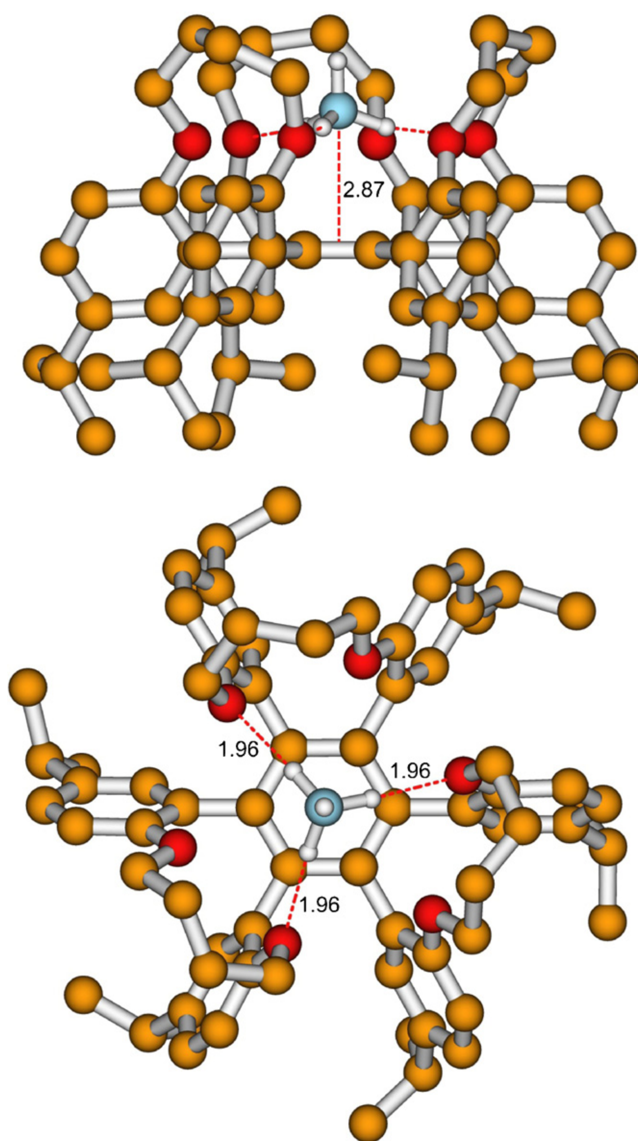

Fig. 6. Two projections of the DFT optimized structure B of the $NH_4\mathbf{R}^+$ complex (B3LYP/6-31G(d)) (hydrogen atoms omitted for clarity except those of NH_4^+). The distance between the mean plane of the bottom benzene ring and the nitrogen atom of NH_4^+ in the $NH_4\mathbf{R}^+$ complex is 2.87 Å. The depth and the diameter of the cavity in $NH_4\mathbf{R}^+$ complex are 2.26 Å and 6.04 Å, respectively. Three hydrogen bonds that are formed between NH_4^+ and receptor **R** are each 1.96 Å long.

cyclodextrins or crown ethers are added to the BGE [14,28,29]. Addition of neutral receptor to the BGE decreases the effective mobilities of central ions due to their complexation with the large neutral receptor molecules. Advantage of such experimental set-up is that the ACE experiments are performed at constant ionic strength. However, in some cases, especially if the receptor is very expensive and/or available in rather small amount, the reverse approach, i.e. application of the receptor as an analyte (sample), and addition of the guest (central ion) in the BGE, is more suitable or even necessary. The latter mode has been employed, e.g. for estimation of binding constants of the complexes of dibenzo-crown ethers [30,31] and macrocyclic depsipeptide valinomycin [21,32] (applied as analytes) with alkali metal ions (added at variable concentrations into the BGE). In this case, the increasing concentration of metal ions in the BGE increases the effective mobility of neutral receptor due to the formation of positively charged receptor–cation complex. This approach is particularly convenient when the receptor absorbs UV light and the central ion is UV-transparent; then commonly available UV-absorption detection can be used and the application of less sensitive detection modes, indirect UV-absorption or conductometry is not necessary. This approach has been employed also in this study, where UV-absorbing hexaarylbenzene-based receptor **R** was used as an analyte and the increasing concentrations of ammonium ion in the form of ammonium chloride were added to the BGE. This approach was favorable because only low amount of the receptor **R** was available. The relative drawback of this mode (performance of ACE experiments at variable ionic strength and a need for appropriate correction of the measured mobilities to constant ionic strength) was not relevant in this study because in the case of relatively strong $NH_4\mathbf{R}^+$ complexes, the concentration of NH_4^+ ions in the BGE was rather low and the changes of ionic strength (less than 1 mM) could be neglected.

In order to perform the ACE measurements as accurately as possible, it is preferable to choose BGE, the constituents of which do not interact with any of the complex components. However, in practice it is often difficult if not impossible to fulfill this condition. In this case, BGEs, the components of which interact with the complex components as weak as possible, should be applied. For that reason, Tris-chloroacetate was employed as the BGE in this study since from the several cationic components of the BGE, $Tris^+$ cation formed the weakest (and rather weak) complex with receptor **R**.

3.2. *Correction of electrophoretic mobilities to reference temperature*

For a correct determination of binding constants, all ACE experiments should be performed at constant temperature and ionic strength. However, the home-made CE device employed in this study is not equipped with an active capillary cooling and therefore the CE experiments were performed at variable ambient temperature (23–26 °C). Additionally, the temperature inside the capillary (even in the cooled system) is increased due to Joule heating. Thus, it was necessary to determine the actual temperature inside the capillary for particular ambient temperature and input power (Joule heating) in order to be able to correct the measured effective mobilities of $NH_4\mathbf{R}^+$ complex to the reference temperature, 25 °C. The earlier developed correction procedure is described in detail elsewhere [32]. Briefly, it is based on electric current measurements in a wide range of applied voltages at the same set-up as that used for CE experiments, only with the exception that the capillary was filled with methanolic solution of a strong electrolyte, 100 mM tetrabutylammonium perchlorate. The electric conductance, G, of this solution in the capillary is changing with temperature mainly due to the temperature dependence of solution viscosity. Thus, viscosity of the solution is strongly correlated with the electric conductance, G. The conductance of the electrolyte solution at the given input electric power, P, (i.e. at given current × voltage product, $P = I \cdot U$) was calculated from the measured current/voltage ratio, $G = I/U$. Taking into account, that in the temperature range 20–30 °C, the viscosity of methanol decreases by 1.34% per Celsius degree [33], the change of solution conductance at given input power was recalculated to the temperature increase, ΔT:

$$\Delta T = T - T_0 = \frac{G - G_0}{0.0134 G_0} \tag{1}$$

where T_0 is the ambient temperature of the air surrounding the capillary, T is the increased temperature inside the capillary at the given input power, G_0 is the conductance of a solution at ambient temperature T_0 (i.e. at the very low input power applied, when the temperature increase in the capillary can be neglected), and G is the conductance of the solution at the given input power applied. Thus, a calibration curve of the temperature increase inside the capillary,

ΔT, vs. input power, P, was obtained, described by the following equation:

$$\Delta T = -2.2067P^2 + 14.462P - 1.089 \quad (2)$$

The effective mobilities measured by CZE at given input power P and actual temperature inside the capillary T ($T = T_0 + \Delta T$) were recalculated to the reference temperature of 25 °C assuming that the mean temperature dependence of mobility will be numerically identical with the temperature dependence of viscosity, i.e. 1.34% mobility increase per Celsius degree [33]:

$$m_{\text{eff},25} = m_{\text{eff},T}[1 - 0.0134(T_0 + \Delta T - 25)] \quad (3)$$

where $m_{\text{eff},25}$ and $m_{\text{eff},T}$ are effective mobilities at 25 °C and at actual temperature T inside the capillary, respectively.

3.3. Determination of the binding constant of R–NH_4^+ complex by ACE

Preliminary experiments have shown that hexaarylbenzene-based receptor **R** forms a relatively strong complex with NH_4^+ cation. Hence, rather low concentrations of NH_4Cl in the BGE (up to 1 mM) were sufficient to observe the shift in migration times caused by the formation of $NH_4\mathbf{R}^+$ complex. Thus, changes of the ionic strengths of BGEs were negligible and no correction of mobilities to constant ionic strength was necessary. The ACE method for the determination of the binding constant involved measurements of effective electrophoretic mobility of receptor **R** as a function of ammonium ion concentration in the BGE. The corresponding apparent binding (stability) constant of the $NH_4\mathbf{R}^+$ complex, K_{NH_4R}, was obtained from the dependence of effective mobility of receptor **R** on the NH_4^+ ion concentration in the BGE using a non-linear regression analysis. The typical electropherograms are shown in Fig. 2. In these experiments, 20 μM receptor **R** and 2.5 mM mesityl oxide (MO) were applied as analyte and EOF marker, respectively, and methanolic solution of 25 mM Tris and 50 mM chloroacetate with increasing concentrations of ammonium chloride was used as the BGE. It can be seen in record (a) in Fig. 2 that even in the absence of ammonium chloride in the BGE, receptor **R** migrated a little bit faster than the EOF marker MO. This observation gives a reason to assume that in the Tris-chloroacetate BGE neutral receptor **R** binds not only ammonium ion but (much more weakly) also $Tris^+$ cation. Thus, the peak R in Fig. 2 represents complex of receptor **R** with $Tris^+$ cation (record a) or with ammonium ion (records b–e), migrating faster than the EOF marker because of the positively charged $NH_4\mathbf{R}^+$ complex formed.

The theoretical treatment of the interacting equilibria and analyte migration behavior in CE with more than one buffer additive has been described by Peng et al. [34] and was also followed in this work. It has been previously reported that receptor **R** forms 1:1 complex with potassium cation [24]. In the current work, it is assumed that NH_4^+ and $Tris^+$ cations react with receptor **R** competitively and receptor **R** can bind only one of them at a time. Since there are no interactions between these two ionic additives, the following equilibria hold:

$$NH_4^+ + \mathbf{R} \rightleftharpoons NH_4\mathbf{R}^+ \quad (4)$$

$$Tris^+ + \mathbf{R} \rightleftharpoons Tris\mathbf{R}^+ \quad (5)$$

The corresponding equilibrium apparent binding constants are:

$$K_{NH_4R} = \frac{[NH_4\mathbf{R}^+]}{[NH_4^+][\mathbf{R}]} \quad (6)$$

$$K_{TrisR} = \frac{[Tris\mathbf{R}^+]}{[Tris^+][\mathbf{R}]} \quad (7)$$

where $[NH_4\mathbf{R}^+]$, $[NH_4^+]$, $[Tris\mathbf{R}^+]$, $[Tris^+]$ and $[\mathbf{R}]$ are the equilibrium concentrations of the $NH_4\mathbf{R}^+$ complex, free NH_4^+ ion, $Tris\mathbf{R}^+$ complex, free $Tris^+$ ion and free receptor **R**, respectively. The effective electrophoretic mobility of receptor **R**, $m_{\text{R,eff}}$, in the presence of NH_4^+ and $Tris^+$ ions can be expressed as

$$m_{\text{R,eff}} = \frac{m_{\text{R}} + K_{NH_4R}[NH_4^+]m_{NH_4R} + K_{TrisR}[Tris^+]m_{TrisR}}{1 + K_{NH_4R}[NH_4^+] + K_{TrisR}[Tris^+]} \quad (8)$$

where m_{R}, m_{NH_4R}, and m_{TrisR} are the electrophoretic mobilities of uncomplexed receptor **R**, $NH_4\mathbf{R}^+$ and $Tris\mathbf{R}^+$ complexes, respectively. When $[Tris^+]$ is constant, Eq. (8) can be simplified to

$$m_{\text{R,eff}} = \frac{m_{\text{R}}^* + K_{NH_4R}^*[NH_4^+]m_{NH_4R}}{1 + K_{NH_4R}^*[NH_4^+]} \quad (9)$$

where m_{R}^* is the effective electrophoretic mobility of receptor **R** in Tris-chloroacetate BGE in the absence of NH_4^+ ions and $K_{NH_4R}^*$ is the apparent binding constant of the $NH_4\mathbf{R}^+$ complex in the presence of $Tris^+$ cation. Nonzero value of m_{R}^* results from the interaction between receptor **R** and $Tris^+$ and its value was determined from the analysis of receptor **R** in Tris-chloroacetate BGE, which did not contain any NH_4Cl. The effective electrophoretic mobilities of receptor **R**, $m_{\text{R,eff}}$, at given NH_4^+ ion concentration are calculated from the measured migration times of the analyte (receptor **R**), $t_{\text{mig,R}}$, and the EOF marker, t_{eof}, respectively, according to following equation:

$$m_{\text{R,eff}} = \frac{L_{\text{tot}}L_{\text{eff}}}{U_{\text{sep}}}\left(\frac{1}{t_{\text{mig,R}}} - \frac{1}{t_{\text{eof}}}\right) \quad (10)$$

where L_{tot} and L_{eff} are the total and the effective capillary lengths, respectively, and U_{sep} is the applied separation voltage. The dependences of the determined electrophoretic mobilities, corrected to reference temperature, 25 °C, on the ammonium ion concentration are presented in Fig. 3. Each data point is a mean of four separate measurements and the error bars represent the standard deviation.

A non-linear regression analysis using the computer program Origin 6.1 (OriginLab Corp., Northampton, MA, USA) was applied to fit the function given by Eq. (9) to the experimental data shown in Fig. 3. In this fitting procedure, the NH_4^+ values (concentrations of the ammonium ion in the BGE) are the independent variable, the $m_{\text{R,eff}}$ values (ACE measured effective mobilities of receptor R) are the dependent variable, whereas the values of binding constant $K_{NH_4R}^*$ and mobility $m_{NH_4R}^*$ of the $NH_4\mathbf{R}^+$ complex are treated as unknown parameters. The best fit is presented in Fig. 3 along with the experimental data. The logarithmic form of the binding constant of $NH_4\mathbf{R}^+$ complex obtained using effective mobilities corrected to reference temperature 25 °C was evaluated as log $K_{NH_4R}^* = 4.03 \pm 0.15$. The ionic radius of NH_4^+ ion is 1.43 Å. The size of the cavity and other structural details of the free receptor **R** as well as the $NH_4\mathbf{R}^+$ complex are described in the following section using the quantum mechanical DFT calculations.

3.4. Structure determination of $NH_4\mathbf{R}^+$ complex by DFT calculations

The quantum mechanical calculations were carried out using the Gaussian 03 suite of programs [35]. The molecular geometries of the studied complexes were optimized at the B3LYP level of density functional theory (DFT) with the 6-31G(d) basis set. Polar solvent employed in the experimental part of this study affects to some extent the detailed structures of the receptor **R** and its complexes with alkali metal ions. However, evidently there is no reliable and universal method of taking into account the polarity of the medium in precise DFT calculations. Nevertheless, our experience with the similar systems shows that DFT calculations made in vacuo give

valuable structural information, often in a very good agreement with the experimental results [36–40].

In the model calculations, the molecular geometries of the parent hexaarylbenzene-based receptor **R** and its complex with NH_4^+ ion were optimized. The optimized structure of a free receptor **R** having C3 symmetry, involving a bowl-shaped cavity, which is comprised of an aromatic bottom (i.e. central benzene ring) and an ethereal fence formed by six oxygen atoms from the peripheral aryl groups, is illustrated in Fig. 4. The depth of the cavity, i.e. the distance between the mean plane of the aromatic bottom and that of the ethereal fence, is 2.15 Å (1 Å = 0.1 nm); the diameter of this cavity in receptor **R** is 6.19 Å (Fig. 4).

The two structures, A and B, obtained by the full DFT optimizations of the cationic complex species $NH_4\mathbf{R}^+$ are depicted in Figs. 5 and 6, respectively, together with the lengths of the corresponding strong linear hydrogen bonds. In both of these structures of the complex $NH_4\mathbf{R}^+$ having also C3 symmetry, the cation NH_4^+ synergistically interacts with the hydrophilic polar ethereal oxygen fence and with the central hydrophobic benzene bottom via cation–π interactions, as shown in Figs. 5 and 6. Moreover, it is necessary to emphasize that the formation of the complex species $NH_4\mathbf{R}^+$ results in the small tapering of the respective cavity and at the same time, in its getting longer, as follows from comparison of Fig. 4 with Figs. 5 and 6. The interaction energies, E(int), corresponding to the structures A and B of the $NH_4\mathbf{R}^+$ complex under study (calculated as the differences between the respective electronic energies of $NH_4\mathbf{R}^+$ and isolated NH_4^+ and **R** species $E(\text{int}) = E(NH_4\mathbf{R}^+) - E(NH_4^+) - E(\mathbf{R})$) are very similar: −783.2 kJ/mol and −788.5 kJ/mol, respectively.

4. Conclusions

In this study, we have demonstrated that combination of an experimental ACE method and theoretical quantum mechanical DFT calculations can provide relevant data on the noncovalent interactions of hexaarylbenzene-based receptor **R** with ammonium cation NH_4^+. From the ACE analyses of receptor **R** at various concentrations of NH_4^+ ions in the background electrolyte, the strength (binding constant) of the $NH_4\mathbf{R}^+$ complex in methanol was evaluated, whereas via DFT calculations the detailed structural features of the $NH_4\mathbf{R}^+$ complex, such as alignment of both complex constituents and interatomic distances within the complex, were obtained.

Acknowledgements

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