Ions interacting with macromolecules.
NMR studies in solution

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Fakultetsopponent: Prof. Monika Schönhoff, Universität Münster, Germany.
To my family
Abstract

Specific ion effects, identified for more than hundred years, play an important role in a wide range of phenomena and applications. Several mechanisms such as direct ion interaction with molecules have been suggested to explain these effects, but quantitative experimental evidence remains scarce. Electrophoretic NMR (eNMR) has been emerging as a very powerful tool for studying molecular association and ionic transport in a variety of systems. Yet its potential in studying specific ion effect has been unexplored. In this thesis, eNMR was in part developed further as an analytical method and was in part used as one of the main techniques to study ions interacting with macromolecules in aqueous and non-aqueous solutions.

The complexation of a large group of cations with poly ethylene oxide (PEO) in methanol was studied with eNMR. The binding of monovalent ions was demonstrated not to follow the Hofmeister order; multivalent cations except barium all showed negligible complexation. As a unifying feature, only cations with surface charge density below a threshold value were able to bind suggesting that ion solvation is critical. The binding mechanism was examined in greater detail for K$^+$ and Ba$^{2+}$ with oligomeric PEO of different chain lengths. Those two cations exhibited different binding mechanisms. K$^+$ was found to bind to PEO by having at least 6 repeating units wrap around it while retaining the polymer flexibility. On the other hand, Ba$^{2+}$ (and, to some extent, (BaAnion)$^+$) needs a slightly shorter section to bind, but the molecular dynamics at the binding site slow considerably.

The binding of anions with poly (N-isopropylacrylamide) in water was quantified at low salt concentration with eNMR and the binding affinity, though very weak, followed the Hofmeister order. This result indicates the non-electrostatic nature of this specific ion effects. The increase of binding strength with salt concentration is well described by a Langmuir isotherm.

The specific ion binding to a protein, bovine serum albumin (BSA), was also studied at pH values where BSA has either net positive and negative charges. Our results show that anions have the same binding affinity irrespective of the surface charge while the binding strength of cations is reversed with the change in net surface charge. This indicates different binding mechanisms for cations and anions.

The ionization of cellobiose in alkaline solutions was measured quantitatively by eNMR. The results show a two-step deprotonation process with increasing alkaline strength.
Supported by results from $^1$H-$^{13}$C HSQC NMR and MD simulation, ionization was proposed to be responsible for the improved solubility of cellulose in alkaline solution. eNMR was also used to characterize the effective charge of tetramethylammonium ions in a variety of solvents. In solvents of high polarity, the results agree well with predictions based on Onsager’s limiting law but for nonpolar solvents deviations were found that were attributed to ion pair formation.

Key words: electrophoretic NMR, diffusion NMR, specific ion effects, Hofmeister, ion binding
Sammanfattning

Specifika joneffekter som har identifierats för mer än hundra år sedan spelar en viktig roll i ett stort antal fenomen och tillämpningar. Flera förslag såsom direkt joninteraktion med molekyler finns vad gäller mekanismen men kvantitativa experimentella bevis är svårt att komma åt. Elektroforetisk NMR (eNMR) har under de senaste åren visat sig vara ett mycket kraftfullt verktyg för att studera molekylär association och jontransport i olika system medan dess potential för att studera specifika joneffekter är relativt outforskat. I denna avhandling har eNMR delvis vidareutvecklats som analytisk verktyg och delvis använts som en av de viktigaste teknikerna för att studera joner som interagerar med makromolekyler i olika lösningsar.

Komplexbildning av en stor grupp katjoner med poly etylenoxid (PEO) i metanol studerades med eNMR. Ordfningen av bindningsstyrkan för monovalenta joner visades inte följa Hofmeisterserien; multivalenta katjoner förutom barium visade försämras bindning. Som en gemensam trend, endast katjoner med ytladdningstäthet under ett visst tröskelvärde kan komplexera med PEO vilket tyder på att jonsolvatisering är en kritisk faktor. Bindningsmekanismen undersöktes i mer detalj för K⁺ och Ba²⁺ joner med oligomera PEO av olika kedjelängd. Det visade sig att dessa två katjoner har olika bindningsmekanismer. K⁺ binder genom att minst 6 enheters lång del av PEO-kedjan lindas runt den, dock behåller den bindande delen sin snabb dynamik. Å andra sidan, Ba²⁺ (och till en viss del (BaAnion)⁺) binder till en något kortare del av kedjan som förblir dock mycket stel vid bindning.


Den specifika jonbindningen till proteinen bovine serum albumin (BSA) studerades vid olika pH i vattenlösning där BSA bär på sig antingen positiv eller negativ nettoladdning. Resultaten visade anjoner har samma bindningsaffinitet oavsett ytlaaddningen medan katjoner affinitet till ytan fick en omvänt ordning vid olika tecken för ytlaaddningen. Denna observation indikerar olika bindningsmekanism för katjoner och anjoner.

Jonisering av cellobios i alkalisk lösnings mättes upp med eNMR. Resultaten visar en två-stegs deprotoneringsprocess med ökande pH. Med stöd från resultat från ¹H-¹³C HSQC
NMR och molekyldynamiksimuleringar föreslås det att jonisering utgör anledningen för den förbättrade löslobilen av cellulosa i alkalisk lösning. eNMR användes också för att karakterisera den effektiva laddningen av tetrametylammonium joner i en mängd olika lösningsmedel. I polära lösningsmedel, samma värde som förutsågs teoretiskt från Onsagers ”limiting law” mättes upp medan i icke-polära lösningsmedel fick man skillnader mellan experiment och teori som tillskrivs till jonparsbildning.

**Nyckelord:** elektroforetisk NMR, diffusions NMR specifika jon effekter, Hofmeister, jon bindning
List of Papers

I. Assessing 2D Electrophoretic Mobility Spectroscopy (2D MOSY) for Analytical Applications
Yuan Fang, Pavel V. Yuchmanov and István Furó

II. Binding of Monovalent and Multivalent Metal Cations to Polyethylene Oxide in Methanol Probed by Electrophoretic and Diffusion NMR
Marianne Giesecke, Fredrik Hallberg, Yuan Fang, Peter Stilbs, and István Furó
The Journal of Physical Chemistry B 2016, 120 (39), 10358-10366

III. Complexing Cations by Polyethylene Oxide. Binding Site and Binding Mode
Yuan Fang, Marianne Giesecke and István Furó
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IV. Anion Binding to Poly(N-isopropylacrylamide). A Quantitative Study by Electrophoretic NMR
Yuan Fang and István Furó
Manuscript in preparation.

V. Binding Mechanisms of Cations and Anions to Proteins. Electrophoretic NMR Studies in Bovine Serum Albumin
Yuan Fang and István Furó
Manuscript in preparation.

VI. Ionization of Cellobiose in Aqueous Alkali and the Mechanism of Cellulose Dissolution
Erik Bialik, Björn Stenqvist, Yuan Fang, Åsa Östlund, István Furó, Björn Lindman, Mikael Lund and Diana Bernin
The Journal of Physical Chemistry Letters 2016, 7 (24), 5044-5048

VII. Ion Association in Aqueous and Non-Aqueous Solutions Probed by Diffusion and Electrophoretic NMR
Marianne Giesecke, Guillaume Mériguet, Fredrik Hallberg, Yuan Fang, Peter Stilbs and István Furó
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I. Participated in all the eNMR measurements together with Pavel V. Yuchmanov and data analysis. Minor contribution to writing.

II. Performed some of the eNMR measurements and data analysis. Minor contribution to writing.

III. Performed all the experimental work and participated in writing.

IV. Planned, performed all the experimental work and participated in writing.

V. Planned, performed all the experimental work and participated in writing.

VI. Performed and evaluated the eNMR measurements. Minor contribution to writing.

VII. Performed and evaluated some of the eNMR measurements. Minor contribution to writing.

Paper of the author not included in the thesis:

Ion transport in polycarbonate based solid polymer electrolytes: experimental and computational investigations

Bing Sun, Jonas Mindemark, Evgeny V. Morozov, Luciano T. Costa, Martin Bergman, Patrik Johansson, Yuan Fang, István Furó and Daniel Brandell

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1. Introduction

When molecules dissolve in solvents, they exist not only in their original form but may also dissociate or aggregate. Solutes that dissociate into charged constituents, called ions can conduct electricity and their solution is termed an electrolyte. Solutes that keep their intact molecular form in solutions do not conduct electricity and are called non-electrolytes. Electrolyte does not only refer to simple salts but also to acids, bases, nucleic acids, proteins or synthetic polymers that have dissociable functional groups. It is encountered in many aspects of our life. Based on composition, living bodies are bags of electrolytes; cells and tissues are mainly electrolyte solutions with added macromolecules. Ions in the physiological electrolyte permit transmitting nerve signals, keep the heart functioning, and let muscle contract and are involved in all activities in a human body. Besides, electrolytes are also playing a critical role in a wide range of industries and products, like batteries, mining, and pulp and paper industry. Understanding electrolytes, their equilibrium and transport properties and their possible interactions are very important not only for controlling industrial processes but also for understanding biological mechanisms in the human body. However, this is not easy since an electrolyte solution is usually constituted by multiple components and there are complex interactions between them. Moreover, the presence of a large number of charged particles in the solution modulates the behavior of other molecules by changing the landscape for all electrostatic interactions, including ion-solvent interactions and solvent-solvent interactions. Despite numerous efforts and progresses made on their physical chemistry during the past decades, a satisfactory theory that could comprehensively describe various phenomena in electrolyte systems is not yet available.

The work in this thesis is mainly directed at the interaction of ions with macromolecules in aqueous and non-aqueous solutions as seen by NMR methods. Starting with introducing ideal and non-ideal electrolytes, the first part of this chapter deals with classical theories, including the Poisson-Boltzmann model, the electrical double layer theory, the electrokinetic theory, and the Debye-Hückel theory. The second part of this chapter touches upon situations where classical theories fail and specific ion effects happen. In particular, ion pairing and ion-ligand complexation will be introduced since those are subjects where specific ion effects were studied in this thesis. The last part of this chapter gives a brief review over the suggested mechanisms of specific ion effects.
1.1. Ideal and non-ideal electrolyte solutions

Ideality in chemistry refers to the case where the interactions between particles are negligible. This usually happens in gases. On the contrary, even in pure liquid there is still significant solvent-solvent interaction. Hence, one talks about ideal electrolyte solutions if the solute concentration is so small so that the solute-solute interactions are negligible. Any change in solute-solute, solute-solvent, or solvent-solvent interactions caused by increasing solute concentration contributes to the non-ideality of the electrolyte. Almost all electrolyte solutions we use are non-ideal. Their non-ideality increases with increasing concentration and manifests itself as, for example, the concentration dependence of association constants or molar conductivities. The non-ideality can be described in terms of an excess free energy in addition to the free energy of an ideal solution, which is in turn re-expressed with activity coefficients of every single ionic species in the electrolyte. For example, the excess free energy of a sodium chloride solution is expressed as

\[
\Delta G_{\text{excess}} = RT\ln\gamma_{Na^+} + RT\ln\gamma_{Cl^-}
\]  

(1.1)

where \( R \) is the gas constant, \( T \) is the absolute temperature, and \( \gamma \) is the activity coefficient of an individual species that can be obtained from activity \( \alpha \) and concentration \( c \) through \( \alpha = \gamma c \). Yet, the activity coefficient of an individual ion cannot be measured directly so instead the mean activity coefficient, which is defined as

\[
\gamma_\pm = (\gamma_{Na^+}\gamma_{Cl^-})^{1/2}
\]  

(1.2)

is usually measured as a property of the electrolyte. For a strong electrolyte the mean activity coefficient approaches 1 as the concentration is approaching to zero which indicates reaching the ideal limit. In turn, non-ideality is indicated by the mean activity coefficient deviating from 1. This seems rather trivial but, as is said in the book Specific Ion Effects, \(^2\) “there is not a single published work in which a prediction of these values (mean activity coefficients) can be found… it was easier to fly to the moon than to describe the free energy of even the simplest salt solution…”. There are many possible reasons for explaining the non-ideal behavior of electrolytes but the most important one is the long-range electrostatic interactions between charged particles. Theories have been developed to account for this in yielding the non-ideal behavior and some of the important and simple theories will be re-capitulated below. Readers are referred to the excellent textbooks available in this field for more details.\(^1\,^8\)
1.1.1. The Poisson-Boltzmann model and the Debye-Hückel approximation

The Poisson-Boltzmann (PB) model was proposed independently by a French physicist Louis George Gouy and a Britain chemist David Leonard Chapman in 1910 and 1913, respectively. It is the simplest model to describe the distribution of ions adjacent to a charged object. Consider a situation where a charged particle $C$ was brought into an electrolyte solution. For simplicity take the electrolyte to be a 1:1 salt solution. Because of the electrostatic interaction $C$ tends to attract ions of the opposite sign (counterions) and repel ions that are of the same sign (co-ions), resulting in a re-arrangement of the ionic distribution around it. So in the vicinity of $C$, ions are not going to be electroneutrally distributed as without $C$, but will have some order that must decay upon increasing distance from $C$. In the PB model, this distribution of ions at equilibrium is described by the Boltzmann distribution equation:

$$n_i(x) = n_\infty \exp\left(-\frac{w_i}{k_B T}\right)$$  \hspace{1cm} (1.3)

where $k_B$ is the Boltzmann constant, $T$ the absolute temperature, $n_i(x)$ is the concentration of the counterions/co-ions at a distance $x$ away from the charged surface and $w_i$ is the work required to take a given ion from infinity to position $x$. Eq (1.3) formally describes the deviation of local ion concentrations from that in bulk $n_\infty$ and makes it dependent on $w_i$. For point charges, that work is due to the electrostatic interaction and could be expressed as $w_i = z_i e \psi$, where $\psi$ is the electric potential at position $x$ ($z_i$ is 1 for 1:1 salts). This electric potential is not only caused by the presence of $C$ but is also influenced by the rearrangement of the mobile ions around it. The variation of electric potential throughout the solution could in turn be described by the Poisson equation:

$$-\nabla^2 \psi = \frac{\rho}{\varepsilon}$$  \hspace{1cm} (1.4)

where $\rho$ is the charge density and $\varepsilon = \varepsilon_0 \varepsilon_r$ is the dielectric constant of solution (constituted by $\varepsilon_0$ the vacuum permittivity and $\varepsilon_r$ the relative permittivity). The PB model is self-consistent in a sense that the potential is defined by the charge density as in (1.4) but the charge density is defined by the number distribution as in (1.3). Hence, combining those equations mathematically expresses this feature as:

$$\nabla^2 \psi = -\frac{1}{\varepsilon} \sum n_\infty z_i e \left[\exp\left(\frac{z_i e \psi}{k_B T}\right) - \exp\left(-\frac{z_i e \psi}{k_B T}\right)\right]$$  \hspace{1cm} (1.5)
The PB model can be used to calculate the electrostatic potential and charge distribution if the surface charge of C and the bulk salt concentration are both known (and are not too high). But (1.5) is not straightforward to solve since it is a nonlinear second-order differential equation and a complete solution could only be obtained numerically. On the other hand, instructive analytical solutions are accessible for the linearized Poisson-Boltzmann equation if the electric term is small compared to the thermal energy, \( z_i e \psi < k_B T \), in which case equation (1.5) can be approximated as:

\[
\nabla^2 \psi = \frac{\sum n_{\alpha} z_i^2 e^2}{\varepsilon k_B T} \psi = \kappa^2 \psi
\]

Equation (1.6) is called the Debye-Hückel approximation with \( \kappa \) defined as:

\[
\kappa^2 = \frac{\sum n_{\alpha} z_i^2 e^2}{\varepsilon k_B T}
\]

This \( \kappa \) is called the Debye-Hückel parameter and its reciprocal value \( \kappa^{-1} \) the Debye length. The solution of this approximation is simply:

\[
\psi(x) = \psi_0 \exp(-\kappa x)
\]

that is, in the Debye-Hückel approximation the electrostatic potential decays exponentially from the value of the potential \( \psi_0 \) at the surface of C. Without mobile ion, the electrostatic potential decreases with the distance as a slower function. The difference arises because the ions in the electrolyte screen the electric charge on C so that ions further away from C “sense” less net charge (because of the collected counterions). The mobile ions within the order of the Debye length are restricted by C through Coulombic force while ions that located further away remain free. The range of screening region is dependent on the valence and concentration of ions. Note that even though the Debye length is frequently used to give a quantitative impression of the screening length it is not that at \( \kappa^{-1} \) away from C the ions distribute randomly like in the bulk. Only at a distance of 3-4 times \( \kappa^{-1} \) does the electrical potential decay to a negligible value. In colloidal systems, the curvature of the surface of C is usually much larger than the Debye length and therefore the surface can be regarded as a plane. The behavior of the electrical potential near a plane is very important regarding the properties and stability of colloids and is discussed briefly in the next section.
1.1.2. The electrical double layer theory

As is discussed above, there is a spatial dependence of ion concentration and electrical potential near charged interfaces. The resulting separation of positive and negative charges in the solution is universal and occurs for both solid-liquid and liquid-liquid interfaces. The distribution of ions creates in turn regions of varying electrical potential and leads to many interesting interfacial phenomena ranging from the formation of opals to the stability of biological cells and, in general, determines a lot of the physico-chemical properties of interfaces. The generally accepted picture for the ion distribution in electrolyte solutions is called the electrical double layer model. Generally, the surface charge of C (that might be contributed by the charge placed upon the particle, by dissociable functional groups or by strongly adsorbed ions) constitutes the first layer. The second layer is composed of mobile ions that are experiencing both thermal motions and Coulombic interactions with C. Ions in the second layer are not fixed in position and can diffuse toward or away from the surface. Hence, this feature is usually called the diffuse layer. There are several theories concerning the double layer with slightly different definitions and terminologies, such as the Helmholtz double layer, Gouy-Chapman model and its modified version by Stern. Here, we present a widely accepted illustration in Figure 1.1.

Figure 1.1 Schematic representation of electrical double layer at a planar charged surface in an electrolyte solution and the corresponding trend of the electrical potential.

The closest layer to the surface is an ion-free region called Stern layer (0<x<d). This region appears since ions have finite sizes and they cannot approach the surface closer than a distance of less than one ion radius. The Stern layer is usually subdivided into two layers by the inner Helmholtz plane (IHP) and the outer Helmholtz plane (OHP). It was
proposed by Grahame that adsorbed bare ions can only locate at the IHP and the nearest approach of a hydrated ion is at the OHP.\textsuperscript{5, 20} The ions at the IHP may be adsorbed on the surface while the ions at the OHP could only interact with the surface through electrostatic interactions. Hence, the Stern layer is usually in the order of one bare ion radius to one hydrated ion radius which puts it around 0.1-0.5 nm. If one assumes that the concept of permittivity is still valid inside the IHP/OHP, the Stern layer behaves like a simple parallel plate capacitor within which the electric potential drops linearly. Outside the OHP is the diffuse layer. In this layer there is an excess amount of counterions. The ions in this region are in exchange with ions in the OHP and with bulk ions outside the diffuse layer. Ions inside the diffuse layer do not interact with the surface as strongly as ions inside the Stern layer because their interactions are partially screened. The distribution of ions in the diffuse layer can be mathematically described by the PB model above with the surface potential set with the potential of OHP.

So far the ionic distribution around an isolated charged surface in electrolyte solutions is described. In reality, there is more to it. When two charged colloids approach they can interact with each other across the ions between them. If they are not too closely located, the electrical potential between them is the superposition of the electrical potential of each individual surface. Though the surface charge is screened to some extent by the ions in the diffuse layer, there is still electrostatic interaction between charged surfaces and this is called the electric double layer force. The electrical double layer force is repulsive if two charged surfaces have the same sign and is getting smaller with increasing salt concentration. The combination of double layer force with the attractive van der Waals force constitutes the basis for Derjaguin–Landau–Verwey–Overbeek (DLVO) theory that can be used to assess colloidal stability on a quantitative manner. Detailed description about DLVO theory could be found in Verwey and Overbeek’s book\textsuperscript{21}.

1.1.3. Electrokinetic theory
In the previous section electrostatic features at equilibrium were introduced. The charge/potential distributions featuring in that description are typically quite difficult to assess experimentally. Electrokinetic measurements introduced here are often useful for this purpose. In electrokinetic experiments, the charged objects are forced to move relative to each other depending on their electrostatic features. By analyzing the way the
charged object responds to a situation where it is subjected to a force, one can acquire information about the particle and its diffuse layer.

Electrophoresis is one of the most studied electrokinetic effects and played (and still plays) a very important role in electrolyte chemistry and protein chemistry. It refers to the movement of charged particles under an electric field $E$ in a solution. If the electric field is homogeneous and not very large, particles attain almost immediately a constant velocity $v_e$ that is proportional to the strength of the applied electric field. In the expression

$$v_e = \mu_e E$$

(1.9)

$\mu_e$ is the electrophoretic mobility, a property of the charged particle that is independent of the applied field strength. Yet, electrophoretic mobility is not enough to characterize the electric state of a charged particle.

To proceed further, one must consider a property called the $\zeta$-potential. When the charged particle is in motion in an electrolyte solution, there is a thin layer of solvent molecules sticking to the surface and moving together with the particle at the same velocity. This layer is contained within a surface called the slipping plane. The position of the slipping plane is not very well defined but is imagined to be rather close to the particle surface. In reality, this is probably not a sharp boundary and its features are probably best described via suitable simulations. Yet, this simple concept is widely spread and for that reason we elaborate it further. Since not only the solvent but also the ions within the slipping plane are supposed to follow the particle, the electric potential sensed by electrokinetic experiments is the potential at the slipping plane – this is the $\zeta$-potential! A large $\zeta$-potential (and, thereby, a large surface charge density as a root cause) was found to be favorable for colloidal stability because for such systems there are big repulsive forces between particles. If the $\zeta$-potential is small, attractive van der Waals interactions may exceed the repulsive double layer force and cause colloidal flocculation or coagulation. For hydrophilic colloids the $\zeta$-potential is important in experimentally determining the charge and iso-electric point of particles. Because of its complex definition, the $\zeta$-potential is determined by the surface nature of particles, the nature of counterions, the solvent and also the electrolyte concentration.

Even though the description of electrokinetic effects is simplified by introducing the $\zeta$-potential, further complications arise. One of these is (confusingly) called the
electrophoretic effect and describes that under the effect of the electric field the
counterions in the diffuse layer migrate to the opposite direction and, while doing that,
drag solvent molecules with them, which reduces the electrophoretic mobility of the
particle under study. Other effects come from ion-ion interactions at finite
concentrations. It is important to take into account these effects and use the appropriate
models to relate velocity to the $\zeta$-potential. 3,7,8

The electrophoretic effect is related to the relative size of the particle to that of the
diffuse double layer. Typically, this is accounted for by Henry’s formula that applies in
the whole range of that ratio:

$$
\mu_e = \frac{2 \varepsilon_0 \varepsilon_r \zeta}{3} f(kr)
$$

(1.10)

where $f(kr)$ is a monotonically increasing function that approaches 1.5 as $kr \to \infty$ and
1 as $kr \to 0$, where $r$ is a measure of size. Henry’s formula is valid providing the $\zeta$-
potential is small and where the applied electric field does not distort the ionic
atmosphere. For spherical particles with radius $r$, an approximate expression for $f(kr)$

$$
f(kr) = 1 + \frac{1}{2 \left(1 + \frac{2.5}{kr(1+2\varepsilon_r (-kr))}\right)^3}
$$

(1.11)

was derived with relative error of less than 1%. 23 Another limitation of Henry’s formula
is that it remains valid at no surface conduction or surface polarization. Usually the
latter condition could be fulfilled if the surface potential is low (usually one assumes
$\zeta < 50 \text{ mV}$). Another way to check if there is surface conduction is to measure mobility
over a range of electrolyte concentrations. If the mobility does not show a monotonic
trend but a maximum, it is taken as a sign that surface conduction could not be
neglected and Henry’s formula is not valid.

In the limit of $kr \to 0$, Henry’s formula yields:

$$
\mu_e = \frac{2 \varepsilon_0 \varepsilon_r \zeta}{3} \zeta
$$

(1.12)

This is called the Hückel-Onsager equation. In the other limit of $kr \to \infty$, one obtains
instead

$$
\mu_e = \frac{\varepsilon_0 \varepsilon_r \zeta}{\eta}
$$

(1.13)
that is called Smoluchowski’s formula and applies to particles (or pores) of any shape as long as the radius of curvature is much larger than the Debye length. In this latter case, the electric double layer is very thin relative to the particle size and the presence of the particle distorts the externally applied electric field - therefore ions in the double layer sense a distorted electric field. The typically cited quantitative limits for Smoluchowski’s formula are $\kappa r > 250$ and $|\zeta| < 50 \text{ mV}$, a condition that could hardly be met even for large biomacromolecules. In the opposite limit of $\kappa r \to 0$, the diffuse layer is much larger than the particle so most of the ions in the diffuse layer are experiencing an undistorted electric field.

To summarize, when converting the mobility to $\zeta$-potential and interpret the result one must take into account all conditions of the studied system and choose the most appropriate model. Otherwise, large errors can be obtained.

1.1.4. The Debye-Hückel theory of non-ideality

We shall now return to our starting point, the non-ideality of electrolytes caused by electrostatic interactions. The treatment we follow here is termed the Debye-Hückel theory\(^1\) and considers the Coulombic force between a particular reference ion in the electrolyte and the rest of the ions present regarded as its ionic atmosphere. These ions are not randomly distributed around the reference ion but have some kind of order caused by the central electrostatic force. To obtain the ion distribution around the central ion, one can exploit the Poisson-Boltzmann model for a charged spherical particle. One then assumes that the non-ideality arise from the interaction of the central ions and its own ionic atmosphere. Here, the lengthy derivation is omitted and the final result of the electrostatic interaction energy between a reference ion sort and their respective ionic atmospheres is presented as:

$$\Delta G_{\text{excess}} = -\frac{z_i^2 e^2 n_i}{4\pi \varepsilon_0 \varepsilon_r} \frac{\kappa}{1 + \kappa r}$$

(1.14)

where $z_i$ is the ionic charge in units of the elementary charge $e$ and $n_i$ is the number of ions of type $i$. $r$ is the closest distance ions can approach each another and is related to the ionic radii (and, if relevant, ion hydration). By summing over all types of ions and to accounting the pair-wise character of ionic interaction and exploiting Eq 1.14, the mean activity coefficient can be obtained as:
\[ \log_{10} Y_\pm = -\frac{A|z_1 z_2| \sqrt{I}}{1 + B r \sqrt{I}} \]  

(1.15)

where \( I \) is ionic strength defined as \( I = \frac{1}{2} \sum n_i z_i^2 \), and \( A \) and \( B \) are two constants with exact expressions as \( B = \left( \frac{2e^2 N}{\varepsilon_0 e_r k_B T} \right)^{\frac{1}{2}} \), \( A = \frac{e^2 B}{2.303 \times 8 \pi \varepsilon_0 e_r k_B T} \). For water at 298 K, \( A = 0.510 \) mole\(^{-1}\) L\(^{-1}\), \( B = 3.288 \) mmol\(^{-1}\) mole\(^{-1}\) L\(^{-2}\). Roughly speaking, the numerator reflects the long-range electrostatic force between ions while the denominator corrects for the contribution of short-range forces. In very dilute solutions, the correction for the finite size of ions can be ignored and equation (1.15) reduces to

\[ \log_{10} Y_\pm = -A |z_1 z_2| \sqrt{I} \]  

(1.16)

This is the Debye-Hückel limiting law.\(^1\)

Within the Debye-Hückel theory, the activity coefficient of each individual species in an electrolyte can be predicted. Tests against experimental results show that the theory predicts the electrolyte behavior well in the dilute electrolyte range, up to \( I \approx 0.001 \) to \( 0.1 \) mole/L while the Debye Hückel limiting law is valid for \( I < 0.01 \) mole/L for a 1:1 electrolyte.\(^1\) The theory fails at far lower concentrations if doubly-charged ions are present.

The shortcomings of the Debye-Hückel theory are numerous. The theory assumes that ions are spherically symmetric, unpolarizable particles and have no specific interactions with the solvent molecules. The solvent is regarded as continuum and structureless with the only relevant property being its dielectric constant. Hence, solvation of different ions is not accounted for, not even at the level of ion-dipole interactions with polar solvents. Furthermore, the Debye-Hückel theory assumes that electrolytes dissociate completely and ions interact with each other only through electrostatic interactions. This is not true at high concentrations and/or for weak electrolytes. In some conditions ions can form ion pairs or complex other species in the solution which is not accounted for. When these factors are dominant, the Debye-Hückel theory fails to predict even qualitatively the trends of non-ideality.
1.2. Specific ion effects

The features of ions in solution that cannot be described by the classical electrolyte theories are summarily called specific ion effects. Classical electrolyte theories include the Debye-Hückel theory, the double layer theory, the DLVO theory, all of which consider the solvent as a continuum and ions as charged spheres. But this assumption may not be justified. For example, the Debye length for a 1:1 monovalent salt in water is 0.8 nm at 0.15 M, that is the range of electrostatic interaction between two particles is about the size of two water molecules (the diameter of a water molecule is around 0.3 nm). Clearly, water cannot be regarded as continuum at high salt concentrations. On the other hand, according to the Debye Hückel theory the only interaction between ions in solution is electrostatic interaction that in turn only depends on the charge and the size of the charged particles. Hence, ions with the same charge and similar size should behave similarly regarding their solutions in equilibrium state and have similar transport properties. However, a huge number of experiments have convincingly shown that the chemical nature of ions is making a large difference.

![Figure 1.2](image)

**Figure 1.2** (a) Activity coefficients of some alkali bromide solutions at different concentrations. (b) Activity coefficients of some alkali acetate solutions at different concentrations. (Adapted with permission from reference 2. Copyright (2009) World Scientific Publishing Co., Inc.)

As an example, Figure 1.2 displays the activity coefficients of alkali salts as a function of salt concentration for a series of ions. The activity coefficient with methanol was also shown as a non-electrolyte reference. The salts exhibit a much stronger non-ideality.
than non-electrolyte. At infinite dilution, all salts have the mean activity coefficient $\gamma=1$. As the salt concentration increases, the activity coefficients become smaller for all of them, as predicted by the Debye-Hückel theory. Upon further increase of salt concentration, above around 0.1 M, the activity coefficients start to increase, yet on a manner that strongly depends on the ion sort. Yet, this is not a simple ion size effect since their order reverses when the anion is acetate instead of bromide! This behavior is totally out of the range that classical theory is able to predict. This is only one case with the simplest example from aqueous salt solutions.

Specific ion effects were observed in many other systems such as solutions of organic molecules, surfactants, polymers, proteins, complex mixtures, colloids and biological systems, almost everywhere when electrolytes are involved in chemical and biological process. New specific ion effects are still being reported. Despite its ubiquitous presence and its wide-ranging influences, this phenomenon has been largely ignored for a long time. Even today, they are often explained with fleetingly mentioning the general concept of “specific ion effects” without any further exploration of the underlying reason. It is only during the past decades that suitable attention has been paid to the specific ion effects at air/water interface, near small molecules, polymers, proteins, colloid particles and designed surfaces with different charge density or hydrophobicity. This attention materialized in theoretical and simulation attempts (see below) as well as experimental tests by many techniques including thermodynamic and, spectroscopic methods.\textsuperscript{24,43}

Experimental tests usually rely on measuring the concentration dependence and/or the cation or anion species dependence of properties, including simple ones, like the density, viscosity, heat capacity, activity coefficient, osmotic pressure, surface tension, etc. One attempt to quantify the specific ion effects in colloidal system is to use the parameter lyotropic number,\textsuperscript{44} connected to the surface charge density of ions. The lyotropic number is believed to be the key factor determining the adsorption strength of ions. Anions usually have larger effects than cations. In many occasions, the efficacy of ions to induce changes follows another and more famous unique order—the Hofmeister series. This is ubiquitous for biological macromolecules and is believed to be responsible for many physiological relevant processes. The details of the Hofmeister series and possible mechanism will be discussed in sections 1.2.3 and 1.2.4. Yet, specific ion effects not following any of these orders are not uncommon. “Reversed order”, “partially reversed order” and even completely different order are continually popping up in
different systems, especially for electrolytes in non-aqueous solutions. The reasons remain unclear. The scope of the work in this thesis includes specific ion effects. In particular, we aimed at specific ion effects near neutral macromolecules, in both aqueous and non-aqueous solutions.

1.2.1. Ion pairs

Electrolytes exist in a solvent not only in dissociated form and non-dissociated form but also somewhere in-between: in loose or tight association with ions of opposite charge. These are held together mainly by electrostatic interaction and move around together as a new entity. Ion pairs are different from a complex: they do not need to have special chemical affinity for each other. Usually, there is no preferred coordination either. Ion pairing is a dynamic process and the paired ions may have quite short lifetime and could fall apart after several collisions. One way to define an ion pair is to say that its lifetime is longer than the time its components need to diffuse away from each other. Free ions and ions in paired form are in equilibrium as for example

\[
\text{Mg}^{2+} + \text{SO}_4^{2-} \rightleftharpoons (\text{Mg}^{2+}\text{SO}_4^{2-})
\]

\[
\text{Ca}^{2+} + \text{OH}^- \rightleftharpoons (\text{CaOH})^+
\]

The ion pair could be neutral or charged (if created by ions of different valences) and can have multiple forms coexisting in a solution at the same time. When two ions keep their individual solvation shells intact in the ion pair they are said to form a solvent-separated ion pair. If two bare ions form an ion pair, it is termed contact ion pair. Two ions can also get rid of part of their solvation shells and form a solvent-shared ion pair. Take MgSO\textsubscript{4} for example: it was indicated to exhibit solvent-separated ion pair, solvent-shared and contact ion pair and even (Mg\textsubscript{2}SO\textsubscript{4})\textsuperscript{2+} in aqueous solution.\textsuperscript{45} Association constants are used to characterize the ion pairing ability. For Mg\textsuperscript{2+} + SO\textsubscript{4}\textsuperscript{2-} \rightleftharpoons (Mg\textsuperscript{2+}SO\textsubscript{4}\textsuperscript{2-}) the association constant \(K_a\) is

\[
K_a = \frac{c(Mg^{2+}SO_4^{2-})}{c(Mg^{2+})c(SO_4^{2-})}
\]

(1.17)

Yet, as methods to quantitatively and separately characterize the nature and amount of ion pairs present are rare, such association constants may have dubious value.
The tendency to form ion pair depends on the nature of ions and the solvent. Ions with higher charge and smaller size exhibit stronger electrostatic interactions and can form ion pairs easier. Solvent acts as a medium that reduces electrostatic interaction and therefore ion pair formation is more common in solvents of low dielectric constant. Most divalent cations were shown to form ion pairs in organic solvents. Ion pair formation can be highly ion specific. For example, metal ions like Ni$^{2+}$, Cu$^{2+}$, Co$^{2+}$, Zn$^{2+}$, Mg$^{2+}$ all have the same charge and similar ion radii. But when they interact with glycinate, a large variation of association constants were found, ranging from $2.75 \times 10^3$ to $4.2 \times 10^8 \text{ M}^{-1}$. Paper VII in this thesis investigated the ion pairing ability quantitatively.

1.2.2. Ion-ligand complexation

Ions can form complexes with neutral or charged molecules called ligands (glycinate in the previous example could be seen both as a counterion but also as a ligand – ion pairs and complexes are partly overlapping categories). The complexed entities are usually metal ion, especially transition metal ions. The ligands or ligand parts associating to the central ion often have lone pair(s) of electrons that could act as donors to the metal ion. The number of ligands attached is called the coordination number, the maximum coordination number of which is determined by the electronic configuration of the metal ion. For example, Cu$^{2+}$ has a maximum coordination number of four. The coordination number is also influenced by the steric hindrance for ligands arranged in the space around the central ion. Complexation is normally assumed not to involve covalent bonding but weaker association bound and the complex is thereby in equilibrium with free ions/ligands. For multiple ligands, the equilibrium can be expressed as stepwise association

\[ M(aq) + L(aq) \overset{K_1}{\rightleftharpoons} ML(aq) \]
\[ ML(aq) + L(aq) \overset{K_2}{\rightleftharpoons} ML_2(aq) \]
\[ \ldots \]
\[ ML_{n-1}(aq) + L(aq) \overset{K_n}{\rightleftharpoons} ML_n(aq) \]

\[ K_1 = \frac{[ML]}{[M][L]}, \quad K_2 = \frac{[ML_2]}{[ML][L]}, \quad \ldots, \quad K_n = \frac{[ML_n]}{[ML_{n-1}][L]} \]  \hspace{2cm} (1.18)
with M representing metal ion concentration and L ligand concentration while $ML_n$ is the complex of one metal ion with n ligands and $K_i$ is the association constant of the i-th step of association. In some cases, the association equilibrium is better expressed by a joint association constant $\beta$ for

$$M(aq) + nL(aq) \Leftrightarrow ML_n(aq)$$  \hspace{1cm} (1.19)

where

$$\beta = \frac{[ML_n]}{[M][L]^n} = K_1K_2K_3 ... K_n.$$  \hspace{1cm} (1.20)

Ion-ligand complexation is important in many fields like protein precipitation, as well as protein crystallization, protein folding, colloidal stability, and gel swelling. Complexes of transition metals are frequently used a lot as catalysts in diverse fields. For example, Rh, Ru and Ir and their complexes are often exploited as water oxidation catalysts.

Complexation is often highly ion specific yet activity and selectivity may not be totally understood. Clearly, the association constants depend on the charge and size of the metal ion as in the ion pair formation process because the ion-ligand interaction is dominantly electrostatic in nature: the positively charged metal ion attracting the negative charged electron density within the lone electron pair of the ligand. Yet, it is also affected by other factors like steric effect etc. The association constant, either joint or stepwise, is related to the Gibbs free energy of formation by

$$\Delta G = -RT\log e K.$$  \hspace{1cm} (1.21)

To suitably define association constants requires one to know the stoichiometric relations. This could be measured by conductivity, calorimetric methods, x-ray crystallography, chromatography and spectroscopic methods, all quite good for strong association. When it comes to weak association, the accuracy is usually not good enough, especially when the ligand is a macromolecule. One reason is that it is difficult to define the binding sites for macromolecules. This represents a problem both for homogeneous polymer chains (with many identical options along the chains to bind to) and for folded heterogeneous chains like proteins where ions have preference for specific sites. When binding to a macromolecule is weak, barely any change can be observed irrespective of the experimental method so it is difficult to locate the binding sites or to quantify binding. Thus, in many cases the binding constants of ion-
macromolecule complexes are based on questionable stoichiometry. The work in this thesis provides an alternative method to characterize the ion binding to macromolecule that is applied to several systems.

1.2.3. The Hofmeister order

In many effects caused by ions, the relative size of the effect of very different nature varies with either the anions or cations within salt species on a similar manner! This order is called Hofmeister series; often, the specific ion effects are (mistakenly) called Hofmeister ion effects. The first systematic study of specific ion effects was by Franz Hofmeister in the 1890s in a series of papers called ‘About the science of the effect of the salts’. In his work he noticed that different salts have different efficacy to precipitate proteins. That first systematic investigation revealed that the salts could be arranged in an order according to their precipitation power irrespective of the proteins, such as hen egg globulin and blood serum. In the following years, this Hofmeister order, exemplified in Fig. 1.3, was re-occurring in simple aqueous electrolyte solution with the activity coefficient, viscosity, heat of solution, refractive index, density, osmotic pressure, etc, all showing significant specific ion effects that followed the Hofmeister order. Hofmeister order is particularly prevalent and dominant in colloidal system where interfacial phenomena are such as surface tension, chromatographic selectivity, the enzymatic activity, ion selectivity of membrane channels, colloidal stability, and protein denaturation.

![Hofmeister series](image)

Figure 1.3 A typical arrangement of ions in anionic and cationic Hofmeister series.

When the Hofmeister series is displayed as in Fig. 1.3, the axis is conventionally marked toward “salting in” and “salting out” species. This arrangement relates to ion effects in
protein precipitation – salts with ions on the left side stabilize protein and decrease the solubility of nonpolar species and while salts with ions on the right side tend to increase the solubility and are usually denaturants. For some time, the existence of Hofmeister effect was attributed to different abilities of ions in arranging water molecules. In particular, the increase/decrease of the viscosity of aqueous solutions along the series were interpreted so that ions on the right side of the anion series have relatively weak ion-water interaction (note: opposite for the cations) compared with water-water interaction while anions on the left side have strong ion-water interactions. Based on that it was believed for a long time that ions could change the structure of water on macroscopic scale and, depending on how ions impact water, some ions are “water structure builders” or kosmotropes and others are “water structure breakers” or chaotropes. With the development of new experimental techniques together with advances in simulations, it could be shown that this picture is not correct and the perturbation caused by ions in water structure extends no further than three layers of water. Yet, the names kosmotrope and chaotrope remained. Currently we have no unifying and generally accepted theory and it looks uncertain if there is one at all. Some of the main proposed mechanisms will be reviewed below.

1.2.4. Review of the mechanisms of specific ion effects

The law of matching water affinity

The law of matching water affinity was proposed by Kim D. Collins to explain the specific ion effects in ion pairing. Collins found that salts composed of ions with similar size have lower solubility in water while salts with different ion size are readily soluble. In addition, when the absolute heat of solution (enthalpy of dissolution) of crystalline alkali halides is plotted as a function of hydration enthalpy difference the result is of volcano shape with a distinct maximum. Based on that it was proposed that the ion-water interaction governs ion-pairing preferences. This is not accounted for in the classical theory where the ions are often treated as point charge and their interaction with solvent molecules is often neglected.
The size effect was ascribed to ions with similar surface charge density interacting with water at similar energy gain/cost. Hence, interaction strength follows the trend small ions-water>water-water>large ions-water. When water is replaced with a small ion of opposite charge, the two ions become closer so that their interaction energy more than compensates for the associated entropy loss. Similarly, large cations and large anions also tend to form ion pairs in water but not because of strong cation-anion interactions but because their reduced interaction with water has low cost. In Hofmeister terms, chaotrope-chaotrope and kosmotrope-kosmotrope ions tend to form ion pairs. This conclusion is not only applicable to ions but could also be extended to polar molecules. Functional groups and side chains of proteins could also be classified as chaotrope or kosmotrope according to their binding ability with ions in water. For example, a carboxylic group is kosmotropic and an amide based functional group is chaotropic. In that way, specific ion effects in proteins could be attributed to the ability to associate with ions of matching absolute enthalpy. Though the law of matching water affinity is frequently referred to, there are only a few reports that report such volcano-shaped trends, and those observations are limited to cations.  

Figure 1.4. Relation of the standard heat of solution of crystalline alkali halides (vertical axis) to the difference between the enthalpies of hydration of the constituting anions and cations in gaseous form (horizontal axis) \(^{60}\). (Reprinted with permission. Copyright 2007 The Biophysical Society)
Solute Partition model (SPM)

Pegram and Record developed a thermodynamic solute partition model. In their model they regard the surface as a separate microphase of finite thickness, with a different local water structure that is determined by the chemical nature of the surface. Based on surface tension or solubility data they then quantified the thermodynamic interaction potential of each individual Hofmeister ion with the surface by determining the partition coefficient between the surface and the bulk. Ions that tend to accumulate at the air/water surface have, for example, high relevant partition coefficients and larger influence on surface tension. Even more interestingly, a good correlation was found for partition at the air/water and protein/water interfaces: those ions that accumulated favorably at the air/water surface tend to remove the water from the protein surface and keep them “salting in” in water while ions that are excluded from air/water surface are also excluded from the protein surface leading to “salting out”. Both trends could be explained by the individual ion’s ability to remove water from the surface or interface to the bulk water.

As is shown in Figure 1.5, route A represents the transfer of a hydrated ion from bulk to the air/water surface. Via this process, the ions distribute between the bulk and the surface-near microphase. On one hand, the surface has low dielectric constant which
promotes the formation of new solvation layers around the ion. On the other hand the ion has to be partially dehydrated in order to be present in the surface region. So the partition of ions depends on the balance between these two driving forces. The same is valid for ions at the bulk-protein surface, which is depicted in Route B. But for that surface the local microphase is heterogeneous in nature. Proteins have both charged regions and nonpolar regions which can interact very differently with ions in addition to the solvation and dehydration effects. Hence, the model proposes that as long as the ion-protein interaction is not the dominating force, the Hofmeister effect involving the bulk-protein interface resembles to that at the air/water surface. This model has been used to analyze and interpret thermodynamic consequences of Hofmeister effects on surface accumulation/exclusion of small solutes, solubilities of hydrocarbons and amides, DNA binding and DNA duplex formation, protein folding and nucleic acid melting, and protein aggregation and phase separation.

**Dispersion force**

In reference to the DLVO theory mentioned above, Boström, Ninham and their coworkers proposed that ion specific short-range dispersion force is the one factor that is missing from current theories. In particular, they considered that it depends on the ion concentration, the polarizability and the electron affinity of the ion, which are determined by the electronic structure of the ion. At low concentration, the electrostatic interaction dominates but at higher concentration the electrostatic interactions are largely screened and dispersion forces take over. Their work shows that adding the dispersion potential between ions and the interface can significantly change the calculated distribution of ions near surfaces and the forces between them. The theory was shown to be able to account for the ion-specific surface tension at the air/water(oil) surface, the counterion condensation near polyelectrolytes, the surface tension and surface potential of biological membranes, binding of peptides to membranes, and ion-specific effects in pH measurements.

**Direct ion interaction with a macromolecule and its hydration shell**

Cremer and his coworkers have proposed this mechanism for explaining salt effects on the lower critical solution temperature (LCST) of poly (N-isopropylacrylamide)
They found a linear salt-concentration dependence of LCST of PNIPAM for kosmotropes and a nonlinear relation for chaotropes. Good correlation was also found between the degree of salt dependence and the hydration entropy or the surface tension of different ions, which they took as a sign for direct ion interaction with the PNIPAM molecule and its hydration shell.

There are three mechanisms, summarized in Fig. 1.6, that were implicated in the chain collapse and resulting LCST of PNIPAM. The first mechanism is that kosmotropes could polarize the water molecules that are hydrogen-bonded to the carbonyl and amide moieties on the side chain. The overall effect is dehydration, particularly that of the amide group. This is supported by the observation that the salt dependence for kosmotropes shows good correlation with the hydration entropy of individual ions. Chaotropic ions usually have smaller polarizing power so they are not able to perturb adjacent water.

The second mechanism is a continuation of the first step on dehydration process. With increasing salt concentration, the dehydration of amide moiety gets stronger and the surface tension between this moiety and the backbone keeps increasing until at some point a microscopic phase separation of the amide group occurs while the rest of the chain, backbone and isopropyl group, remained hydrated. It is this partially collapsed chain that facilitates the phase transition of the whole chain. This mechanism is both for kosmotropes at high salt concentration but also for chaotropes. In electrolyte solutions simple inorganic ions usually increase the surface tension by \( \gamma = \gamma_0 + m \sigma \).
ions could also decrease the water surface tension) where $\gamma_0$ is the surface tension of pure water, $\gamma$ is the surface tension of salt solution, $m$ is the molar concentration of salt and the coefficient $\sigma$ is the molar surface tension increment. This value is often used to characterize the hydrophobic interaction. As the backbone and isopropyl group are hydrophobic while the amide functional group is hydrophilic, ions with higher molar surface tension increase the surface tension on PNIPAM water surface thereby tend to precipitate PNIPAM more easily. Correlation between molar surface tension and salting out/in was found only for chaotropes and concentrated kosmotropes. For dilute kosmotropes there is no such relation, the reason of which remains unclear. The third mechanism is direct ion-polymer interaction. The anion is believed to bind directly to the amide group of PNIPAM so the chain associated with it helps to keep the water shell around the side chain and to salt in the molecule. The binding of anion to the side chain is Langmuir-isotherm type.

While this proposed mechanism was used many times for rationalizing the Hofmeister effect in surfactants, protein and peptides, it lacks solid theoretical foundations (as is solely based on the correlations observed), a quantitative mathematical model, and simplicity (as it required three separate types of interaction).

**Molecular dynamics simulations**

Thus far, not a single proposed mechanism is able to describe anywhere near the full spectrum of experimental evidence for Hofmeister behavior. It is thereby possible/likely that there is actually no single and unifying mechanism but instead a complex mixture of contributions. Hence, molecular dynamics simulation augmented with suitable representation of intermolecular interactions can be the valid tool to test and predict Hofmeister-type behavior. Simulations have a broader ability to validate possible mechanisms also because interconnected pieces of information, such as the ion distribution profile, the water density, the intermolecular interactions and other physical chemical properties such as the surface tension, the surface potential, etc can be jointly analyzed. Another advantage with simulations is that they could explore perfect ideal surfaces. Although proteins are more interesting for practical purposes, they are usually not good objects for understanding the physical chemical mechanisms both because of their heterogeneous nature and their complex secondary structure. A large number of
simulations with ideal model surfaces have already provided in recent years remarkable contributions to understanding the Hofmeister mechanism. 36-40
2. Experimental part

2.1. Principles of NMR

A rotating object possesses a property called angular momentum. It is represented by a vector that points along the axis around which the object rotates. Elementary particles like proton, neutron, electron, photon, all have angular momentum. For several of them, this appears not because of rotation of a spatially extended body, but is instead an intrinsic property of the particle. This intrinsic property is called spin. It is known from quantum mechanics that angular momentum is quantized, so is the spin. It is characterized by a spin quantum number $I$ that could be either integer or half integer depending on the particular particle. Regarding nuclei, $I$ depends on the nuclear composition. For example, $^2$H has one proton and one neutron, and a total spin quantum number $I = 1$. In general, nuclei with even number of protons and neutrons have $I = 0$ and are not NMR-active.

Particles can also have another property, a magnetic moment. For each elementary particle and for each atomic nucleus, the magnetic moment $\mu$ and the spin angular momentum $J$ are precisely related as:

$$\mu = \gamma J$$ (2.1)

where $\gamma$ is called gyromagnetic ratio that can be either positive or negative. If one places the particle in a magnetic field, the magnetic moment interacts with the field with an interaction energy given by $E = -\mu \cdot B$. The scalar product in this expression indicates that the relative orientation of magnetic moment/angular momentum with the magnetic field results in different interaction energies. The orientation of the spin and, thereby, the magnetic moment relative to the external field is characterized by another spin quantum number $m$ that takes values from $-I$ to $I$ in integer steps. As the interaction energy is orientation-dependent, and the orientation is quantized by $m$, the interaction energy is also going to be quantized. In the absence of magnetic field, all spins with the same $I$ have the same energy. When a magnetic field is applied, available energies for the spins split into $(2I+1)$ slightly different energy levels signifying the different orientations. This is called Zeeman splitting. NMR is the spectroscopy that observes and exploits the Zeeman splitting for nuclear spins.
Knowing that spin is both a magnetic moment and an angular momentum, it is now
time to consider its dynamics inside the magnetic field. If a magnetic moment is not
aligned with an external magnetic field, it experiences a torque:

\[ \vec{L} = \vec{\mu} \times \vec{B} \]  \hspace{1cm} (2.2)

Classical physics yields that the time derivative of angular momentum \( \vec{J} \) is determined by
the torque \( \vec{L} \) applied to it:

\[ \frac{d\vec{J}}{dt} = \frac{d(\vec{r} \times m \vec{v})}{dt} = \frac{d\vec{r}}{dt} \times m \vec{v} + \vec{r} \times \frac{d(m \vec{v})}{dt} = \vec{r} \times m \vec{a} = \vec{r} \times \vec{F} = \vec{L} \] \hspace{1cm} (2.3)

For an object that possesses both angular momentum and a magnetic moment, these
two equations jointly yield

\[ \gamma \frac{d\vec{J}}{dt} = \frac{d\vec{\mu}}{dt} = \gamma \vec{L} = \gamma \vec{\mu} \times \vec{B} \hspace{1cm} \text{integrate over all spins} \hspace{1cm} \frac{d\vec{M}}{dt} = \gamma \vec{M} \times \vec{B} \] \hspace{1cm} (2.4)

which results in a rotation around the direction of the magnetic field at a constant
angular frequency:

\[ \vec{\omega} = -\gamma \times \vec{B} \] \hspace{1cm} (2.5)

This particular mode of motion is called precession and the particular frequency is
termed resonant frequency or Larmor frequency. A schematic Figure 2.1 illustrates
precession.

Figure 2.1. Illustration of precession of a single spin in the magnetic field.
As a spin precesses it sweeps a cone at a constant angle relative to the magnetic field. The angle of procession is determined by the initial direction of spin. If the spin direction is parallel to the direction of the magnetic field (henceforth called the z axis), it stands still.

In NMR we do not detect the behavior of a single spin, but the net effect of the whole ensemble of nuclear spins in the sample. Besides \( B_0 \), nuclear spins also feel small fluctuating fields created by molecular motion and the coupling of spin to their environment (electrons and other spins). Hence, the orientation of individual spins can change. Given enough time spent in the magnetic field, the spin system (all spins in a sample) reaches a new thermal equilibrium state in which there is a slight population difference of spins aligned with and against \( B_0 \). The integration over all the sample spins yields a net nuclear magnetization along the magnetic field. The build-up process of this is called longitudinal relaxation. If we excite or interact with a spin system so that it leaves its thermal equilibrium state, its magnetization along the z-axis comes back to the thermal equilibrium state through this relaxation mechanism. This process can also be described by the one term in the so-called Bloch equation:

\[
\frac{dM_z}{dt} = -\frac{M_z - M_0}{T_1}
\]  

(2.6)

where \( M_0 \) is the equilibrium magnetization, and \( M_z \) is the z component of net magnetization at time \( t \), while \( T_1 \) is the longitudinal relaxation time. Its counterpart is transverse relaxation time \( T_2 \). Transverse relaxation is the process by which any magnetization in the x-y plane (the plane perpendicular to the z axis) disappears after having been created by some excitation process since in the equilibrium state the only magnetization is parallel to \( z \) after perturbation. The behavior of the magnetization in the transverse plane is described by the other terms of the Bloch equation:

\[
\begin{align*}
\frac{dM_x}{dt} &= -\frac{M_x}{T_2} \\
\frac{dM_y}{dt} &= -\frac{M_y}{T_2}
\end{align*}
\]  

(2.7)

Both relaxation mechanisms are affected by fluctuating couplings. The reason why spin couplings such as the dipole-dipole interaction, quadrupole interaction and chemical shift anisotropy interaction fluctuate is mainly a consequence of molecular motions. Additional terms are needed to take into account other factors such as diffusion.
The solution of the Bloch equations is precessing magnetization in the x-y plane that diminishes by the time constant $T_2$. NMR detects the precession of the bulk magnetization since that generates a voltage in a coil that surrounds the sample. To set suitable initial condition for precession, the magnetization must be tilted away from the z axis. This is achieved by applying a time-dependent magnetic field $B_1$ in the x-y plane. The time-dependence is typically periodic, and to have an effect its frequency must be suitably close to the Larmor frequency. The effect of such a field switched on for a period of $t_p$ is to turn the magnetization by a specific angle:

$$|\theta| = |\omega t_p| = |-\gamma B_1 t_p|$$  \hspace{1cm} (2.8)

called the flip angle. This feature is called in NMR the radiofrequency (rf) pulse. Besides its length in degrees it is also characterized by its so-called phase that decides around which axis is the magnetization tilted.

In order to have the maximum signal one applies a 90˚ rf pulse to an equilibrium sample by which $M$ is tilted by 90˚ into the x-y plane. Precessing there, the magnetization creates a time-dependent voltage that, after having been recorded, constitutes the NMR signal. The characteristic frequency within the signal is around the Larmor frequency. Small deviations from that frequency appear because, for example, the electronic environment changes the magnetic field at the site of the atomic nuclei. This latter feature is called chemical shift and is the major contributing reason for the appearance of NMR spectra. For practical reasons, the time-dependence of the NMR signal is measured with reference to an average frequency and thereby we only detect the relative frequencies contained in the NMR signal that is usually referred to as free induction decay (FID). A typical NMR pulse sequence together with FID is shown in Figure 2.2. Fourier transformation of the FID signal provides the NMR spectrum.
As mentioned earlier, longitudinal relaxation affects the $z$ magnetization while, as we discussed, NMR signal requires that magnetization to be turned into the $x$-$y$ plane. For this reason, the method for measuring the longitudinal relaxation time $T_1$ is based on the inversion recovery pulse sequence $180^\circ - \tau - 90^\circ$. Here, the $180^\circ$ pulse first turns the magnetization to $-z$ axis, and then during the period $\tau$ the magnetization recovers toward its equilibrium state. To follow this recovery, the $90^\circ$ pulse creates the signal that will be proportional to the value of the $z$-magnetization at time $\tau$. By repeating the experiment several times at different $\tau$ values the time dependence is obtained from which $T_1$ can be extracted. In contrast, the $T_2$ relaxation time is measured by another method called spin echo. To comprehend the NMR phenomenon mathematically and in full detail, the readers are referred to suitable textbooks.\textsuperscript{78-80}

### 2.2. Diffusion NMR

Diffusion is the stochastic motion of molecules in liquid. In an isotropic homogeneous solution, the random motions driven by the internal thermal energy is called self-diffusion. Another kind of diffusion is mutual diffusion. It is driven both by the internal thermal energy and the concentration gradient. These two values are quite different at intermediate solute concentration range but in the dilute limit, mutual diffusion is approaching self-diffusion. Measuring the self-diffusion coefficient $D$ is becoming interesting for more and more studies since it provides information about the overall molecular size, shape and also about the structural changes and molecular association.

Typically, experiments do not measure the diffusion coefficient directly but the resulting probability distribution of molecules that, in our case, contain the nuclear spins. In homogeneous bulk material the probability distribution for reaching a new position $r_1$ for a point-like initial state at $r_0$ after time $t$ is:

$$P(r_0, r_1, t) = (4\pi D t)^{-\frac{3}{2}} \exp \left(-\frac{(r_1-r_0)^2}{4Dt}\right)$$

(2.9)

$D$ is the only parameter that characterizes this probability distribution. The longer the diffusion time, the wider the probability distribution and, in three dimensions, the mean square displacement is proportional to the diffusion time $t$ and diffusion coefficient as:

$$\langle (r_1 - r_0)^2 \rangle = 6Dt$$

(2.10)
All discussion below is for free diffusion, that is motion not being restricted by the boundaries. For the latter case, readers are referred to Callaghan and Price books for more detailed discussion. For free diffusion, the relation between the diffusion coefficient and the molecular structure is given by the Debye-Einstein theory $D = \frac{k_B T}{f}$, where $k_B$ is the Boltzmann constant, $T$ is the temperature and $f$ is the frictional factor where $f$ is determined by molecular size, shape and the neighbouring environment. For a spherical particle of hydrodynamic radius $r$ in a solution of viscosity $\eta$, the frictional factor is $f = 6\pi \eta r$ which yields the Einstein-Stokes equation:

$$D = \frac{k_B T}{6\pi \eta r} \tag{2.11}$$

The technique we present below by which NMR accesses the self-diffusion coefficient is readily accessible at most NMR facilities and with no specific requirements, such as isotope labeling. It is especially useful for investigating systems with multiple components where the chemical shift provides the way to measure diffusion for each separate component. The most widely used diffusion NMR technique is based on pulsed field gradients on which we focus in the following text.

Recall that the Larmor frequency is proportional to the external magnetic field. If $B_0$ is the same over the whole sample volume, all spins have the same Larmor frequency. But when the magnetic field strength is spatially dependent, such as $B(z) = B_0 + g \cdot z$, where $g$ is called the gradient, spins at different positions precess at different frequencies $\omega(z) = -\gamma B(z)$. Hence, if the gradient, for simplicity, is such that the field varies only in the $z$ direction and is switched on for a duration $\delta$ (this is called a gradient pulse) the position $z$ of all spins are encoded in their different relative phases of precession:

$$\phi(z) = -\gamma B(z)\delta = -\gamma g z \delta \tag{2.12}$$

In this expression, $g$ is the magnitude of gradient, also called the gradient strength.

The phase factor gained during a gradient pulse forms the basis for diffusion NMR based on spin echo methods. The basic pulse sequence is illustrated in Figure 2.3.
In this pulse sequence, the 90° pulse turns the magnetization to x-y plane. Over the subsequent gradient pulse, spins at different positions precess differently and gain phase factor $\phi = \gamma g z \delta$ that depends on their position at the time of the gradient pulse. The net nuclear magnetization at this moment is the vector sum over all spins pointing in different directions, which yields zero. Then the 180° pulse reverses the distribution of the nuclear magnetization components in the x-y plane so that the sign of the gained phase factors changes. Assuming all spins retain their position during time $\Delta$, the spins gain the same phase factor as during that of the first gradient pulse that sets their total gained phase to zero $\phi(2\tau) = 0$. In other words, all spins point in the same direction in the x-y plane after the second gradient pulse. This means large net magnetization and thereby large detected NMR signal. This re-appearance of the NMR signal is the ‘spin echo’.

The condition of having zero total phase is based on the molecules containing the spins not having moved during time $\Delta$. Yet, if molecules do move, they contribute to the magnetization in the x-y plane with different relative phases. The signal, again proportional to the sum of all magnetization from all molecules, can be expressed as an integral over all possible displacements, whose probability is expressed by equation (2.9). Hence, one obtains the expression which shows that the signal is attenuated as a consequence of diffusion described by:

$$E(\Delta) = \iint P(r_0, r_1, \Delta) \exp[i \gamma g (r_1 - r_0) \delta] dr_0 dr_1$$

(2.13)

Assuming the system is homogeneous so that the starting point $r_0$ makes no difference, Eq (2.13) could be simplified as:
\[ E(\Delta) = \iint P(R, \Delta) \exp(i\gamma g R \delta) dR \]  

(2.14)

It is obvious that the signal attenuation and the phase distribution functions are Fourier conjugates. Hence, one obtains the expression:

\[ E = \exp \left( -\frac{\phi^2}{2} \right) = \exp \left[ -\gamma^2 g^2 D \delta^2 (\Delta - \frac{\delta}{3}) \right] \]

(2.15)

In the short gradient pulse (SGP) approximation one assumes that the gradient is so short that there is no significant molecular displacement during it. In the SGP limit equation (2.15) simplifies to:

\[ E = \exp(-\gamma^2 g^2 \delta^2 D \Delta). \]

(2.16)

By fitting these expressions to the experimental signal attenuation upon increasing gradient strength \( D \) can be obtained. It must be stressed that this result is valid for an isotropic diffusion. In case of restrictions and/or anisotropy more complex results are obtained.

Although the signal attenuation caused by relaxation is well separable from the diffusion-induced attenuation, the diffusion experiment introduced above is still restricted by the transverse relaxation time compared to which the diffusion time cannot be too long otherwise the signal is lost. To remedy this situation, the stimulated echo pulse sequence was introduced with three rf pulses as shown in Figure 2.4.

\[ \begin{align*}
\pi/2 & \quad \tau_1 \quad \pi/2 \\
\pi/2 & \quad \tau_2 \quad \pi/2 \\
\delta & \quad \delta \\
\Delta & \quad \Delta
\end{align*} \]

Figure 2.4. A pulse sequence of stimulated echo type.

In the stimulated echo, the diffusion time includes periods during which either the transverse \( \tau_1 \) in Fig. 2.4 or the longitudinal \( \tau_2 \) relaxation is dominant. Usually \( \tau_1 \) is very short and just allows a gradient pulse to be applied, so the majority of signal
evolution time happens under the effect of longitudinal relaxation. Since $T_1$ is longer, sometimes very much so, than $T_2$, the signal that is left after a given diffusion time is more than that in the spin echo experiment in Fig. 2.3, even though the stimulated echo signal without gradient is only half of that for the spin echo.

Diffusion is not only useful in characterizing molecular properties, but also a very good tool in analyzing and separating components in an unknown mixture based on their different diffusion coefficients. The technique used is called diffusion-ordered spectroscopy (DOSY). Basically, this consists of any of the diffusion experiments presented above but the signal processing and presentation differs. After having obtained a stack of spectra that are attenuated to different degrees under different gradient strength, one applied inverse Laplace transform to extract the attenuation spectrum for the signal at different NMR frequencies. The result is a two dimensional (2D) spectrum with chemical shift in the first dimension and diffusion coefficients and their distribution in the second dimension. This method is very useful in identifying different components in mixtures, especially when phenomena like molecular binding and association occurs. Many successful applications exist in biofluids, colloid science and drug development.

2.2.1. Electrophoretic NMR (eNMR)

eNMR is possible thanks to the ability of the methods using pulsed field gradients to detect the displacement of NMR active species in the gradient direction. Stejskal who invented current diffusion NMR methods was also the first to propose the possibility to measure flow by PGF NMR method. In his paper in 1965 he carefully described the theoretical principle and got the exact relationship between the suitable experimental parameter and flow. Despite the fact that the principle was clear, the first attempt to measure flow including electrokinetic flow with gradient-based NMR methods was in 1972. It was not until ten years later the first successful and quantitative measurement of ionic velocity was conducted in a German group by Holz and Müller. In the following years continued efforts extended the eNMR technique from proton to other nuclei, like $^7\text{Li}$, $^{133}\text{Cs}$. Both the configuration of cell and the data analysis methods were modified and improved in order to overcome the difficulties encountered. The term ‘electrophoretic NMR’ was actually introduced by Johnson and his coworkers and they also performed 2D eNMR measurement with potential for determining the
content of mixtures. The methodology has undergone continuous improvements and becoming an important tool to investigate simple electrolytes, surfactants, polymers, micelles, biomolecules and ionic liquids. Several good reviews exist.\textsuperscript{90,91}

In an eNMR experiment an electric field is applied parallel to the direction of the applied gradient. The electric field sets the charged particles into motion and they attain a velocity along the gradient direction that one intends to measure. The velocity is related to other electrokinetic parameters like electrophoretic mobility, zeta potential and surface charge density as discussed in section 1.1.3.

In contrast to the diffusion process where the displacements and thereby the acquired phase factors (see above) are random, the flow is a coherent motion. Hence, the positions of the spin-bearing species are all changed by the same amount and therefore the total magnetization gains a net phase shift connected to the net displacement during the diffusion time. In case of flow with a constant velocity $v_e$, the displacement during time $\Delta$ will be $v_e\Delta$ that results in a phase factor

$$\phi = \gamma g \delta v_e \Delta$$

that leads to a complex modulation of the NMR signal in the form

$$S(2\tau) = M_0 \exp\left(-\frac{2\gamma}{T_2}\right) \exp(-\gamma^2 g^2 \delta^2 D \left(\Delta - \frac{\delta}{3}\right)) \exp(i\phi)$$

(2.18)

As shown by the equation above, the signal also suffers the diffusional attenuation since, of course, thermally driven diffusional motion proceeds independently from the electrostatically driven electrophoresis. In contrast to a diffusion NMR experiment, in an eNMR measurement one increments not $g$ but, typically, $E$ (and less usually $\delta$ or $\Delta$) and detect the complex modulation factor of the signal.

For a particle moving by steady velocity under an electric field, the Coulomb force $F_e = Eq = Eze$ is balanced by the friction force $F_f = f v_e$ where $f$ is the molecular friction constant yielding:

$$v_e = \frac{Eze}{f}$$

(2.19)

For a spherical particle of radius $r$, $f = 6\pi \eta r$ in the Einstein-Stokes approximation which yields:
\[
\mu_e = \frac{v_e}{E} = \frac{ze}{6\pi}
\]  
(2.20)

However, this result is valid only at infinite dilution because at finite ion concentrations the electric field not only affects the charged particle, but also the ions in its diffuse layer. In that case, the mobility is connected to Henry’s formula\(^\text{92}\) as:

\[
\mu_e = \frac{ze}{6\pi\eta} \frac{f(\kappa r)}{(1+\kappa r)}
\]  
(2.21)

Similarly to that in section 1.1.3, in the limit of \(\kappa r \to 0\), one obtains:

\[
\mu_e = \frac{ze}{6\pi\eta r}
\]  
(2.22)

while in the other limit of \(\kappa r \to \infty\),

\[
\mu_e = \frac{ze}{4\pi\eta r^2} = \frac{\sigma_0}{\eta k}
\]  
(2.23)

where \(\sigma_0\) is the surface charge density. In the limit of either small \(\kappa r\) or low ionic concentration and by exploiting that the self-diffusion coefficient can be delivered by the Einstein-Stokes equation, one obtains:

\[
\mu_e = \frac{ze D}{k_B T}
\]  
(2.24)

Hence, in that limit one can measure not only the mobility and zeta-potential, but also the charge of individual particle. Yet, that charge also involves the counterions that are hydrodynamically coupled to the probed particle. For that reason, one obtains not the nominal charge but an effective charge that is usually smaller than the nominal charge. Only in some favorable cases can one characterize the effect of counterions in such detail that the nominal charge becomes available.

One point that needs attention is that the electrophoretic velocity is, by some measures, very small compared to the diffusional motion. Take for example tetramethylammonium chloride (TMACl) from Paper VII. For 2 mM TMACl in heavy water, the diffusion coefficient of TMA\(^+\) is \(1.03 \times 10^{-9} \text{ m}^2/\text{s}\) and the electrophoretic mobility is \(3.96 \times 10^{-8} \text{ m}^2/\text{Vs}\). Sampling the diffusive motion at every 10 ns,\(^\text{93}\) the mean displacement (recall eq. (2.10)) can be estimated to \(\approx 8 \text{ nm}\), and the average speed characterizing the diffusion of TMA\(^+\) is roughly 0.8 m/s. In comparison, with an electric field of 100 V/cm the electrophoretic velocity is \(\approx 4 \times 10^{-4} \text{ m/s}\). Hence, on the molecular scale electrophoresis is but a small bias to random thermal motion. Standing
in the middle of an electrolyte, we could not notice it at any particular moment. Yet, on larger length scales the electrophoretic motion may dominate because of its coherent nature which makes displacement increasing in direct proportion to time (compare that to eq. (2.10)).

Another point to consider is that as the target molecule migrates relative to the solvent molecules, the old equilibrium is broken and a new diffuse double layer forms within under nanoseconds. Yet, during 1 ns the net electrophoretic displacement is negligible \(4 \times 10^{-13} \text{ m} \) and therefore at any moment the particle and its diffuse layer is in equilibrium.

Finally, for eNMR the drift time is limited by \(T_1 \) to usually below a second. During that time interval, the average distance the TMA\(^+\) ion travels in the gradient direction is in the order of 100 \(\mu\text{m} \). This restriction may make eNMR less sensitive than conventional capillary electrophoresis (whether this happens or not depends mainly on the applied gradient strength that in turn is limited by the particle diffusion coefficient). On the other hand, for the same reason motion is limited far below the NMR sample size.

### 2.2.2. One dimensional eNMR experiments

In principle the electrophoretic displacement could be measured by varying either one of \(\delta, \Delta \) or \(E \) and detecting the phase modulation of the NMR spectral peak belonging to the species of interest. Multiple species can be detected at the same time provided that (i) they present NMR peaks that are sufficiently well resolved and (ii) their relaxation time and diffusion behavior do not differ significantly. The exact methodology used varies as there are several ways to reduce the various artifacts.

Rather early one adopted the U-tube type cell for eNMR.\(^{85,86,89,94,95}\) This cell had the advantage that (i) the electrodes feeding the cell from the top remained outside the detection range and (ii) any gas produced by electrolysis at the electrodes could escape without disturbing the signal detection region. Yet, it had also serious disadvantages. First and foremost, the flow at the two different sides was opposite and thereby one lost the complex modulation (recall eq.(2.18)) that was replaced by the cosine modulation of the intensity:

\[
\frac{s(2\tau)}{s_{\text{ref}}(2\tau)} = \cos \left( \gamma g \delta \Delta \mu E \right) \tag{2.25}
\]
It is not only so that the cosine modulation is less sensitive to small phase factors, but one also loses the sign of the modulation. In addition, the flow through the horizontal part of the cell created a magnetic field gradient in the z direction in addition to the well-defined applied gradient, which caused additional error. Finally, the cell had a low filling factor and thereby provided small NMR signal. The amplitude analysis method is not accurate enough for detecting weakly charged species because cosine modulation is insensitive to small phase changes. Hence, the method was not suitable for heteronuclei whose electrophoretic phase factor is usually much smaller than that of the proton because of their small gyromagnetic ratio $\gamma$.

More recently a cylindrical cell was designed to overcome the shortcomings of the U-tube cell. That cell has several advantages over the U-tube. First, a cylindrical cell has a large filling factor which significantly improves the signal. Besides, the flow in cylindrical cell is unidirectional so that the sign of displacement is reserved. Over the course of this work, a cell design that uses a conventional 5 mm NMR tube was employed. The electric field was applied by two palladium (Pd) electrodes inserted into the tube, one of which crossed the whole sample volume. The end of electrodes was wound to a round spiral that fitted just with the tube diameter and an insulation sheath outside the metal wire made sure that one obtains a well-defined homogeneous electric field along the tube in conductive samples. The Pd electrode is proved to be advantageous in suppressing gas formation upon electrolysis. Hence, during experiments with low current, bubble formation is negligible as can be easily tested by eye. Furthermore, a radiofrequency filter was developed which reduced the rf noise picked up from the wire crossing the sample space. With all these changes, the signal-to-noise ratio improved by one order of magnitude as compared to the U-tube system. The cell also has the advantage that it is easily accessible and adapts well to commercial NMR probes. More recently, this cell design was further modified by placing an array of capillaries on the bottom of the cell. The capillaries are bundled together by a thin glass tube and placed symmetrically in the center of the cell. The space between capillaries is filled with a gel so that solution could only transport through the capillary cores. This design suppresses background bulk flow effect caused by Joule heating and resulting thermal convection. Hence, it has been used in ionic liquid where the mobility of molecules is very low while the conductivity of the sample is very high.
Figure 2.5. The configuration of the cell with conventional NMR tube and a modified cell with a bundle of capillaries.

With the cell configuration as in Fig. 2.6a, the experiment consists of collecting spectra acquired at different duration or voltage of the electric field pulse; the detailed procedure was described earlier. Since non-charged particles should not show any electrophoretic phase effect (any phase effect they show arises from undesired bulk flow like convection), the phase of the signal of interest is measured relative to their phase. In practice, this is done by phasing the signal from non-charged reference species to pure absorption shape, then starting from there phasing the signal of interest to pure absorption shape and recording the required phase difference. This latter number is the requested phase modulation as provided by eq.(2.18). An example for the phase modulation with the reference signal set absorptive is shown in Figure 2.6 (a).

Figure 2.6. (a) Phase modulation of charged species relative to the constantly zero phase of neural species in the presence of electric field in an eNMR experiment. (b) The phase variation with electric field strength. The red line shows a linear fit.
The relative phase change is linearly proportional to the varied parameter ($\delta, \Delta$ or $E$). Fitting the phase change by eq.(2.17) provides the electrophoretic mobility (or, $\zeta$-potential). Thereafter, if the self-diffusion coefficient was also measured, the effective charge per molecule can also be obtained via eq.(2.24).

### 2.2.3. Two dimensional eNMR experiments

The eNMR data could also be analyzed by two-dimension Fourier transformation yielding 2D NMR spectra. In this way, a whole stack of spectra recorded at different voltages or durations are processed simultaneously. First, the recorded FIDs are Fourier transformed with respect to the acquisition time $t_2$ in one dimension and then Fourier transformed with respect to the electrophoretic modulation by increasing voltage or duration of the electric field pulse. The different components at the same chemical shift are then resolved by their different mobilities without any physical separation. This method is called Mobility Ordered SpectroscopY (MOSY) and has been shown in Paper I to present a superior advantage in analysing complex mixtures with severe signal overlap.

### 2.2.4. Error sources

One of the most serious problems in eNMR is the heating produced by the applied current. In conventional NMR probes, the air used for temperature regulation cools the sample tube from outside while Joule heating warms the sample volume. The result is a temperature gradient, which causes in turn a density gradient and convection. Since heating is directly proportional to the applied current, eNMR is restricted to samples that are not too conductive.

Another source of convective flow is from electro-osmosis. The NMR tubes used often have silanol (-SiOH) groups on their surface. With electrolytes inside these functional groups get deprotonated and gain a negative surface charge that in turn collects a diffuse layer of positive counterions in its vicinity. When the electric field is applied, the cations in this layer respond, start to move and drag the surrounding solvent with themselves. The motion of the solvent propagates into the liquid volume and, in the end, the whole sample volume may be set into convection. This is called electro-osmosis. As is discussed before, typical electrolytes only show very small displacement on the timescale
of eNMR measurement. Hence, flow effects may cause significant error. Bulk flow can be evaluated by looking at the behavior of neutral solute in the electrolytes - bulk flow is signified by phase modulation for neutral species. If the flow is slow, the flow pattern is simple and more regular and in that case the phase of neutral species can be subtracted from the apparent phase shift of the charged particles in order to make a first-order correction for the flow artifact. However, if the bulk flow is large, the flow pattern becomes both time-dependent and chaotic that makes correction impossible.

There are several ways to control the bulk flow. The most direct way is to suppress the electroosmotic flow is to attach covalently to the tube surface a non-charged polymer. The coating both reduces the solute-silica adsorption and moves the slipping plane further out so that electro-osmosis is eliminated. The coating is typically made of non-ionic hydrophilic molecules that are stable enough in solutions against different pH conditions. Linear polyacrylamide is most widely used and the procedure of treatment is mature. The coating procedure and its influence in reducing electro-osmosis were carefully described and systematically studied. Usually a siloxane group (Si-O-Si-C) is formed when linking the tube surface groups with polyacrylamide, but this arrangement is not stable under alkaline conditions. In a modified scheme one replaces the siloxane group with a Si-C bond, which is more stable against hydrolysis. Methyl cellulose and polyimide coatings are two more examples to be used in eNMR.

To reduce the convection, gelling agents were used to stabilize the solution. However, it is hard to guarantee if there is no interaction between the gelling agent and the electrolytes. Indeed, it is preferable that one measures electrophoretic mobility without introducing new components into the system. One method is to place capillary arrays in the tube to reduce bulk flow. The capillary bundle has (i) a large surface area so that the system has fast heat exchange rate and (ii) the small tube diameter suppresses the formation of convection cells and thereby suppresses convective flow.

In addition to capillaries, a convection-compensated pulse sequence was developed to detect electrophoretic migration in the presence of convection. The pulse sequence is shown in Figure 2.6.
Figure 2.6. Pulse sequence of double stimulated echo used in eNMR.

It is composed of two spin echo sequences with switched polarity of the electric field. In this sequence, the accumulated phase as a result of convective flow is cancelled by the second half, provided that the convective flow pattern is stable over the drift time. On the other hand, as the polarity of electric field is also switched in the middle of the sequence, both the flow direction (and the coherence order) changes resulting in the cumulative accumulation of the electrophoretic phase factor. So the overall effect is that the phase factor from convection is cancelled but the phase from electrophoretic flow is accumulated. This pulse sequence suppressed artifacts caused by convective flow.

A similar pulse sequence with double stimulated echo was developed later\textsuperscript{106} which also had the same function to remove convective artifacts. The double spin or stimulated echo sequences are most effective when the convective flow pattern does not change during the drift time. But long drift times are often required over which convection changes. Hence, further efforts were made\textsuperscript{118} that resulted in the CPMGER (Carr-Purcell-Meiboom-Gill with Electric field Reversal) pulse sequence with very rapid polarity switching. This pulse sequence is applicable to highly conductive samples like ionic liquids without the need to have a neutral reference molecule which carries the same nuclei.
3. Summary of results

3.1. Methodology

The spectral resolution is always a problem when studying multi-component systems, especially in the field of metabolomics and drug discovery with a very large number of molecular components. Two-dimensional NMR serves as a useful tool for improving spectral resolution/selectivity without the need of physical separation. In Paper I, a 2D NMR method of Mobility Ordered SpectroscopY (MOSY) was investigated as a tool for chemical analysis. The principles of MOSY were already introduced in section 2.2.3. Shortly, it is an eNMR measurement where results are presented in 2D form after a second Fourier transformation. Hence, the first spectral dimension is over chemical shifts and the suitably scaled second dimension is over the electrophoretic mobility. Since different molecules have their unique electrophoretic mobilities that depend, among other things, on the pKa of dissociable functional groups, the pH and viscosity of the solution, different molecules provide spectral manifolds grouped at different positions along that second spectral dimension. Hence, we get separation even though the chemical shifts may overlap.

The performance of MOSY was tested in two model systems: an amino acids mixture and a drug tablet dissolved in water. The latter sample was often chosen as a model target for potential methods, such as LC-NMR, for metabolomics and drug components analysis. The results of MOSY are shown in Figure 3.1 together with the corresponding results obtained by the better-known Diffusion Ordered SpectroscopY (DOSY) method. In the DOSY method, the second dimension is along self-diffusion coefficients and is created by inverse Laplace transformation. For the drug sample, the MOSY spectrum has significantly improved the selectivity with one component completely resolved and other two components partially separated. The $^1$H NMR spectrum of each individual component could be extracted from the MOSY spectrum and the components were determined to be a-acetylsalicylic acid, p-acetaminophen and c-caffeine. The same sample was also studied by DOSY in Paper I and a combination of capillary zone electrophoresis (CZE) or gradient capillary electrochromatography chromatography (gradient CEC) coupled with NMR. A comparison of the results shows that MOSY has a performance similar to that of CZE-NMR or gradient CEC-NMR where the acetylsalicylic acid is completely separated while acetaminophen and caffeine are partially separated.
Figure 3.1 (a) $^1$H DOSY spectrum of dissolved Thomapyrin® tablets at pD=9.0. (a: acetylsalicylic acid; p: acetaminophen; c: caffeine.) (b) $^1$H MOSY spectrum of dissolved Thomapyrin® tablets at pD=9.0. (c) $^1$H DOSY spectrum of mixed amino acids solution at pD=6.2. (a: L-asparticacid; s:L-serine; l: L-lysine) (d) $^1$H MOSY spectra of mixed amino acids solution at pD=6.2.

All three methods are superior to DOSY which shows no separation at all probably because all the components are small molecules of similar size. Yet the MOSY method has a clear advantage over the coupling methods CZE-NMR and CEC-NMR because the sample is easier to handle, the sample volume required is much smaller and the external instrumentation required is less extensive and expensive. Changing pH could
modulate the separation picture, as is particularly clear for the investigated amino acid mixture that can be seen as a model for metabolomic applications.

3.2. Specific cation binding to polyethylene oxide

Papers II and III concern the cation binding to polyethylene oxide (PEO), a frequently used model system soluble in many solvents including water. This system is also practically interesting because PEO is used to dissolve salts as part of solid polyelectrolyte batteries and its conductivity performance is highly related to the complexation of cations with the ether oxygen. Yet the details of complexation and mechanism of conduction are not well understood. Particular cations binding to PEO were previously reported in solvents of low polarity, such as methanol and acetonitrile, but there was no systematic study of specific cation effects in nonaqueous solutions. That was the motivation for Papers II and III.

![Figure 3.2](image)

Figure 3.2. The effective charge of PEO probed by eNMR and diffusion NMR experiments in d4-methanol at 2 mM monomeric concentration of polymer and 2 mM salt concentration.

Hence, the complexation of a series of metal cations with PEO in methanol was investigated by diffusion and eNMR techniques. The effective charge of PEO chain which is gained upon cation complexation was obtained in the presence of different
salts and the result is shown in Figure 3.2. The positive charge of PEO indicates it is the cations that are predominantly complexed by PEO and the complexation capability is highly ion specific. For monovalent cations, the binding strength increased in the order \( \text{Li}^+ << \text{Na}^+ < \text{K}^+ \approx \text{Rb}^+ \approx \text{Cs}^+ \) while multivalent cations do not bind with the notable exception of \( \text{Ba}^{2+} \) that displays a binding strength similar to that of \( \text{K}^+ \). Clearly, the binding strength is not directly related to the valence of cations and is not following the Hofmeister order, but was found to be related to the charge density of different ions. The ions with surface charge density lower than 0.10-0.15 Å\(^{-2}\) tend to bind to PEO while ions with higher charge density do not bind. We proposed that the solvation of the ions is important in the binding process. To be complexed with PEO, the cations have to be partially de-solvated. Ions with high charge density bind solvent molecules more tightly which makes it energetically unfavorable to be complexed by PEO.

To get a more detailed picture of cation-PEO complexation on the molecular level, an additional study was carried out in Paper III on two selected cations - \( \text{K}^+ \) and \( \text{Ba}^{2+} \). In that study, short-chain PEO was used instead of long-chain PEO because the binding constants of cations binding to long chain PEO was found to decrease strongly with salt concentration that indicated a significant electrostatic repulsion between bound ions.

![Figure 3.3](image)

**Figure 3.3.** The effective charge of PEO oligomers of different molecular weight and chain end at 2 mM monomeric concentration and salt at 2 mM concentration in d4-methanol.
Thus, the binding constants obtained were not solely dependent on the affinity of the cation to oxygens at the binding motif of PEO. By using short chain PEO we could avoid this problem.

Moreover, the mechanism of specific ion binding could be better understood if the binding mode were known. Hence, the binding of K\(^+\) and Ba\(^{2+}\) were studied with PEO oligomers of chain length varying from 4 to 25 monomers and also with different chain ends, hydroxyl and methyl. Some results are shown in Figure 3.3.

The binding strength of cations with short-chain PEO is stronger than that with long chain PEO in terms of the number of cations bound per repeating unit but the relative binding strength remains the same, with Li\(^+\) < K\(^+\) < Ba\(^{2+}\). Barium has slightly higher binding affinity to PEO than potassium for all oligomer lengths. In contrast to the similar binding strength with long-chain PEO, the variation of binding strength as a function of the length of PEO oligomer for K\(^+\) and Ba\(^{2+}\) shows different trends: binding with K\(^+\) gets progressively stronger with increasing chain length but remains constant with Ba\(^{2+}\), indicating different binding modes for those two cations. To study the binding modes both \(^1\)H NMR spectra and spin relaxation of PEO were measured. The results showed that K\(^+\) binding involves the polymer chain wrapping around the metal cation and forming a quasi-cyclic structure. At least 6 units were needed to have a significant binding so that the enthalpy gain upon binding is enough to compensate for the entropy loss connected to polymer chain rearrangement. The number of ions binding to PEO increases with increasing chain length with up to two K\(^+\) that could bind to the same chain when that was 24 monomers long. The PEO chain retains its flexibility upon K\(^+\) binding and the bound K\(^+\) could exchange rapidly with K\(^+\) free in the bulk solution.

Ba\(^{2+}\) binding to PEO is rather different from that for K\(^+\). First, barium could bind to PEO both in the form of Ba\(^{2+}\) and as ion pair (BaAnion)\(^+\). At least 5 ether oxygen atoms are participating in the binding. The chain segments are directly and rigidly coordinated with Ba\(^{2+}\) or (BaAnion)\(^+\) as indicated both by the \(^1\)H NMR and the \(^1\)H shorter relaxation rate, which also yield that bound and free Ba\(^{2+}\) or (BaAnion)\(^+\) are in slow exchange with each other.
3.3. Specific anion binding to poly (N-isopropylacrylamide)

Despite its importance and long history, the mechanism of Hofmeister effect is still under debate. Several popular mechanisms for Hofmeister effect were reviewed in section 1.2.4. Direct anion binding was believed to be the main mechanism for salting in, yet it only received limited and indirect experimental tests. Since eNMR provides a sensitive way to study specific ion binding with macromolecules in salt solutions it was natural to consider its use for that purpose. Hence, in Paper IV we investigated anion binding to Poly (N-isopropylacrylamide) (PNIPAM) with Mn=120,000. This polymer, often considered as a model for proteins, is neutral with no dissociable functional groups over the pH range 3-11 which simplifies analysis.

As for PEO, the effective charge of PNIPAM in different salt solutions was measured by combining eNMR and diffusion NMR with result illustrated in Figure 3.4. In relation to PEO, there are two differences. First, the effective charge is now negative as it is anions that bind. Secondly, the effective charge is very low (ca 1 charge per 500 monomers) and therefore the ion-ion interaction among bound anions has a negligible effect. The binding, though very weak, is anion specific on a manner that follows the Hofmeister order \( \text{SCN}^- > \text{ClO}_4^- > \text{I}^- > \text{Cl}^- > \text{F}^- \approx \text{SO}_4^{2-} \approx 0 \) with all the chaotropic anions
binding to PNIPAM to different degrees but none of the kosmotropic anions doing so. This result provides solid proof that there is specific ion binding even at very low salt concentration 10 mM and it gets stronger at higher concentrations. Regarding the weak binding at low salt concentration, no such data was available previously and the reason why it remains hidden is probably that the interaction of ions with PNIPAM is too insignificant to cause any change in thermodynamic behavior or other spectroscopic variables. This highlights the sensitivity of our characterization method.

The results above provided trends, but the mechanism of specific ion binding remained unclear. In order to understand that more, the concentration dependence of ion binding to PNIPAM was studied with sodium thiocyanate as salt. As is shown in Figure 3.5, the binding gets stronger with increasing salt concentration, approaching apparent saturation towards 0.5 M. This behavior could be perfectly described by the weak Langmuir-Stern adsorption isotherm model $z = \frac{NC}{1+KC}$ where $z$ is the effective charge of PNIPAM molecule; $N$ the maximum effective charge per molecule; $K$ the binding constant and $C$ is the molar concentration of salt. The fitting results showed that the binding constant of SCN$^-$ with PNIPAM is 8.8 M$^{-1}$ and the maximum number of anions that could bind is 7. A linear correlation of the partition free energy with the free energy of transferring the anions from water to methanol, which mimics the PNIPAM surface,
suggests that solvation effect of anions is the main determining factor for specific anion binding. When an anion binds to the PNIPAM surface, there is an energy cost for the transfer of the anion from water to the polymer surface but the same transfer releases ordered water molecules from the surface. The free energy contributed by these two factors may be the driving force for the specific anion binding to PNIPAM. This is not the first time that entropic effects are proposed to be responsible for Hofmeister order but it is the first time that the suggestion is made with regards to the mechanism of ion binding.

3.4. Specific ion binding to proteins

In Paper V the specific ion binding was also studies in aqueous bovine serum albumin (BSA) solutions. While there is agreement in the literature about Hofmeister effect near neutral surfaces, the Hofmeister effect near charged or polar surfaces is much more complex. Partial or complete reversal of Hofmeister order was observed in many different cases and the reason still remains unclear. Thus we chose BSA as a model molecule to study specific cation and anion effects systematically both at negatively and positively charged surfaces.

Ion binding of a series of salts with BSA was measured both at pH below and above the isoelectric point. Specific anion binding was observed in both conditions. When the BSA has a net negative charge, the magnitude of anion binding is similar to that with PNIPAM indicating the same driving force. When the BSA is positively charged anions are much strongly bound and binding is to a large part driven by the electrostatic interactions with the oppositely charged surface. In both cases, the chaotropes bind more than kosmotropes and the binding strength follows the Hofmeister order SCN$^-$ >ClO$_4^-$ >Cl$^-$ >F$^-$. Thus the net charge at two pH conditions has the opposite trends which might be responsible for reversed Hofmeister order when investigating other physical chemical properties. The cation effect is different. The chaotropes display higher affinity to BSA when the protein is positively charged but lower affinity when it is negatively charged so that their binding reverses with reversed sign of the net surface charge. Under both conditions reversed Hofmeister order was observed. This indicates that the law of matching water affinity is the major mechanism for cations. When the BSA is positively charged, there is an excess of amino acid group in dissociated states in the solution. The amino acid functional groups are regarded as kosmotropes and have a
preference towards interacting with kosmotropic ion. For negatively charged BSA, there are more carboxylic groups that are chaotropic in nature and interacting more with chaotropic ions. This study indicates that cation effect and anion effect are governed by different interacting mechanisms.

3.5. Ionization of cellobiose and the mechanism of cellulose solubility

Cellulose is one of the most abundant renewable materials from nature, but its application is limited by its poor solubility. It is soluble in strong alkaline solutions only in a narrow range of concentration and temperature, yet the solubility is still not satisfying. To be able to solve this problem, it is necessary to understand the dissolution mechanism of cellulose. In Paper VI cellobiose was used as a soluble model of cellulose and its ionization in alkaline solution was tested at different alkaline strengths. The result shows that with increasing alkaline concentration the cellobiose deprotonates in two steps. Further test with $^1$H-$^{13}$C 2D HSQC NMR have shown that the deprotonated hydroxyls are mainly hemiacetal OH and most probably 'O2 in the glucose unit at the nonreducing end. MD simulations applied for short cellulose chains with appropriate net charge have shown that charging of chain reduces the extent of chain aggregation and facilitates dispersion into the solution.

3.6. Ion association in aqueous and non-aqueous solutions

In Paper VII the effective charge of tetramethylammonium (TMA) ions for salts dissolved at low concentration in different solvents was measured by diffusion NMR and eNMR. The deviation of effective charge from the nominal charge was calculated and compared to the theoretical values calculated from the Onsager limiting law. The result showed that the Onsager limiting law predicts well the relaxation and electrophoretic effect in solvents of high polarity, including water and DMSO. However, in solvents of low dielectric constants such as acetonitrile and ethanol there is big discrepancy between the experimental values and the values predicted from the Onsager limiting law. This has been explained by the Onsager limiting law not accounting for ion pairing in those solvents. It has also been indicated that ion pairing is not entirely described by solely considering the dielectric constant of the solvent.
### 4. List of frequent abbreviations and symbols

<table>
<thead>
<tr>
<th>Symbol</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>a</td>
<td>activity</td>
</tr>
<tr>
<td>B</td>
<td>magnetic field</td>
</tr>
<tr>
<td>c</td>
<td>concentration</td>
</tr>
<tr>
<td>D</td>
<td>self-diffusion coefficient</td>
</tr>
<tr>
<td>DLVO</td>
<td>Derjaguin–Landau–Verwey–Overbeek</td>
</tr>
<tr>
<td>DOSY</td>
<td>diffusion ordered spectroscopy</td>
</tr>
<tr>
<td>e</td>
<td>elementary charge</td>
</tr>
<tr>
<td>E</td>
<td>electric field</td>
</tr>
<tr>
<td>E(Δ)</td>
<td>spin echo attenuation</td>
</tr>
<tr>
<td>eNMR</td>
<td>electrophoretic NMR</td>
</tr>
<tr>
<td>f</td>
<td>molecular friction coefficient</td>
</tr>
<tr>
<td>FID</td>
<td>free induction decay</td>
</tr>
<tr>
<td>FT</td>
<td>Fourier transform</td>
</tr>
<tr>
<td>g</td>
<td>magnetic field gradient strength</td>
</tr>
<tr>
<td>ΔG</td>
<td>Gibbs free energy difference</td>
</tr>
<tr>
<td>I</td>
<td>ionic strength, spin quantum number</td>
</tr>
<tr>
<td>k_B</td>
<td>Boltzmann constant</td>
</tr>
<tr>
<td>K_a</td>
<td>association constant</td>
</tr>
<tr>
<td>m</td>
<td>spin quantum number</td>
</tr>
<tr>
<td>M</td>
<td>magnetization</td>
</tr>
<tr>
<td>MOSY</td>
<td>mobility ordered spectroscopy</td>
</tr>
<tr>
<td>PB</td>
<td>Poisson Boltzmann</td>
</tr>
<tr>
<td>PNIPAM</td>
<td>poly (N-isopropylacrylamide)</td>
</tr>
<tr>
<td>r</td>
<td>particle size</td>
</tr>
<tr>
<td>R</td>
<td>gas constant</td>
</tr>
<tr>
<td>T</td>
<td>Temperature</td>
</tr>
<tr>
<td>T_1</td>
<td>longitudinal relaxation time</td>
</tr>
</tbody>
</table>
$T_2$  transverse relaxation time
$\nu_e$  electrophoretic velocity
$z_i$  ion valence
$\gamma$  activity coefficient, nuclear gyromagnetic ratio
$\gamma_{\pm}$  mean activity coefficient
$\delta$  duration of the gradient pulse
$\Delta$  diffusion time
$\epsilon$  dielectric constant of solution
$\epsilon_0$  vacuum permittivity
$\epsilon_r$  relative permittivity
$\zeta$  zeta-potential
$\eta$  viscosity
$\kappa^{-1}$  Debye length
$\mu$  magnetic moment
$\mu_e$  electrophoretic mobility
$\sigma_0$  surface charge density
$\tau$  a delay in a pulse sequence
$\psi$  electric potential
$\omega$  Larmor frequency
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6. References


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