Zwitterionic Separation Materials for Liquid Chromatography and Capillary Electrophoresis

Synthesis, Characterization and Application for Inorganic Ion and Biomolecule Separations

by

Wen Jiang

Akademisk Avhandling

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Zwitterionic Separation Materials for Liquid Chromatography and Capillary Electrophoresis
Synthesis, Characterization and Application for Inorganic Ion and Biomolecule Separations

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Abstract  Liquid Chromatography (LC) and Capillary Electrophoresis (CE) are modern analytical techniques that play very important roles in many areas of modern science such as life science, biotechnology, biomedicine, environmental studies, and development of pharmaceutics. Even though these two techniques have existed and been subjected to studies for several decades, the developments of new separation materials for them are still very important till now in order to meet the different new demands for improvement from other disciplines in science.

In this doctoral thesis, several novel covalently bonded sulfobetaine type zwitterionic separation materials are synthesized for the application in LC and CE. These materials carry both positively charged quaternary ammonium groups and negatively charged sulfonic groups, which result in a very low net surface charge compared to conventional separation materials with only anionic or cationic functional groups. Consequently, it is possible to employ these materials for separation of different ionic species under mild conditions. The surface properties have also been characterized, mainly by elemental analysis, sorption isotherm, ζ-potential measurements, and spectroscopic methods.

By using packed zwitterionic columns for liquid chromatography, small inorganic anions or cations, and acidic or basic proteins can be independently and simultaneously separated in a single run using optimal sets of separation conditions. This is a unique property compared to conventional ionic separation material for LC. When fused silica capillaries coated with zwitterionic polymer are used for capillary electrophoresis, good separations can be achieved for solutes as different as inorganic anions, peptides, proteins, and tryptically digested proteins.

Keywords  Zwitterionic, stationary phase, separation, liquid chromatography, capillary electrophoresis, sulfobetaine, modification, covalently bonded, graft polymerization, ζ-potential.

ISBN  91-7305-543-3
To XiaoLei, ChenChen and My Parents
The symbol in the middle of the illustration is a well-known Chinese Yin Yang symbol. Sometimes it is called Tai-Chi (太極) symbol. The Tai-Chi is from I-Ching (易經), which is the greatest foundation of Chinese philosophy. It is developed from the natural phenomena of our universe observed by ancient Chinese around 700 B.C.. Accordingly, although the universe is changing every day, it also has seasonal and annual cycles. From these cycles the unchanging rules (I-Ching) are created and applied on human activities.

In the symbol the light color area which indicates more sunlight is called Yang (Sun), and the dark color area has less sunlight (more moonlight) and is called Yin (Moon). Yang is like man, Yin is like woman. Yang would not grow without Yin. Yin could not give birth without Yang. Yin is born (begins) at Summer Solstice and Yang is born (begins) at Winter Solstice. Therefore one little circle Yin is marked on the Summer Solstice position, and another little circle Yang is marked on the Winter Solstice position.

Anions and cations are respectively called Yin ions (阴离子) and Yang ions (阳离子) in Chinese. The word of Zwitterion is then expressed as 阴阳离子. According to the principle of I-Ching, the Yin and Yang are correlated with each other and should be in a good balance. In this thesis, the Yin and Yang balance is a major concern in order to synthesize zwitterionic materials with good charge balance.

Some of above information is from http://www.chinesefortunecalendar.com/yinyang.htm.
This doctoral thesis is based on the following papers and manuscripts, which are hereafter referred to in the text by their roman numerals.

I. Covalently Bonded Polymeric Zwitterionic Stationary Phase for Simultaneous Separation of Inorganic Cations and Anions

   Wen Jiang and Knut Irgum

II. Synthesis and Evaluation of Polymer-Based Zwitterionic Stationary Phases for Separation of Ionic Species

   Wen Jiang and Knut Irgum


   Wen Jiang and Knut Irgum

IV. Characterization of Polymeric Zwitterionic Stationary Phase and Application for Protein Separation

   Wen Jiang and Knut Irgum
   Submitted to J. Chromatography A.

V. Control of Electroosmotic Flow and Wall Interactions in Capillary Electrophoresis Capillaries by Photograftered Zwitterionic Polymer Surface Layers

   Wen Jiang, Justina Ngum Awasum and Knut Irgum
   Analytical Chemistry, 2003, 75, 2768-2774.

VI. New Way to Synthesize Zwitterionic Capillary and Its Applications for Determination of Inorganic Anions and Peptides

   Wen Jiang and Knut Irgum
   Manuscript for Analytical Chemistry.
Related patents by the author but not included in this thesis:

1. **New Capillary Comprising Covalently Bonded Zwitterionic Sulfo betaine Groups**
   *Wen Jiang and Knut Irgum*

2. **Novel Column Packing Material**
   *Wen Jiang and Knut Irgum*
### Abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Full Form</th>
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<tbody>
<tr>
<td>AIBN</td>
<td>Azobisisobutylironitrile</td>
</tr>
<tr>
<td>ATRP</td>
<td>Atom Transfer Radical Polymerization</td>
</tr>
<tr>
<td>BGE</td>
<td>Background Electrolyte</td>
</tr>
<tr>
<td>BME</td>
<td>Benzoin Methyl Ether</td>
</tr>
<tr>
<td>CE</td>
<td>Capillary Electrophoresis</td>
</tr>
<tr>
<td>CEC</td>
<td>Capillary Electrochromatography</td>
</tr>
<tr>
<td>CGE</td>
<td>Capillary Gel Electrophoresis</td>
</tr>
<tr>
<td>CITP</td>
<td>Capillary Isotachophoresis</td>
</tr>
<tr>
<td>CZE</td>
<td>Capillary Zone Electrophoresis</td>
</tr>
<tr>
<td>DMA</td>
<td>Dimethyl Amine</td>
</tr>
<tr>
<td>DMAP</td>
<td>4-(Dimethylamino) Pyridine</td>
</tr>
<tr>
<td>DVB</td>
<td>Divinyl benzene</td>
</tr>
<tr>
<td>ECH</td>
<td>Epichlorohydrin</td>
</tr>
<tr>
<td>EDMA</td>
<td>1,2-Ethylene Dimethacrylate</td>
</tr>
<tr>
<td>EIC</td>
<td>Electrostatic Ion Chromatography</td>
</tr>
<tr>
<td>EOF</td>
<td>Electroosmotic Flow</td>
</tr>
<tr>
<td>GC</td>
<td>Gas Chromatography</td>
</tr>
<tr>
<td>HEMA</td>
<td>2-Hydroxyethyl Methacrylate</td>
</tr>
<tr>
<td>HILIC</td>
<td>Hydrophilic Interaction Chromatography</td>
</tr>
<tr>
<td>HPLC</td>
<td>High Performance Liquid Chromatography</td>
</tr>
<tr>
<td>HV</td>
<td>High Voltage Power Supply</td>
</tr>
<tr>
<td>IC</td>
<td>Ion Chromatography</td>
</tr>
<tr>
<td>IEC</td>
<td>Ion-exchange Chromatography</td>
</tr>
<tr>
<td>IR</td>
<td>Infrared</td>
</tr>
<tr>
<td>MEKC</td>
<td>Micelle Electrokinetic Capillary Electrophoresis</td>
</tr>
<tr>
<td>MS</td>
<td>Mass Spectrometry</td>
</tr>
<tr>
<td>pI</td>
<td>Isoelectric Point</td>
</tr>
<tr>
<td>PS</td>
<td>1,3-Propane Sultone</td>
</tr>
<tr>
<td>S/N</td>
<td>Single to Noise Ratio</td>
</tr>
<tr>
<td>SPE</td>
<td>3-[N,N-Dimethyl-N-(Methacryloyloxyethyl)-ammonium] propanesulfonate</td>
</tr>
<tr>
<td>TC</td>
<td>Thionyl Chloride</td>
</tr>
<tr>
<td>UV</td>
<td>Ultraviolet</td>
</tr>
</tbody>
</table>
## List of Symbols

<table>
<thead>
<tr>
<th>Symbol</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>ζ</td>
<td>zeta potential</td>
</tr>
<tr>
<td>µ&lt;sub&gt;a&lt;/sub&gt;</td>
<td>apparent mobility</td>
</tr>
<tr>
<td>µ&lt;sub&gt;eo&lt;/sub&gt;</td>
<td>electroosmotic flow mobility</td>
</tr>
<tr>
<td>µ&lt;sub&gt;ep&lt;/sub&gt;</td>
<td>electrophoretic mobility</td>
</tr>
<tr>
<td>E</td>
<td>electric field strength</td>
</tr>
<tr>
<td>F&lt;sub&gt;ep&lt;/sub&gt;</td>
<td>accelerating force</td>
</tr>
<tr>
<td>F&lt;sub&gt;fr&lt;/sub&gt;</td>
<td>friction force</td>
</tr>
<tr>
<td>q</td>
<td>charge of particle</td>
</tr>
<tr>
<td>r</td>
<td>radius of the charged particle</td>
</tr>
<tr>
<td>v&lt;sub&gt;ep&lt;/sub&gt;</td>
<td>velocity of movement</td>
</tr>
<tr>
<td>ε</td>
<td>dielectric constant</td>
</tr>
<tr>
<td>η</td>
<td>viscosity of the solution</td>
</tr>
</tbody>
</table>
# Table of Contents

1. Introduction .................................................................................................................................. 1

2. Overview of Liquid Chromatography for Inorganic Ions and Proteins, and Capillary Electrophoresis ......................................................................................................................... 3

   2.1. Ion Chromatography ........................................................................................................... 3

      2.1.1. Suppressed Ion Chromatography ............................................................................. 3

      2.1.2. Non-Suppressed Ion Chromatography .................................................................... 5

   2.2. Ion-exchange Chromatography for Protein ......................................................................... 5

   2.3. Capillary Electrophoresis .................................................................................................... 6

      2.3.1. Electrophoretic Mobility ......................................................................................... 7

      2.3.2. Electroosmotic Flow ............................................................................................... 7

      2.3.3. Surface Modification ............................................................................................... 8

   2.4. Liquid Chromatography for Inorganic Ions and Proteins vs. Capillary Electrophoresis ................................................................................................................................. 9

3. Covalently Bonded Zwitterionic Separation Materials for Liquid Chromatography ............................................................................................................................... 11

   3.1. Introduction .......................................................................................................................... 11

   3.2. Synthesis of Covalently Bonded Sulfo betaine Type Zwitterionic Separation Materials for LC ...................................................................................................................... 13

      3.2.1. By Direct Surface Reaction ..................................................................................... 13

      3.2.2. By Surface Graft Polymerization .......................................................................... 15

   3.3. Matrices for Synthesis of Zwitterionic Separation Material .............................................. 17

4. Covalently Bonded Zwitterionic Separation Materials for Capillary Electrophoresis ................................................................................................................................. 19

   4.1. Introduction .......................................................................................................................... 19

   4.2. Synthesis of Zwitterionic Capillaries for CE ..................................................................... 20

5. Characterization of Zwitterionic Separation Materials .................................................................. 23

   5.1. Spectroscopy Method .......................................................................................................... 23

   5.2. ζ-potential ........................................................................................................................... 24

      5.2.1. Double layer and ζ-potential .................................................................................. 24

      5.2.2. ζ-potential Measurement by Photon Correlation Spectroscopy ......................... 25
5.2.3. Salt Effect on the \( \zeta \)-potential of Zwitterionic Separation Materials...... 26

5.3. Elemental Analysis................................................................................................. 26

5.4. Sorption Isotherm ................................................................................................. 27

6. Application of Zwitterionic Separation Materials for Liquid Chromatography (Paper I-IV)........................................................................... 29

6.1. Separation of Inorganic Ions ............................................................................ 29

6.2. Separation of Biological Macromolecules ....................................................... 33

7. Application of Zwitterionic Separation Materials for Capillary Electrophoresis (Paper V and VI)................................................................. 35

7.1. Separation of Inorganic Ions............................................................. 35

7.2. Separation of Biological Molecules.................................................................. 36

8. Concluding Remarks............................................................................................ 40

9. Acknowledgements-Tack!.................................................................................... 41

10. References........................................................................................................... 43
1. Introduction

Analytical samples of natural origin are usually complex mixtures consisting of many different compounds. When there is a need to know what is really inside a mixture, a separation technique should be used. Even though the classic separation methods such as liquid extraction or centrifugation are still used nowadays, they are just applied for macroscale fraction separation such as sample preparation. How can we then gain access to the microscale information in each fraction? Ultimately, new powerful separation and detection techniques are required to fulfill the task. The modern High Performance Liquid Chromatography (HPLC) and Capillary Electrophoresis (CE) are two of these new techniques, which can meet the demand. Since they were invented several decades ago, they have been applied and play very important roles in many scientific areas, e.g. life science, biomedicine, biotechnology, environmental analysis, and development of new pharmaceutics, etc. However, there is still a room for development of these techniques to meet the different new demands and requests for improvement from other disciplines in science.

Along the development of HPLC, the technique has been separated into several subtechniques, based on the separation modes, for example, Reverse-Phase (RPLC), Ion-Exchange (IEC), Immobilized Metal Affinity (IMAC), Hydrophobic Interaction (HIC), Hydrophilic Interaction (HILIC) and Normal Phase Chromatography. All of these techniques indubitably use liquid as carrier, and the separation material in column varies according to the separation mode. CE is a totally different technique from the HPLC, because the separation principle is based on the electrophoretic migration of samples in a capillary instead of the sample partition between the stationary and mobile phases. It has also been separated into groups according to the separation modes, which include Capillary Zone Electrophoresis (CZE), Capillary Gel Electrophoresis (CGE), Micellar Electrokinetic CE (MEKC), Capillary Isoelectric Focusing (CIEF), and Capillary Isotachophoresis (CITP). The separations are carried out in the capillary in CE, but ideally this capillary should not take active part in the separation, apart from providing a tubular confinement for the electrophoretic separation. Because wall interactions increase with decreasing capillary diameter, there is an enormous concern with the wall chemistry. In brief, even though there are so many separation materials that have been well developed for different separation modes, new separation materials are still very interesting to develop, because very complex samples call for additional new separation dimension with selectivities orthogonal to old chemistry to be fully resolved.

This thesis deals with the synthesis of several novel sulfobetaine type zwitterionic separation materials for liquid chromatography and capillary electrophoresis. These materials carry both positively charged quaternary ammonium groups and negatively charged sulfonic groups, and have been characterized by various spectroscopic techniques, elemental analysis and \( \zeta \)-potential measurements. When particle type
witterionic separation materials are packed into HPLC columns, they show the ability of simultaneous separation of inorganic anions and cations, and also acidic and basic proteins in a single run. This is a unique property compared to a classic separation material for ion-exchange chromatography. When capillary type zwitterionic separation materials are used for CE, the wall interaction and electroosmotic flow in capillary are reduced. Consequently, it shows good separations of inorganic anions, peptides, proteins and tryptically digested proteins with very high efficiency and resolution.
2. Overview of Liquid Chromatography for Inorganic Ions and Proteins, and Capillary Electrophoresis

As described above, High Performance Liquid Chromatography includes several different separation modes, but this thesis will only focus on the introduction of ion-exchange chromatography. Alternatively, following the conventional nomenclature, I will use ion chromatography (IC) instead of ion-exchange chromatography for small inorganic and organic ions, and continue to use ion-exchange chromatography for large biological macromolecules.

2.1. Ion Chromatography

Classical ion exchange separations were developed to modern ion chromatography through a research breakthrough by Small, Stevens and Bauman in 1975 [1]. Their improvements based on a low capacity stationary phase and using suppressed conductivity for detection lays the foundations for modern IC systems, especially for suppressed IC. Small inorganic and organic anions and cations can be rapidly and efficiently separated and sensitively detected by this technique. Since the suppressed IC needs a suppression technique after the chromatographic separation, Fritz et al. lately developed a new IC method called non-suppressed IC [2]. This method can also provide sensitive separation for common small inorganic and organic ions without suppressor. The details on the suppressed IC and non-suppressed IC will be briefly described in the later sections. Several textbooks [3-6] and review papers [7-16] give more historical evaluation and future aspects.

Over years of technique development, IC has been divided into several groups from its origin by their separation mechanism, e.g., ion-exchange chromatography, ion-exclusion chromatography and ion-pair chromatography. Therefore, IC should not be consider as a single, specific analytical technique but rather as a collection of LC techniques used to separate inorganic anions and cations, as well as low molecular weight water-soluble organic acids and bases [3-6, 13]. It has also become a robust separation technique for the routine analysis of small ions. As the separation of inorganic anions is the most important part in this doctoral study, the introduction will be mainly focused on anionic separations.

2.1.1. Suppressed Ion Chromatography

A typical suppressed IC system for the separation of anions usually comprises a HPLC pump, an injector, a suppressor, a conductivity detector, and a data acquisition system, as shown in Figure 1. The eluent is typically prepared from sodium hydroxide or sodium carbonate/bicarbonate, which have reasonable elution powers for anions and also provide a possibility of being fully or partially converted to their corresponding acids in an ion exchange process, using a cation exchanger in the
hydrogen ion form. The hydroxide and carbonate ions are hence converted to water or carboxylic acid in the suppressor to reduce the total conductivity of background, which in turn increases the detection sensitivity. By varying the concentration and composition of eluent, the separation time and to some extent the selectivity for different anions can be adjusted.

**Figure 1.** Scheme of a suppressed Ion Chromatography system.

The columns are normally packed with 5-50 µm particulate separation material with positively charged functional groups such as quaternary/tertiary ammonium groups bonded onto the material surface. When anions are injected into the column, they will interact with the positively charge groups by electrostatic interaction. This interaction is a dynamic equilibration between the stationary phase and the mobile phase, because the counter ion in the eluent will compete with the sample anions for the oppositely charged sites on the material surface. The separation is finally depending on the capacity of the stationary phase, the charge and size of the analyte, and the type and concentration of the eluent solution. The capacity of the ion exchanger should not be too high, because it results high partitioning, which makes the analyte peak appear later in the chromatogram. A strong eluent would then be required in order to obtain a reasonable separation, and this increases the load of the suppressor. There are several classic types of chromatographic materials available for IC, but the most popular and widely used one is the pellicular agglomerated polymeric ion exchangers due to the historical development reason. It is also because this type of material has better mass transfer and the desired low capacity; higher efficiency and resolution can thus be easily accomplished.

The suppressor is used to reduce the total background conductivity of the eluent before the conductivity detector, and then get better sensitivity for the injected samples. In 1975, Small et al. first used a column type suppressor which was packed by an ion-exchanger with opposite charge to that of the analytical column [1]. This type of suppressor is restricted by the capacity and needed offline regeneration, and also increases the bandbroading. In the early 1980s, Stevens et al. [17, 18] developed the hollow-fiber membrane type suppressor, which reduced the band-broadening and can be regenerated continuously. Even though the hollow-fiber membrane suppressor has
been used since its invention till now, it starts to be replaced by a flat micro-membrane type suppressor due to its higher ion exchange capacity and efficiency. In the late 1990s, a new conceptual suppressor called electrolytic suppressor was first demonstrated by Strong and Dasgupta [19], who used electrolysis of water to produce hydronium ions for the suppression reaction. Its commercial version is called “Self Regenerating Suppressor”. There are also some other type of suppressors, which will not be discussed here. More detailed information can be found in some recent reviews papers [20, 21] and some fundamental textbooks [3-6].

2.1.2. Non-Suppressed Ion Chromatography

In 1979, Gjerde, Fritz and Schmuckler first reported another IC approach, non-suppressed ion chromatography [2]. As can be inferred from the name, there is no suppressor in this system. However, the sensitive conductivity detection can still be achieved by using aromatic carboxylic acids such as benzoate or phthalate acid as eluent to separate anions due to the low limiting equivalent ionic conductance of these eluent ions. The packing materials for the non-suppressed IC columns were synthesized from the copolymers of styrene and divinyl benzene (DVB); further by short time of sulfonation or amination to produce negatively and positively charged groups on the particle surface. This makes the materials with final capacity as low as around 10-30 µequ/g.

2.2. Ion-exchange Chromatography for Protein

Protein separation and purification are important in the biological study. Although there are numerous separation and purification methods available for proteins, ion-exchange chromatography (IEC) accounts for more than 75% of all chromatographic separations and purifications of proteins [22]. This is because most proteins are zwitterionic molecules and show charge in different aqueous solution. Therefore, they can be separated according to the different electrostatic interaction between the stationary phase and proteins. This method is simple and efficient. The chromatographic system typically consists of a gradient HPLC pump, a sample injector, an ion-exchange column, an UV detector, and a data acquisition system. The separation column is normally packed with the anionic or cationic exchanger, which typically contains functional groups such as tertiary/quaternary ammonium or sulfonic/carboxylic acid on the matrix material surface.

Normally, the ion-exchange column is first equilibrated with a low concentration buffer solution called starting buffer solution. When the charged proteins are injected into the column, the proteins with opposite charge as ionic sites are attracted to the ion-exchanger via electrostatic interaction; however, the ones with the same charge as the ionic sites are flushed out off the column by the mobile phase. This attraction interaction varies with the pH and the concentration of the starting buffer solution, the nature of the ion-exchange material and the charge properties of protein itself. The
higher the protein charge, the stronger the interaction will be. The salt gradient elution starts when the injection valve is switched for injection. This salt solution usually contains the starting buffer solution and high concentration of salt solution such as 1 M NaCl. The sodium ions or chloride ions in the gradient salt solution then compete with the protein to occupy the ionic sites on the ion-exchanger. The elution curves are often steep and the protein retention changes dramatically when a certain concentration of salt is reached. Reginer et al. and Stålberg et al. give detailed and somewhat different accounts of the separation mechanism [23-25].

2.3. Capillary Electrophoresis

Electrophoresis is originally defined as the movement of charged particles driven by an electric field in a liquid medium. Its historical developments can be traced back to 1907, when the Russian physicist Reuss made an experiment using clay particles. This is regarded as the first observation of electrophoresis and electroosmosis [26]. However, it was not utilized as an analytic technique until introduced by Arne Tiselius around 1930. He thereafter won the Nobel Prize in 1948 for his research of moving boundary electrophoresis, which led to separations that revealed the complex nature of serum proteins [27]. Nonetheless, Tiselius also encountered the problems of the poor resolution of proteins, large sample demand, and large convection current from Joule heating, which are mostly due to the large inner diameter of the glass tubes used in classical electrophoresis. Hjertén [28], Virtanen [29] and Mikkers et al. [30] later did the ground works to reduce the capillary size down to 300 µm or 200 µm on the study of open tube free solution electrophoresis. However, the future was still not so bright for this electrophoresis technique at that time, compared to the rapid development in HPLC and gel slab based electrophoresis. In 1981, Jorgenson and Lukacs published a pioneer work by using a capillary with 75 µm i.d., which led the classic electrophoresis into a modern high performance capillary electrophoresis (HPCE). This narrow capillary with its high surface area to volume ratio allows efficient dispersion of the heat generated from the applied high voltage, and obtain high efficient separations of amino acids, dipeptides, and amines [31].

With years of research and development on this technique, and also the introduction of a commercial automated CE instrument in 1989, CE started to become a more popular separation technique. Numerous original papers, review papers, and books have been published, especially in the last ten years [32, 33]. It has been applied to the separation of a wide range of solutes, e.g., inorganic compounds, small organic molecules, and large biomolecules in applications ranging from water monitoring, drug and food industry, clinic study, etc, using different separation modes [33-36]. The best known application of CE is DNA sequencing by capillary gel filtration in the Human Genome Project. Figure 2 shows a typical modern HPCE instrument setup, which consists of a 20-100 µm capillary, a high voltage power supply up to 30 kV, a
UV light source and detector, a set of separation buffers and samples, and a data acquisition system.

![Diagram of Capillary Electrophoresis system.](image)

**Figure 2.** Scheme of Capillary Electrophoresis system.

### 2.3.1. Electrophoretic Mobility

The electrophoretic mobility of an ionic analyte is the key issue for the electrophoretic separation in CE. When a charged particle is placed in an electric field, it will be accelerated and start to move toward the electrode with opposite charge. This accelerating force (\(F_{ep}\)) and the velocity of movement (\(v_{ep}\)) can be defined as:

\[
F_{ep} = qE \\
v_{ep} = \mu_{ep}E
\]

where \(q\) is the charge of particle, \(\mu_{ep}\) the electrophoretic mobility, and \(E\) the electric field strength.

As the friction force (\(F_{fr}\)) is a function of the velocity of the moving particles and the friction coefficient \(f\) (defined by Stokes equation as \(f = 6\pi\eta r\)); therefore, it is expressed as:

\[
F_{fr} = f v_{ep} = 6\pi\eta r v_{ep} = 6\pi\eta r \mu_{ep} E
\]

where \(\eta\) is the viscosity of the solution and \(r\) is the radius of the charged particle.

When the particle starts to move under the influence of the electric field, the fraction force increases with increasing speed of particle and it finally reaches a magnitude equal to the accelerating force at a steady speed, i.e., \(F_{ep} = F_{fr}\).

Finally, the electrophoretic mobility can be defined as:

\[
\mu_{ep} = \frac{q}{6\pi\eta r}
\]

### 2.3.2. Electroosmotic Flow

Electroosmotic flow (EOF) is one of the most important phenomena for the CE. It was first discovered by Helmholtz in the 1800s [37]. With the continuous study after
Helmholtz, it was found that the EOF is mainly caused by the electric double layer in the interface between the capillary surface and the solution. Silanol groups naturally exist on the inner surface of fused silica capillary. When pH is above 3, the silanol groups dissociate and form a negatively charged wall surface. The cations in the buffer solution are attracted to the capillary wall by the electrostatic field and then form an electric double layer. After applying high voltage to the capillary, the mobile cations in the diffuse layer along with solvating solvent molecules are dragged toward the cathode and form the bulk flow called electroosmotic flow (EOF). When increasing the pH of the separation buffer solution, the EOF increases due to the high surface charge from dissociation. The EOF is also affected by many other parameters, e.g., temperature, ionic strength, organic solvent; it can be generally expressed as:

$$\mu_{\text{eof}} = \frac{\varepsilon \zeta}{\eta}$$  \hspace{1cm} (5)

where $\varepsilon$ = dielectric constant, $\zeta$ = zeta potential, $\eta$ = viscosity.

In a native capillary, the EOF is very large and acts as an “electric pump” that helps to transfer the sample toward the detection window for detection. However, it is not always good to have high EOF, because the ionic sample will not have sufficient time to stay in the capillary and achieve separation. Therefore, the capillary surface should be modified in many cases.

2.3.3. Surface Modification

Apart from the reasons of suppressing EOF and obtaining high efficient separation, another one for modifying the capillary surface is to prevent electrostatic interaction between the negative charged fused silica capillary and cationic samples, especially for biological macromolecules such as proteins. Although there is a wide possibility of choosing CE capillaries from different materials, fused silica is still the most popular one. The major advantage of using fused silica is its hydrophilic surface which prevents wall adsorption by hydrophobic interaction for large biological molecules. There is also a wide variety of chemical reactions that can be carried out on silica surfaces, which has been extensively investigated in the production of silica-based HPLC separation materials and GC columns. Other capillary materials such as poly(tetrafluoroethene) (PTFE) [30], polypropylene (PP) [38-46], poly(methyl metharylate) (PMMA) [47], poly(ether ether ketone) (PEEK) [48] and other polymeric capillaries [49] have been studied and show lower EOF than the fused silica capillaries. Nevertheless, they also show disadvantages such as low UV transparency and highly hydrophobic surface properties which consequently also need surface modification [50]. Accordingly, they are still in the research and developing stages.

There are numerous scientific papers demonstrating different ways to modify the inner surface of fused silica capillaries. Studies show that the suppressed EOF helps to improve the separation efficiency and resolution in many applications. The surface modifications can be generally separated into two groups, dynamic coating and
covalently bonded surface modification. For the dynamic modification, the positive charged or neutral compound such as inorganic salt, amine, surfactant, or polymer is added in the background electrolyte. The negative charge on the capillary surface is either neutralized or shielded by the additives. For the covalent bonded surface modification, appropriate functional groups are covalently bonded on the capillary inner surface, using the silanol groups as anchor groups. These function groups can be either single functional molecule, or ionic and neutral polymer. More detail information about the surface modification can be found in several comprehensive review papers [50-55] and textbooks [32, 37, 56-58].

2.4. Liquid Chromatography for Inorganic Ions and Proteins vs. Capillary Electrophoresis

In CE, samples are separated under high voltage electric field according to their mass to charge ratio. In contrast to the separation by HPLC is mostly due to the sample partition between the stationary and mobile phases. Because mass transfer is not involved in the separation process, CE has a potential for much faster and more efficient separations than HPLC. Moreover, since HPLC uses hydraulic pressure established by a mechanic pump to delivered eluent and CE uses EOF as “electric pump”, flow profiles are different in a HPLC column and CE capillary. This is illustrated in Figure 3, where A is a parabolic laminar flow profile existing in the passageway of a LC column, whereas B is the flow profile in capillary under electroosmotic flow, which shows a very flat profile.

Figure 3. The flow profiles in a LC column (A) and CE capillary (B).

Harris gives a very simple and clear explanation of these phenomena based on the theoretical aspects in his analytical textbook [59]. As we know the approximate van Deemter equation is:

\[ H = A + B/u_x + C u_x \]  

\[ (6) \]

\( H \) is plate height; \( u_x \) is linear flow rate; \( A, B, C \) are the constants for a given column and stationary phase. It can be seen from this equation that the major effects on bandbroadening in a chromatographic system are from the multipaths effect term \( A \), the longitudinal diffusion term \( (B/u_x) \), and the term \( (C u_x) \) which relates to the mass transfer limitations between the stationary and mobile phases. In a CE system, the terms \( A \) and \( C u_x \) can eliminated from the equation since they are related to the column
packing and the interaction between solutes and a stationary phase (none of which exist in CE), and the plate height then depends solely on the \((B/u_s)\) term. Hence, the potential of obtaining good separation efficiency is higher for CE.

Even though LC and CE are two totally different analytical techniques, it is still very interesting to compare them and foresee the future trends in separation science [14, 37, 60]. When comparing ion chromatography and capillary electrophoresis for the analysis of small ionic analytes, modern CE generally has the advantages of faster separation, low sample and separation solution consumption, higher efficiency and resolution, and a simpler system setup compared to IC – at least in principle. However, CE has the problems of low reproducibility, inferior robustness, and high demand on the operator’s knowledge, compared to LC. Therefore, although these two techniques look competitive one to another, they are complementary nowadays. Lucy [61] applied Laitinen’s “Seven Ages of an Analytical Method” to review the development of ion chromatography, and placed it at 6th age. Applying the same philosophy, Haddad [60] then positioned CE at ages 2nd-5th. He points out that CE is much less developed than IC. This also means we are comparing two techniques at different stages of development. Accordingly, CE should have large latent capacity to overtake IC. Chen et al. also demonstrated that CE has many attractive features for the separation of small organic acids, compared to IC [62].

When comparing ion-exchange chromatography and CE for the biological macromolecule analysis such as protein, CE also shows much high separation efficiency and other advantages over IEC owing to the different separation mechanisms, i.e., IEC uses sample partition principle to achieve the protein separation; in contrary, CE separate proteins according to their charge and mass. However, if a preparative scale separation is needed, IEC is very easy to be enlarged, whereas it is quite difficult for CE so far. Shen and Smith recently review the high-efficiency capillary separation techniques included CE and capillary IEC for the modern proteomics study, they shows the IEC is just primarily used for the sample fractionation [63]. It also has the possibility to combine IEC and CE system for the two dimensions separation in proteomics study in order to increase the dynamic separation range and sensitivity of complex samples.
3. Covalently Bonded Zwitterionic Separation Materials for Liquid Chromatography

3.1. Introduction

Liquid chromatography has been separated into several groups according to the separation mode, e.g., Reverse-Phase, Normal Phase, Immobilized Metal Affinity, Size-Exclusion, Ion-Exchange, Hydrophobic Interaction, and Hydrophilic Interaction Chromatography, and etc. The reason for this plentitude of separation modes is that liquid chromatography is a separation technique which is driven mainly by selectivity, rather than efficiency. Fortunately, the liquid separation medium offers almost endless ways of adjusting the eluents; therefore, the final retention is a combination of the affinity of the stationary phase for the solutes and the solvophobic forces that act by driving the solutes out of the solution and onto the stationary phase. For a given number of stationary phases, the potential for selectivity tuning is therefore much larger than in technique such as gas chromatography, where the carrier gas does not play an active role in the separation, except in a few special modes. The major difference between the separation modes in LC is the use of the separation materials with different surface ligands, in combination with suitable mobile phase compositions. The separation modes are thus designed for separation of different species according to the solute properties, and all modes have their advantages and drawbacks. There is no single sub-technique that can be used to carry out the separation of all kinds of solutes, and in real samples one often runs into the problem of having solutes with properties that do not match any separation technique well, or where certain solutes require conditions that are vastly different from those required by other solutes. Therefore, even though so many separation modes exist nowadays, it is still worthwhile to exploit new and innovative selectivity dimensions, in order to develop new separation materials.

Chromatographic separation with zwitterionic modified materials is one of these new LC separation modes studied in recent years. Although some studies of liquid chromatography with stationary phases carrying zwitterionic functionality appeared in the 1980s [64-74], these works did not catch much interest and relatively few works appeared over the ten year period following the initial work by Knox et al.. However, the interest in zwitterionic groups was renewed by the study from Hu et al., who established the zwitterionic functionality by rinsing the ODS (Octadecyl Silica) column with sulfobetaine type zwitterionic surfactant. By this way, the zwitterionic surfactant was physically adsorbed on the ODS material surface by the interaction between the C18 groups in column and the hydrophobic tail of the surfactant. The positively charged ammonium group and the negatively charged sulfonic group coexist as one functional moiety in the head of the zwitterionic surfactant. Inorganic anions and cations can be separated through interaction with this dynamically coated
layer using water only as eluent [75-78], albeit Hu et al. have extensively used the electrolyte solutions as eluents in their more recent studies [79-83]. Other types of zwitterionic surfactants such as the phosphocholine [84, 85] and carboxybetaine [86] can also be used for coating for the chromatographic study. Hu et al. named this novel chromatographic separation method as “electrostatic ion chromatography” [75], but Cook et al. recently proposed that the name of “zwitterion ion chromatography” would be more appropriate in view of the separation mechanism [87]. Zwitterionic ion chromatography is different from other methods based on dynamic coating of hydrophobic columns, such as ion interaction chromatography [64-68], and micellar liquid chromatography [71], by not requiring the addition of surfactant in mobile phase. Therefore, it can be concluded that the zwitterionic modification via dynamic coating technique is simple and less expensive. Another important type of zwitterionic stationary phase called “immobilized artificial membrane chromatography” (IAMC) has also been widely studied by Pidgeon’s group since the 1990s [73, 88-90], but the objectives of this method is to assess the interaction of solutes with an immobilized layer in partially organic aqueous eluents, where the sorbent covalently bonded with phospholipid type ligands are intended to mimic the sorption properties of biological membranes. This thesis deals with sulfobetaine type zwitterionic materials, since the ion and protein separations that were investigated require more or less fully aqueous eluent.

The main drawback of dynamically coated columns is that they usually have an inferior stability compared to covalently bonded columns, due to loss of functional moieties from the dynamically attached layer. This is especially problematic in the separation of samples containing biological macromolecules, because they may strip off the adsorbed surfactant through the hydrophobic interaction. Therefore, the surfactant often should be added to mobile phase to keep the system stable [91, 92]. Another problem encountered is the separation of hydrophobic solutes by zwitterion-exchange chromatography or hydrophilic interaction chromatography, where an organic solvent should be used in eluent [74, 93, 94]. Moreover, the separation of anions is according to their ion-pair with the cations in the sample solution, which is difficult for the peak identification and quantitative analysis. So it is very interesting to carry out studies comparing covalently bonded and dynamically coated zwitterionic materials with the same functional groups. When searching the literature, it was found that there are only few covalently bonded zwitterionic separation materials have been developed based on silica particles [70, 72, 74, 88-90, 93, 95-98]. As methacrylate polymer materials are known to be less hydrophobic than poly(styrene-DVB) materials and more stable than silica material over a wide pH range. Particles based on methacrylates were therefore selected initially as substrates in the attempt to synthesize a new sulfobetaine type covalently bonded zwitterionic material (Paper I-II). Additional historical information on separation materials with various zwitterionic
functionalities and material conformations can be found in a comprehensive review paper by Nesterenko and Haddad [99].

The schematic illustration in Figure 4 shows the functional group constitution of a typical zwitterionic material along with several typical zwitterionic functional groups. This thesis focuses on materials with sulfobetaine type zwitterionic functional groups, which belongs to the strong/strong category, i.e., both the negative and positive groups retain their charge over the entire operational pH range. In other words, the overall zwitterionic moiety maintains a zero net charge.

![Schematic illustration of a typical zwitterionic material and typical zwitterionic functional groups](image)

**Figure 4.** Schematic illustration of a typical zwitterionic material (left) and Typical zwitterionic functional groups for the zwitterionic modification (Right).

### 3.2. Synthesis of Covalently Bonded Sulfobetaine Type Zwitterionic Separation Materials for LC

#### 3.2.1. By Direct Surface Reaction

The 2-hydroxyethyl methacrylate/ethylene dimethacrylate (HEMA-EDMA) type of macroporous material (Spheron® or Separon®), invented by the group of Čoupek in former Czechoslovakia in 1970s, was studied by various surface modification reactions using the 2-hydroxyethyl moieties on the particle surfaces as the anchor groups [100-102]. HEMA-EDMA is a more hydrophilic separation material compared to poly(styrene-DVB), and also has a far better stability in alkaline solution compared to porous silica. Our group has long experience of using this kind of material as the supporting matrix for chemiluminescence detection [101, 103, 104]. We therefore choose it as matrix material for the synthesis reported in **Papers I** and **II**. The details about the matrix material will be described in next section.

In **Paper I**, the surface functionality was established by a two-step reaction, in which HEMA-EDMA copolymer beads were first activated with epichlorohydrin, whereafter 2-dimethylaminoethanesulfonic acid inner salt was coupled to the epoxide groups on the activated beads in a quarternizing reaction, as it can be seen in Figure 5.
This resulting material carried strong/zwitterionic pendant groups. However, the material did not show a good charge balance because the stoichiometry of nitrogen:sulfur is about \(1:0.85-0.90\) in molar ratio from the elemental analysis. The separation properties for inorganic ions on this material are very different from the dynamically coated zwitterionic materials reported in the literature, and this difference is attributed to the charge imbalance in the zwitterionic functionality in Paper I.

![Figure 5](image-url)

**Figure 5.** Representation of the synthesis reactions leading to the zwitterionic separation material based on the cross-linked 2-hydroxyethyl methacrylate polymer beads. [Paper I]

With the aim of achieving a material with good charge balance and suitable for the separation with water only as eluent, new synthetic routes were chosen in Paper II. Three different zwitterionic materials were synthesized based on polymethacrylate polymer particles. In details, materials designated S300-ECH-DMA-PS or S300-TC-DMA-PS involved activation of the 2-hydroxyethyl groups of the HEMA-EDMA material with epichlorohydrin or thionyl chloride, respectively, followed by dimethylamination and quaternizing 3-sulfopropylation with 1,3-propane sultone; the third route was accomplished by attaching methacrylate moieties to the 2-hydroxyethyl moieties through a reaction with methacrylic anhydride, followed by graft photopolymerization of the zwitterionic monomer \(3-[N,N\text{-dimethyl-N-}(\text{methacryloyl-oxyethyl})\text{-ammonium}]\) propanesulfonate (SPE), initiated by benzoin methyl ether under 365 nm light. The synthesis of S300-TC-DMA-PS in the study can be seen in Figure 6, whereas the other two synthesis routes can be found in Paper II.

![Figure 6](image-url)

**Figure 6.** Synthesis steps used in the preparation of the zwitterionic stationary phase S300-TC-DMA-PS. [Paper II]
Elemental analyses of nitrogen and sulfur were also performed in this study, and it was found that the nitrogen:sulfur molar ratios differ substantially for these three materials (see Table 1, Paper II), and S300-TC-DMA-PS shows the best charge balance. Further chromatographic evaluations show that this charge balance will finally affect the separation properties. More details on the separation will be discussed in the later sections. The 3-sulfopropylation reaction in the synthesis of the first two materials is the critical step for obtaining a zwitterionic material with good charge balance. Although this reaction is widely used in preparation of zwitterionic monomers [105-111], there are only a handful of applications employed to produce zwitterionic chromatographic materials [74, 95].

3.2.2. By Surface Graft Polymerization

In classic polymer chemistry, graft polymerization is originally described as a polymerization procedure to make a new polymer segment on the top of the matrix polymer chain and form block copolymer by a covalent link [112]. However, the use of graft notion has not been restricted only to polymeric matrices; it is common to refer to grafting for most processes where polymer chains are attached to or grown from almost all kinds of material such as polymer, metal, silica [113]. The reaction can be achieved by using plasma, UV, ozone, photo or thermal initiators [114, 115]. The graft polymerization technique is also not new in chromatography; its application in ion-exchange chromatography of proteins was the subject of considerable discussions a few years ago, when “tentacle” resins were a hot topic [116-124]. Applications in capillary electrophoresis can also be easily found, where the graft polymerizations are used for covalent bonded surface modification [50, 53, 125]. Generally, graft polymerization is divided into two categories, graft “onto” or graft “from”, depending on whether the growing polymer chains start from the matrix polymer or elsewhere and later attached to it [114, 115, 126]. Although Davis et al. give a third graft category, graft “through”, I will still keep the discussion based on two categories of graft polymerization.

Graft “onto” solid polymeric matrices is usually carried out with the matrix suspended in a mixture containing monomer, initiator and solvent. The polymerization starts in the mixture solution, and the preformed telomer can randomly reacted onto the matrix via the active groups on surface. This method has been used in Paper II, where the SPE monomer is grafted onto the polymeric HEMA polymer particle via methacrylate anchor groups, as shown in Figure 7. The resulting material can be used for the separation of inorganic anions using perchloric acid or perchlorate eluent. Viklund et al. also used the graft “onto” technique to prepare monolithic type zwitterionic separation materials [127, 128]. However, the elemental analysis for the grafted zwitterionic separation material in Paper II shows that the quaternary ammonium and sulfonic group is in the molar ratio of 1:0.77, which we supposed it may be a result of the first surface activation step with amine type catalyst.
In Paper III, we chose a graft “from” method via the peroxide initiating groups attached onto the silica surface with the intention of synthesizing the zwitterionic materials with better charge balance. Graft “from” polymerizations are usually carried out with the matrix material with bonded initiator groups suspended in monomer solution and solvent. By this way, polymer chains grow from the matrix surface and result in a higher surface coverage compared to the graft “onto” method [115]. Tsubokawa’s group has worked for a long time on the graft polymerization on different materials via pendant initiating groups such as azo, peroxide, benzylium perchlorate, amino, or peroxcarbonate groups [129-134]. More new interesting chemistry for graft “from” polymerization, for example, Atomic Transfer Radical Polymerization (ATRP) [135-137] or living radical polymerization by iniferter [138, 139], have also been broadly used to get grafted polymer brushes on the matrix surface. The detailed synthesis route for the graft “from” polymerization in Paper III is shown in Figure 8. By this approach, the final material shows a good charge balance with a nitrogen:sulfur molar ratio of about 1:1.01. Satisfactory separations are also obtained for inorganic anions and proteins by varying the separation eluents.

3.3. Matrices for Synthesis of Zwitterionic Separation Materials

During the early development of chromatography, inorganic materials were used as separation material. The first and most well known experiment on column chromatography was done by the Russian botanist M.S. Tswett, who separated green leaf pigments into colored bands on calcium carbonate, using CS₂ as solvent [140]. After a century of development, the separation materials for LC have experienced from ground particles with irregular shape to monodisperse spheres, from inorganic material to polymer type, and from particle size of several hundred micrometers to only a few micrometers. Nowadays, the most popularly used chromatographic LC materials are spheric macroporous particles, for example, silica, metal oxides,
polysaccharides, poly(styrene-DVB), and polymethacrylates [141, 142]. New packing materials such as perfusive packings [143-146], non-porous particles [147-150], and monolithic materials [151-161] have also become popular recently [162]. Separation materials are normally surface modified to get different functional groups according to diverse separation mechanisms of chromatography [163, 164]. As the particles I have used in my studies were HEMA-based methacrylate polymeric particles and silica, which are intended to be modified with zwitterionic functional groups for separation of ionic solutes. So I will give more details on these two types of material in the application for ion chromatography.

![Figure 8. Scheme of synthesis of zwitterionic separation material by graft “from” approach. [Paper III]](image-url)

Most polymer particles for IC separation are synthesized by copolymerization of either styrene-DVB, or methacrylate type monomers and crosslinkers such as HEMA and EDMA. The resulting porous polymeric particles have relatively good mechanic properties and are characterized by their pore size distribution and surface chemistry. The particle and pore size of the macroporous separation material can be adjusted with changing the monomer and crosslinker composition, type and concentration of porogen, polymerization temperature, stirring speed, and etc. Generally, polymer particles made from styrene and DVB have higher mechanic strength and more hydrophobic surface properties compared to methacrylate type particles. This hydrophobic interaction causes some problem in the separation of organic ions, and also polarizable inorganic anions [165]. Haddad and Jackson provide a more detailed comparison of methacrylate and styrene based materials, and also of silica material [4]. A recent study by Jackson et al. also shows that the methacrylate latex based agglomerated separation materials give much better selectivity for the oxyhalide anions, and good separation of fluoride ion from water dip [166, 167].
Silica particles are typically prepared by a sol-gel process, i.e., the hydrolysis and condensation of silicic acid, or more commonly suitable metal alkoxides such as tetramethoxysilane in alcoholic solutions with acid catalyst [168]. Silica particles have advantages over polymer type materials, e.g., excellent mechanic stability and no swelling or shrinkage in solution even with organic solvents. Surface modification of silica is also facile by the reaction through the silanol groups on surface [168, 169]. All these advantages make silica material to be the most popular packing material for modern HPLC. However, silica suffers from instability in high pH solutions, and can only be used in a narrow pH range from pH 2 to 8 due to the risk of hydrolytic cleavage. This makes silica less popular compared to polymeric particles for IC, because aqueous solutions of sodium hydroxide or sodium carbonate/bicarbonate are usually used as eluent for this method. By covering polymer on the silica surface, the stability of material can be improved [163].
4. Covalently Bonded Zwitterionic Separation Materials for Capillary Electrophoresis

4.1. Introduction

Many studies have involved in the surface modification of fused silica capillaries to reduce the EOF and wall adsorption in order to improve the separation efficiencies of various samples. In relation to zwitterionic modifications for these proposes, it can be first traced back to the study by Bushey and Jorgenson, who separated model proteins with buffers containing high concentration of zwitterionic salts [170]. The organic zwitterionic salts show very low conductivity which allows the separation to be carried out under higher voltage; moreover, the zwitterionic salts also increase the viscosity of separation buffer, which also helps to reduce the EOF. Other works related to Micellar Electrophoretic Capillary Electrophoresisis using zwitterionic surfactants are also very interesting. Swedberg found the selectivity was enhanced by adding nonionic and zwitterionic surfactant in the separation buffer [171]. Hansen et al. used zwitterionic and nonionic surfactants for the separation of basic drug substances with similar structure by MEKC [172]. Fürtös-Matei et al. evaluated the MEKC separation of 13 dynorphin peptide analogs with anionic, cationic, and zwitterionic (CHAPS) surfactants. It was found that the CHAPS-MEKC system can fully separate these 13 peptides analogs [173]. Lookart et al. utilized five zwitterionic surfactants with different hydrocarbon tails for the separation of barley hordeins [174]. All these applications show different improvements on the selectivity and resolution by using zwitterionic surfactants.

When a relatively low concentration of zwitterionic surfactant was used for dynamic surface modification in capillary zone electrophoresis, the EOF was extensively suppressed as well. This is a simple and low cost way of tuning the EOF and has been studied by several research groups and applied to improve the separations of peptides [175, 176], proteins [177-182], and inorganic anions [183-187] in recent years. Nevertheless, as the surfactant is mixed in the background electrolyte, it adds background interference; especially its use will be restricted with mass spectrometric detection. In order to get rid of this problem and also keep the attractive features of zwitterionic modification, Lucy et al. recently reported a semi-permanent phosphocholine coating on the capillary with double chained zwitterionic surfactant [188, 189]. Due to its strong sorption to the wall, no zwitterionic surfactants are added into the separation buffer; the EOF is also widely suppressed and good separations of proteins are obtained. However, flushing with zwitterionic surfactant is necessary after each run in order to get reproducible results; this will increase the total separation time.
Based on our knowledge of preparing zwitterionic separation materials for LC, we found the study of covalent bonded zwitterionic surface modification for CE to be highly interesting, both to investigate the basic separation properties and to compare with capillaries dynamically coated with zwitterionic surfactants. When we searched the literature, we found almost no study on covalently bonded zwitterionic modification in the CE capillary, except one patent application in 1992 by Swedberg and McManigill [190]. Although they announced the possibility to suppress the EOF in CE capillaries by a surface modification with a phosphocholine type silylation regent, unfortunately, there were no detailed results showing the improvement on CE separation.

Figure 9. Reaction scheme for the synthesis of the CE capillary grafted with SPE zwitterionic polymer layer. [Paper V]

4.2. Synthesis of Zwitterionic Capillaries for CE

In Paper V, a two-step scheme (Figure 9) was chosen to modify the capillary surface by grafting a zwitterionic polymer layer on the inner surface, using a photoinitiated polymerization technique. The silanol groups on the native capillary first react with 3-methacryloyloxy-propyl trimethoxysilane (MAPTMS), which produce methacrylic anchors on the surface covalently bonded through a Si-O-Si-C linkage [191].
After filling the capillary with the grafting solution containing SPE monomer and photo-initiator (Benzoin methyl ether, BME), the polymerization was started by 365 nm UV light for one hour. A transparent capillary was used here because of the photopolymerization approach, which is a faster free radical polymerization compared to thermal one. During the polymerization step, the surface attached methacrylic groups will be incorporated in the growing polymer chains by a grafting procedure, in which the same polymer chain may incorporate several surface methacrylic groups leading to multipoint attachment.

According to the EOF measurements, the EOF is suppressed to 1/6 of that in a native capillary. The EOF was also measured on a MAPTMS activated capillary in order to ascertain the reason for the EOF suppression. As the EOF on the MAPTMS activated capillary was similar as the native capillary, we propose that the suppression is not due to a reduction of the silanol group density, instead to the charge shielding or dissipation in the graft polymer layer, which has been discussed in the Paper III. Moreover, as it can be seen from Figure 10, positively charged compounds, especially solutes with large molecule size such as proteins, show considerable electrostatic interaction with the negative charged wall for a native capillary; this makes the separation efficiency and resolution very poor. With zwitterionic grafted capillaries, the negative charge from capillary wall is diluted in the zwitterionic polymer layer, and thus the electrostatic interaction becomes weaker.

![Figure 10](image-url). A diagram on the wall interaction before and after the surface graft polymerization of zwitterionic polymer.

In Paper VI, a new zwitterionic capillary was synthesized by a thermo-initiated graft polymerization on a polyimide coated fused silica capillary. This is because the transparent type capillary used in Paper V is very fragile and difficult to be handled in the operation, and also it can not be used in a CE instrument with liquid cooling system. In contrary, the polyimide coated capillary is very rigid and has been popularly used in all kinds of CE and GC instruments. However, as the coating is not UV transparent, different synthetic way had to be chosen for producing zwitterionic polymer layer on the inner surface of capillary. It was then synthesized by thermal grafting polymerization, where the photo initiator BME was replace by water soluble initiator V85. Thermal polymerization usually is slower than photo polymerization. In
this study, the thermal graft polymerization was then carried out at 85 °C for three hours. The EOF study was also carried out on this capillary, and it was suppressed about two third compared to the one in a native capillary. However, it has less suppression compared a photo grafted capillary. It may owe to the different polymerization rate, the solvent effect or some other polymerization conditions, which soon will be evaluated.
5. Characterization of Zwitterionic Separation Materials

5.1. Spectroscopy Method

The spectroscopy methods described here for characterizing zwitterionic functionality include Infrared and Raman Spectroscopy, which have been used in the synthesis of particle type zwitterionic materials.

Infrared spectroscopy is based on investigation of molecular vibration and rotation states under radiation with light in the infrared region. As we know, molecules consist of a group of atoms which are connected each other by covalent bonds, and rotate and vibrate in a 3 D coordination system. When irradiated by photons of the appropriate energy, the molecule undergoes vibrational and rotational transitions by absorbing light. Different bonds have their specific absorption of light at certain wavelengths. By this method, we can identify changes in the molecule structure. In Paper I-III, FTIR was used to check the formation of epoxide groups and zwitterionic functional groups on the surface of HEMA polymer particles. The overlaid FT-IR spectra are present in Figure 11. As the quaternary and tertiary amine groups do not show specific absorbance in IR, we therefore just study the appearance of epoxide and sulfonic groups. It can be seen from Figure 10 that epoxide groups appear at around 850 and 910 cm\(^{-1}\) and sulfonic groups at around 1039 cm\(^{-1}\).

![Figure 11. Overlaid FT-IR spectra of unfunctionalized Spheron 300 cross-linked HEMA sorbent (lower), after epichlorohydrin activation (middle), and after final coupling of DMAES to the activated particles (upper). [Paper I]](image)
Raman spectroscopy is complementary to IR. It works by measuring the Raman scattering radiation, which is the result of a frequency shift corresponding to vibrational transitions, when light irradiates on the molecule. As it differs from IR by the wavelength of the incident light, Raman spectroscopy can give some information which can not be obtained by IR spectroscopy. In Paper I, we used Raman in an attempt to positively identify quaternary and tertiary ammonium groups, and also to get further support for the existence of the sulfonic acid groups. It was found that the peak at 1032 cm\(^{-1}\) can be assigned to the sulfonic acid, but no conclusive information was obtained that could verify the ammonium groups. Figure 12 shows the overlaid FT-Raman spectra of unfunctionalized particles and zwitterionic functionalized one.

![Figure 12. Overlaid FT-Raman spectra of unfunctionalized Separon HEMA S 300 before (lower) and after activation and final coupling of DMAES to the activated particles (upper). [Paper I]](image)

5.2. ζ-potential

5.2.1. Double layer and ζ-potential

In liquid medium, charged particles attract ions with opposite charge (counter ions) and develop an electric double layer. As can be seen from Figure 13, this double layer consists of a tightly adsorbed inner layer called the Stern layer, and a loosely adsorbed outer layer called the diffuse layer. For particles with net negative charge in electrolytes of moderate ionic strength, cations are attracted to the surface and enriched in the inner layer due to electrostatic forces; the concentration of cations gradually decreases with distance from the shear surface between the inner layer and outer layer to the bulk solution. The potential formed between the shear surface and bulk solution is called the ζ-potential. It reflects the charge property of a charged
particle interacting with the surrounding electrolyte. The main factors governing the magnitude of the ζ-potential are the charge density of the surface, and the electrolyte type and concentration. When we study the ζ-potentials in different aqueous electrolyte solutions, we get qualitative information about the change of ζ-potential; this in turn gives an indication of the strength of ionic interactions, for example, the interaction between protein and a charged surface [192]. It is also very important to study the ζ-potential of capillary surfaces, because it is directly connected to the EOF, which can be seen from Equation 5.

![Diagram of an electric double layer and ζ-potential on a charged surface.]

**Figure 13.** Schematic presentation of an electric double layer and ζ-potential on a charged surface.

### 5.2.2. ζ-potential Measurement by Photon Correlation Spectroscopy

There are several ways to measure the ζ-potential, and it has traditionally been measured by observing the movement of charged particles in an electric field using microscopy. The charged particles are attracted towards the electrode with opposite charge to the surface charge, and their velocity in the electric field depends on the particle surface charge (or zeta potential), and the voltage applied. Therefore, the velocity can be measured and the ζ-potential can be determined by Smoluchowski equation (Equation 5). However, this method is normally more time-consuming and provides lower accuracy for the measurement of slow moving particles due to the long measuring time. It also involves a substantial manual works.

In this thesis, a Malvern Zetasizer 4 Photon Correlation Spectroscopy instrument was used [193]. The basic operation principle is described as follows: The diluted particle solution is transferred to a measuring cell, where the particles are electrically driven across the interference fringes from two crossing laser beams. Light reflected from the moving particles is then collected by a photomultiplier. In the setup, one of the laser beams is oscillated by a mirror attached to a piezo-electric crystal which is actuated by an AC voltage with 10-100 Hz modulation frequency. For particles that are static in solution, the light collected by the photomultiplier has the same frequency as the modulation frequency. When the particles move under the influence of the electric field, the collected frequency of scattering light is shifted by the Doppler Effect, which
in turn can be transformed to velocity, electrophoretic mobility. Finally the \( \zeta \)-potential can be determined.

5.2.3. Salt Effect on the \( \zeta \)-potential of Zwitterionic Separation Materials

Discussion of the effects of inorganic salts on the surface charge of zwitterionic separation materials occurs in most of the papers in this thesis. The reason is that the charge property of the separation materials will substantially influence the final separation property. The interaction between the ionic functional groups and added salt in aqueous solutions follows the Hofmeister series, which was originally studied by Franz Hofmeister in 1888 in order to rank ions on the efficiency of precipitating egg white protein [194-196]. For the study the solution properties of sulfobetaine type zwitterionic polymer, this series of anions is described as: \( F^- < Cl^- < NO_3^- < I^- < SCN^- < ClO_4^- \), which is closely parallel to the classic Hofmeister series. The series can rank the interaction between “soft” quaternary ammonium groups and anions [197-203]. \( ClO_4^- \) is a largely polarizable anion and defined as a “soft” ion. The “soft” anions prefer to bind on the “soft” cations [204]. This is why \( ClO_4^- \) has the strongest interaction with quaternary ammonium group in all ranked anions. Apart from the study in polymer and protein solution, this series has also been applied to study chromatographic separations [127, 128, 205-208], micellar solutions [209-214].

Unlike other polyelectrolytes and neutral polymers, zwitterionic polymers show “anti-polyelectrolyte” properties, i.e., they do not dissolve in pure water and require an electrolyte solution to dissolve at room temperature. On the other hand, since the material possesses both negative and positive charged groups, the charge properties will be affected by both the anions and cations in solution. However, most cations, with the exception of some hard cations, have less effect on the net charge of sulfoalkylbetaine zwitterions due to the low polarizability of the sulfonic acid moiety. In Papers II-IV, the \( \zeta \)-potentials of the synthesized sulfoalkylbetaine type zwitterionic materials are measured in different acid, base, and salt solutions. It was found that the perchlorate ion has stronger interaction with quaternary ammonium group compared to the chloride ion; which in turn means that the perchlorate ion induces a more negative \( \zeta \)-potential on the zwitterionic separation material. The effect of the tested cations on \( \zeta \)-potential follows the order: trivalent (\( Ce^{3+} \)) > divalent (\( Mg^{2+} \)) > monovalent (\( Na^+ \)).

5.3. Elemental Analysis

By this method, the ion exchange capacity for the sulfobetaine type zwitterionic separation material can be estimated from the content of nitrogen and sulfur, which sequentially can be used to judge the charge balance on the material.

Modern elemental analysis of organic compounds is usually performed by a CHN (carbon, hydrogen and nitrogen) and S (sulfur) analyzer based on the principle of
combustion. Standard and sample are weighed into a tin capsule and placed in an autosampler where the sample drops in a packed combustion tube at about 1000 °C. When the sample is inside the tube, oxygen is injected to the helium carrier gas. The tin capsule is oxidized and the temperature rises momentarily to about 1800 °C by the exothermic effect, which ensures a complete disintegration of sample. The combustion gases containing the oxidized species of the analytes, CO₂, H₂O, NOₓ, SO₂ and SO₃, are then swept into a reduction chamber containing a copper catalyst at 650 °C, where NOₓ are reduced to N₂ and SO₃ to SO₂. The gases are separated by the principle of gas chromatography and measured by TCD (Thermal Conductivity Detector) [215, 216]. Calibration is established by measuring a series of standards (acetanilide), and evaluated by the method of least squares. In order to control the drift, the standard substance is analyzed after every fifth sample. In this doctoral study, both polymeric and silica particles with or without zwitterionic modification are tested. The amount of nitrogen and sulfur of zwitterionic separation material could be accurately determined in both sample types.

![Figure 14. Conductivity “blank” titration of an unfunctionalized Separon HEMA S300 at low perchloric acid concentration (filled squares) with titration data for the zwitterion functionalized material added for comparison (open triangles). [Paper I]](image)

### 5.4. Sorption Isotherm

The sorption isotherm is commonly applied in the study of dynamic equilibration between solid material and surrounding liquid. In the ion-exchange chromatography, the sorption isotherm studies the correlation between the absorbed concentration of ionic species in ion exchanger and the one in external solution under specified conditions and at constant temperature. Accordingly, a plot of the equilibrium concentration in the stationary phase vs. the one in the mobile phase is usually drawn.
The shape of the adsorption isotherm reflects the equilibration of solutes between the two phases, which can thus be used to determine the chromatographic behavior of the solute such as tailing, fronting, or overload.

In Paper I, the sorption isotherm was obtained by conductometric titration of the suspension solutions containing polymer particle material. The material with or without zwitterionic functional groups were both titrated by different acid and salt solutions. The conductivity of solution was recorded after each aliquot addition of titrant. The concentration of titrant left in solution can be calculated from the change of the total conductivity, and the concentration absorbed by the polymer particles can be calculated using the total titrated salt minus the one left in solution. Figure 14 shows one of the important sorption isotherm experiments in Paper I, called “blank” titration of unfunctionalized HEMA-EDMA material. This study will help us to ascertain that the acid consumption was not due to the functional groups present in the methacrylate polymer matrix, instead to a property of introduced zwitterionic functionalization. It can be see that the unfunctionalized particle has less acid adsorbed onto the zwitterionic material surface. Another sorption isotherm study is the acid effect on the same functionalized material. It can be seen from the profile of two curves in Figure 15, more perchloric acid are absorbed onto the material compared to hydrochloric acid, which indicates perchloric acid has stronger interaction with the zwitterionic functionalized material. This also follows the Hofmeister series effect.

![Figure 15. Sorption efficiency for perchloric acid (upper curve) and hydrochloric acid (lower curve) upon titrating the zwitterionic material recovered from the column below the saturation region. [Paper I]](image-url)
6. Application of Zwitterionic Separation Materials for Liquid Chromatography (Paper I-IV)

6.1. Separation of Inorganic Ions

In Paper I, one zwitterionic separation material was synthesized based on the HEMA type methacrylate polymer particles, and packed into PEEK columns by the dry packing method (low packing efficiency). Chromatographic evaluation started by the separation of inorganic ions with water only as eluent, in an attempt to see if the material has the same separation properties as the dynamically coated zwitterionic separation materials [75]. Hu et al. found that the dynamically coated materials are capable of simultaneous separation of inorganic anions and cations with water as eluent, through a proposed “ion-pair interaction” separation mechanism. In this mode, sodium salts with different anions show several peaks in the chromatogram, according to their combination with the anions.

However, our covalently bonded zwitterionic material does not follow that rule. It can only accomplish the separation between NaCl and CaCl₂ with water only as eluent, which is shown in Figure 4, Paper I. It was therefore necessary to investigate if other eluents were applicable to achieve better separation of inorganic ions with this zwitterionic separation material. Finally it was found that the material was capable of separating inorganic anions and cations, both independently and simultaneously, using aqueous solutions of perchloric acid or perchlorate salts as eluents. The retention of cations and anions on this material depended on the individual ions in the analyte, not on the ion-pairs, as reported for the dynamically coated zwitterionic column. This study also revealed that both the concentration and the water structure related properties of the eluent ions were important for the retention of ionic species. When Paper I was published in 1999, this new kind of zwitterionic material had two main advantages over zwitterionic surfactant coated columns; one is that it is suitable for quantitative analysis, because the cations and anions can be separated as single peaks eluting at retention times independent of their counter ions; the other is that it can be used over a far wider pH range compared to silica-based columns. The main drawback is that no retention difference was obtained between different anions and only small differences for cations when water was used as eluent. A later study by Hu et al. shows that zwitterionic stationary phases based on dynamically modified reversed phase columns have similar separation properties as the material synthesized in this paper, when perchloric acid is used as eluent [82].

In Paper II, three zwitterionic stationary phases were synthesized for the separation of inorganic ions, and compared with the material presented in Paper I. All three materials are packed by a slurry packing method (high packing efficiency). As can be seen from Figure 16 these three materials are also capable of simultaneously...
separating inorganic anions and cations using aqueous solutions of perchloric acid or perchlorate salts as eluent, albeit with markedly different selectivities. We attribute the results to the different stoichiometries of nitrogen and sulfur ratio (see Table 1 in Paper II), which finally affected the surface charge and separation properties. This can be seen from the investigations on the correlation between the concentration of perchloric acid and the retention time of inorganic anions and cations with all materials, as shown in Figure 17. On the S300-TC-DMA-PS and S300-MAA-SPE materials, the retention times increased for cations and decreased for anions with increasing eluent concentration. These profiles are quite similar to the one shown in Paper I, and the materials are characterized with zwitterionic separation properties. Yet the S300-ECH-DMA-PS material shows the retention times of both anions and cations decreased with increasing eluent concentration, which follows the rule of conventional ion chromatography. These results demonstrate the importance of choosing appropriate synthesis conditions in order to prepare covalently bonded zwitterionic separation materials with an acceptable charge balance.

Figure 16. Chromatogram from the separation of mixtures containing a) 1mM each of RbCl, NaNO₃ and NaBr on the material S300-ECH-DMA-PS using 1 mM perchloric acid at a flow rate at 1 mL/min as eluent; b) 2 mM NaCl, 2 mM KBr and 1 mM Ca(NO₃)₂ on S300-MAA-SPE using 1 mM perchloric acid as eluent; c) 1 mM each of Na₂SO₄, CaCl₂, Ca(NO₃)₂, and 2 mM KBr on column S300-TC-DMA-PS using 1.5 mM magnesium perchlorate at 1 mL/min as eluent. Direct conductivity detection was used in all chromatograms. [Paper II]

Sodium bicarbonate was also evaluated as eluent on S300-TC-DMA-PS column, which has better charge balance, by suppressed IC mode, and compared with the dynamically coated column. It was observed that all of the tested anions had longer retention times using bicarbonate eluent than perchlorate eluent. This follows the normal elution rules for ion chromatography, i.e., the perchlorate ion should have a higher eluting ability than the bicarbonate ion. We also found that the retention time for anions decreased with increasing sodium bicarbonate eluent concentration from 5 to 20 mM, but the sensitivity to the eluent concentration appears to be substantially less than in conventional ion exchange elution.
In **Paper III**, a “tentacle” type of zwitterionic separation material KS-TC-TBHP-SPE was synthesized by surface graft polymerization on the spherical silica particles. This resulted material had a good charge balance since the nitrogen:sulfur molar ratio is 1:1.01. Its separation property was studied using the solution of perchloric acid or perchlorate salt as eluent. The plot of retention time versus the concentration of perchloric acid (Figure 1, **Paper III**) shows that this material is similar to the other zwitterionic separation materials in **Paper I** and **II**, except the S300-ECH-DMA-PS material. Therefore, it had more characteristics of a zwitterionic column, because the retention time of anions decreases and that of cations increases with increasing concentration of perchloric acid. In Figure 18, a representative chromatogram from the simultaneous separation of several inorganic ions with perchlorate salt is shown.
At the beginning of the study on zwitterionic separation materials, we always try to use the principle of normal ion chromatography or the “ion-pair interaction” mechanism to explain the phenomena we discovered in Paper I-III. Nevertheless, it is not so easy to match them together. In Paper I, it was found that the other anions also showed a general behavior on increasing the concentration of HClO₄: an initial decrease followed by a leveling off of retention times above 10 mM HClO₄. This observation, paired with the relatively large difference in retention time between the anions, even as the effect of increasing the eluent concentration had ceased at the highest HClO₄ tested. Consequently, we assumed that the separation mechanisms may be other than “conventional” ion exchange. As the anions are eluted out the column according to the increasing chaotropic properties in the Hofmeister series, i.e., in the order of smaller hydrated radii, we then assumed that an exclusion mechanism might be involved in the separation. An increased perchlorate concentration results in increasing cationic exchange capacity of the zwitterion up to approximately 10 mM of a perchloric acid eluent. Above this concentration the material appears to become saturated, and the retention times of both cations and anions become practically independent of the HClO₄ concentration.

With the exception of our predicted mechanism, there are also several other mechanisms suggested for separation of inorganic ions on the different zwitterionic separation materials. Hu et al. suggested an “ion-pair interaction” [75] and a “binary electric double-layer” mechanism [217]. Okada et al. proposed a “partition and ion-pair” mechanism for the well hydrated small ion and poorly hydrated large ions.
respectively. Macka et al. used histidine as isoelectric mobile phase to explore the separation for Electrostatic Ion Chromatography (EIC). Furthermore, Cook et al. have recently done an excellent work, with systematic experiments, to explore the separation mechanism for EIC [87]. They demonstrate that the separation of anions is due to the combination of chaotropic interaction and ionic repulsion, which is somewhat similar as we described in Paper I. In detail, the negatively charged sulfonic groups in the out part of zwitterionic separation material acts as Donnan membrane, which repels anions to move close to the material surface; the positive charged ammonium groups in the inner part of zwitterionic material attract the anions to move close to the surface according to their chaotropic selectivity, which follows the Hofmeister series.

6.2. Separation of Biological Macromolecules

In Paper III, two acidic proteins (ovalbumin and conalbumin) and three basic proteins (α-chymotrypsinogen A, cytochrome C, and lysozyme) were used as model proteins, and evaluated on the KS-TC-TBHP-SPE material. It was shown that all these five proteins could be separated using gradient elution according to a mechanism, which is predominantly of an ion-exchange nature. It was further found that the zwitterionic material acts as cationic ion-exchange material because of the distal position of the sulfonic acid with respect to the quaternary ammonium group. This cationic interaction was similar to the materials prepared by the copolymerized monolithic zwitterionic separation material and the grafted zwitterionic monoliths prepared by other members of our group [127, 128]. The correlation between the retention time of proteins and the pH or gradient conditions of the different salt solutions used as eluents was also studied. When pH is around 5, these five proteins can be simultaneous separated in one run (see Figure 4 in Paper III).

In Paper IV, the material S300-TC-DMA-PS prepared as in Paper II was studied for separation of same model proteins as above. This because the material, which is synthesized by a direct co-polymerization of a zwitterionic monomer and a cross-linker into a porous monolith [128], shows very weak interactions between the proteins and the zwitterionic functional groups, and is capable of separating the proteins under very mild conditions. However, we found a markedly stronger retention for proteins on two other zwitterionic materials, which were synthesized by graft polymerization of zwitterionic polymer onto the pore surface of monolithic copolymer [127] or silica particles (the one in Paper III). It was therefore interesting to investigate whether the exceptionally low retention seen for the copolymerized zwitterionic material was due to the functional groups being present only at the surface. In contrary to the copolymerized material, the grafted materials have the functional groups situated on non-crosslinked chains, which are attached to the substrate surface. It was also interesting to see the difference between a material with zwitterionic functional groups directly polymerized into the polymer matrix in a
methanolic solution, where there is a risk of burial of the active groups in the polymer matrix, and one with the functional groups located mainly on the surface through a post-polymerization functionalization scheme. The synthesis in this work was thus designed to produce functional groups that are identical to the groups in the copolymerized monolith, which can be seen from Fig. 1 in Paper IV. When the model proteins were injected into the packed column, good separation was achieved by optimizing the buffer pH and type of salt, as well as the gradient elution procedure, as shown in Figure 19. However, the final results show this material still shows stronger retention compared to the copolymerized zwitterionic monolith.

![Figure 19](image)

**Figure 19.** Optimized separation of a protein mixture containing 2 mg/ml of ovalbumin and conalbumin, 1 mg/ml of α-chymotrypsinogen A and lysozyme, and 0.5 mg/ml of cytochrome C. Linear gradient from 0 to 15 % A to B in 10 min, keeping 15 % B for 5 min, from 15 to 100 % B in 5 min, maintaining 100 % B for another 6 min; A: 10 mM phosphate buffer; B: 10 mM phosphate buffer plus 0.5 M NaCl and 0.1 M NaClO₄; Flow rate: 1 ml/min; UV detection at 280 nm. [Paper IV]

The salt effect is very important in the study of protein separation on sulfobetaine type zwitterionic separation material, because the material has both sulfonic and quaternary ammonium groups in a single pendant moiety. The charge properties are affected by the different salt solutions, which have been discussed in the section of ζ-potential study. The general trend on the interactions of salt-protein and salt-separation material in solution follows the Hofmeister series. When the gradient salt was changed from NaCl to NaClO₄, it can be rationalized that the surface charge of the positively charged protein will be less positive and that of zwitterionic material will be more negative. According to the separation principle for the ion-exchange chromatography of protein, these two factors will give opposite effects on the retention time of the positively charged proteins. However, in Paper IV, the gross result is found that the interaction between the perchlorate ions and zwitterionic functionalities outweighs the effect of perchlorate on the proteins, since the retention time increased.
7. Application of Zwitterionic Separation Materials for Capillary Electrophoresis (Paper V and VI)

7.1. Separation of Inorganic Ions

The first work demonstrating the separation of inorganic anions in CE was done on PTFE capillaries by Mikkers et al. in 1979. Lately, Jones and Jandik published a pioneer work on ion analysis using EOF modifier (a cationic surfactant) and chromate (background electrolyte) in separation buffer for indirect UV detection; 30 small inorganic and organic anions were well separated in 3.1 minute [37, 218]. They also studied the peak capacity for CE and IC in separating small anions, and showed CE is 10 times higher. As we know, the apparent mobility ($\mu_a$) for a small anion is dependent on both the electrophoretic mobility ($\mu_{ep}$) and EOF mobility ($\mu_{eof}$), which shows opposite direction, i.e., $\mu_a = \mu_{ep} - \mu_{eof}$ if the absolute value are used. Thus, the high peak capacity and separation efficiency can be obtained only when EOF is extensive suppressed. From this point of view, different methods included dynamic modified with different additives and covalent bonded surface modification are studied to suppress the EOF and achieve better separation performances [15, 32, 37, 58, 218-220].

![Figure 20](image.png)

**Figure 20.** Electropherogram from the separation of eight inorganic anions. Experimental conditions: detection, 214 nm; applied voltage, -25 kV; capillary, 50 $\mu$m i.d. by 50/57 cm; separation buffer, 35 mM phosphate buffer, pH 7. [Paper V]

In Paper V, we use a novel covalent bonded zwitterionic capillary prepared by a photo grafting of zwitterionic polymer. It was found the EOF with this capillary was suppressed to about 1/6 of that in a native fused silica capillary using identical separation conditions. A typical electropherogram can be seen in Figure 20, which shows good separation on a mixture of eight anions. In order to exam the separation
mechanism, the limiting equivalent ionic conductance and migration time was plotted in Figure 21 as in other studies [188, 218]. From this reciprocal plot, it can be concluded that the separation in this covalently bonded zwitterionic capillary is mainly dependent on the limiting equivalent ionic conductance, and hence electrophoretic mobility of the solute ions.

**Figure 21.** Limiting equivalent ionic conductance versus migration time of six inorganic anions at three concentration levels with pH 7. The separation conditions are the same as in Figure 20. [Paper V]

In Paper VI, the same inorganic anions mixture as in Figure 20 was evaluated on a thermal grafted zwitterionic capillary, in order to compare the one in Paper V. It was found that all eight anions in the mixture were well separate (see Figure 1, Paper VI). Nevertheless, the migration times for all anions by this capillary are more or less three times longer than the results by photo grafted capillary. We assumed that it is due to the less suppression of EOF by this capillary. As it has been discussed in the synthesis section, the graft conditions may result this. Therefore, more experiments will soon be carried out to see what the key reason. The detection limit of Bromate ion was also tested, and it was about 10 µM.

### 7.2. Separation of Biological Molecules

Proteins play crucial roles in the biological process. Therefore, it is not only important on its original name, protein, which means “of first rank” in Greek [221]. On the other hand, the peptides are also important because there is a lot of evidence suggesting that small polypeptides (i.e., below 40 amino acids) or small organic molecules (below 2000 Da) can act as protein regulatory elements, mimicking the biological functions of the much larger protein hormones or growth factors [222, 223]. When we want to study the function of a specific protein or peptide, purification and
separation is of primary importance, because we need to have pure protein or peptide as tools to study them in complex biological matrices.

Proteins are traditionally analyzed by the slab-gel electrophoresis according to their physicochemical properties such as pI, hydrophobicity, and molecule weight. However, compared to a CE system, slab-gel electrophoresis is usually operated without an instrumental automation and the analysis time is longer due to the lower electric fields applied [35, 224, 225]. Even with the fully automated two-dimensional polyacrylamide-gel electrophoresis, it can also not meet the demand for the modern proteomics research owing to its limitation of dynamic range and sensitivity for a more complicated protein mixture [63]. Therefore, new separation techniques such as the CE and capillary LC are extremely interesting for the contemporary proteomic studies. Righetti reviewed several interesting examples on the analysis of peptide and proteins by CE from a vast number of studies in life science, biomedical and biotechnology in recent years [226]. He also described the importance of surface modification of fused silica capillary for these kinds of studies. The application of CE for the analysis of biotechnology-derived therapeutic proteins, e.g., human insulin and immuno-globulins, is reviewed by Patrick and Lagu [227]. There are also several other important review papers which give more information related to the separation of proteins and peptides by CE technique [34, 36].

![Figure 22](image)

**Figure 22.** Separation of five common peptides, Gly-His-Lys, bradykinin, angiotensin II, neurotensin, and Gly-Arg-Gly-Asp. Experimental conditions: detection, 214 nm; applied voltage, 20 kV; capillary, 50 µm i.d. by 40/47 cm; separation buffer, 40 mM ammonium acetate buffer, pH 5. [Paper V]

In Paper V, by using the photo grafted zwitterionic capillary, a rather mild separation conditions can be used to separate a mixture of five model peptides can be separated with high efficiency (309,000 N/m for Gly-Arg-Gly-Asp). A characteristic electropherogram is shown in Figure 22. When the same capillary was used to separate
the mixture of five model proteins by an optimized separation conditions, the separation efficiency can reach to 435,000 N/m for α-chymotrypsinogen A. The electropherogram is shown in Figure 23. According to the ζ-potential studies in Paper III and IV, the surface charges of polymeric or silica particles still show negative, which is attributed to the distal arrangement of the zwitterionic functional group. Consequently, the basic proteins owning large molecule size and positive charge will still have the wall adsorption problem. Magnesium ions were then chosen to be added in the separation buffer, so as to neutralize part of the negative surface charge and reduce the protein adsorption. This reason for this success is because the electrostatic interaction between the Mg²⁺ and the sulfonic groups in zwitterionic polymer layer, which in turn results a less negative ζ-potential corresponded to the low EOF. The correlation between the EOF and added salts in the aqueous solution was also studied and shown in Figure 4, Paper V.

Although the above peptide mixture was also tested on thermal grafted zwitterionic capillary in Paper VI, three peptides show significant peak tailing. All these five peptides have shorter migration times compared the ones for photo grafted capillary. This is another indication for the low EOF suppression because the µₑ has the same direction as µₑ₀, which makes µₑ faster.

Peptide mapping is one of the powerful tools for the protein identification and characterization in modern proteomics study. The analysis of proteins is done by analyzing a number of cleaved peptides instead of entire proteins, since the cleavage action of the proteins by trypsin, chymotrypsin and other protelytic enzymes is well known. The small peptides from the cleavage process can be easily separated by
chromatographic or electrophoretic methods and detected by the mass spectrometry, by which the fragments can be searched and identified from the database. The peptide mapping can also supply with fingerprint information on various proteins [228-230]. In **Paper VI**, a tryptically digested protein kit, which contains digests of lysozyme, cytochrome c, haemoglobin, myoglobin, carbonic anhydrase, conalbumin, ovalbumin, $\alpha$-amylase, bovine serum albumin, catalase, lactic dehydrogenase, and immuno-$\gamma$-globulin (IgG), was separated by the thermal grafted zwitterionic capillary. As we have discussed the less effective EOF suppression on this capillary, $\text{Mg}^{2+}$ ions needed to be added in separation buffer in order to get rid of the residual wall interference. Good separations can be accomplished, and all of them have their own characteristic profiles, which are shown in Figure 4 in **Paper VI**. Figure 24 demonstrates a typical separation of hemoglobin digests.

![Figure 24](image_url)

**Figure 24.** Electropherogram from the separation of tryptically digested Hemoglobin. Experimental conditions: detection, 214 nm; applied voltage, 20 kV; capillary, 50 $\mu$m i.d. by 50/57 cm; injection, electrokinetic injection for 10 s at 10 kV; separation buffer, 40 mM phosphate buffer, pH 2.5, plus 15 mM $\text{MgCl}_2$. [Paper VI]
8. Concluding Remarks

- Several sulfobetaine type zwitterionic separation materials were synthesized based on methacrylate polymers and silica particles according to different synthesis routes. Procedures employed over the duration of this work yielded materials that carry both positively charged ammonium groups and negatively charged sulfonic groups in various balance. The materials synthesized in the final works showed the separation characteristics typical of zwitterionic surfaces.

- The surfaces of zwitterionic material can be characterized by the techniques such as elemental analysis, $\zeta$-potential measurement and spectroscopy methods.

- Covalently bonded zwitterionic separation materials for IC provide possibilities to separate inorganic anions and cations, independently and simultaneously, using aqueous solution of perchloric acid and perchlorate salts as eluents. The materials are also capable of separating biomacromolecules such as proteins when optical conditions were found for the pH and the salt type and concentration in the gradient elution scheme.

- Two types of CE capillaries were produced by modifying their surfaces with zwitterionic polymer through photo-initiated or thermo-initiated graft polymerization. The resulting materials show high suppression of the electroosmotic flow, which is useful and necessary for the CE separations. Wall interactions for proteins are also minimized by the zwitterionic modification.

- By using zwitterionic capillaries, good separation of inorganic anions, peptides and proteins standard samples can be achieved, as well as the tryptically digested proteins.
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10. References


Kawai, T.; Saijo, K.; Lee, W. Protein binding to polymer brush, based on ion-exchange, hydrophobic, and affinity interactions, *J. Chromatogr. B* 2003, 790, 131-142.


