Maria E. Bohlin

Method development for affinity capillary electrophoresis of $\beta_2$-glycoprotein I and biological ligands
Method development for affinity capillary electrophoresis of $\beta_2$-glycoprotein I and biological ligands
Maria E. Bohlin. *Method development for affinity capillary electrophoresis of β2-glycoprotein I and biological ligands*

Dissertation

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“No doubt the final success is, in most cases, a consequence of a series of consecutive advances and improvements and, therefore, not concentrated in one or a few days/…/”

Stellan Hjertén
Abstract

The final goal of this study is to establish a microscale analysis method that allows solution phase characterization of interactions between \( \beta_2 \)-glycoprotein I (\( \beta_2 \)gpI) and some of its ligands. Human \( \beta_2 \)gpI is a phospholipid- and heparin-binding plasma glycoprotein. The physiological role of the protein in normal blood coagulation is not entirely known, nor is its role in autoimmune diseases characterized by blood clotting disturbances (thrombosis). Quantitative binding data of \( \beta_2 \)gpI interactions with some of its ligands may help elucidating the mechanisms behind these diseases and in the development of new approaches for diagnostics, prevention, and therapy.

In this thesis, capillary electrophoresis (CE) was used as methodological platform for the interaction studies. The analysis of peptides and proteins by CE is desirable due to low sample consumption, possibilities for non-denaturing and highly effective separations. The first objective of this thesis was to find an approach to prevent charge dependent adsorption of \( \beta_2 \)gpI to the inner surface of the capillaries. Analyte adsorption at the negatively charged inner surface of fused silica capillaries is detrimental to interaction analyses. This phenomenon is especially pronounced in the analysis of basic proteins and proteins containing exposed positively charged domains, such as \( \beta_2 \)gpI. A new strategy to suppress these solute-wall interactions was devised, investigated and optimized. This strategy exploits the pH hysteresis behavior of fused silica surfaces, by simply performing an acidic pretreatment of the capillary. The results in this thesis show that the acidic pretreatment efficiently prevents protein adsorption.
List of papers

I  Capillary electrophoresis-based analysis of phospholipid and glycosaminoglycan binding by human β₂-glycoprotein I
   *Maria E. Bohlin, Ewa Kogutowska, Lars G. Blomberg, Niels H.H. Heegaard.*

II  Utilizing the pH hysteresis effect for versatile and simple electrophoretic analysis of proteins in bare fused-silica capillaries
   *Maria E. Bohlin, Lars G. Blomberg, Niels H.H. Heegaard.*

III  Effects of ionic strength, temperature and conformation on affinity interactions of β₂-glycoprotein I monitored by capillary electrophoresis
   *Maria E. Bohlin, Lars G. Blomberg, Niels H. H. Heegaard.*

IV  Estimation of the amount of β₂-glycoprotein I adsorbed at the inner surface of fused silica capillaries after acidic, neutral and alkaline pretreatments
   *Maria E. Bohlin, Ida Johannesson, Gunilla Carlsson, Niels H. H. Heegaard, Lars G. Blomberg*
   Manuscript to be submitted.
### Abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Definition</th>
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<tbody>
<tr>
<td>α-C Trg</td>
<td>α-chymotrypsinogen</td>
</tr>
<tr>
<td>AA</td>
<td>acrylamide</td>
</tr>
<tr>
<td>ACE</td>
<td>affinity capillary electrophoresis</td>
</tr>
<tr>
<td>APS</td>
<td>antiphospholipid syndrome</td>
</tr>
<tr>
<td>β2gpI</td>
<td>β2-glycoprotein I</td>
</tr>
<tr>
<td>BGE</td>
<td>background electrolyte</td>
</tr>
<tr>
<td>CAC</td>
<td>critical aggregation concentration</td>
</tr>
<tr>
<td>CE</td>
<td>capillary electrophoresis</td>
</tr>
<tr>
<td>CE-FA</td>
<td>frontal analysis capillary electrophoresis</td>
</tr>
<tr>
<td>CMC</td>
<td>critical micellar concentration</td>
</tr>
<tr>
<td>Cyt c</td>
<td>cytochrome c</td>
</tr>
<tr>
<td>DLS</td>
<td>dynamic light scattering</td>
</tr>
<tr>
<td>DMA</td>
<td>dimethylacrylamide</td>
</tr>
<tr>
<td>EKC</td>
<td>electrokinetic chromatography</td>
</tr>
<tr>
<td>EOF</td>
<td>electroosmotic flow</td>
</tr>
<tr>
<td>FASS</td>
<td>field amplified sample stacking</td>
</tr>
<tr>
<td>FITC-anti-β2gpI mAb</td>
<td>fluorescein isothiocyanate-labeled human antibody against β2gpI</td>
</tr>
<tr>
<td>LUV</td>
<td>large unilamellar vesicles</td>
</tr>
<tr>
<td>Lys</td>
<td>lysozyme</td>
</tr>
<tr>
<td>MLV</td>
<td>multilamellar vesicles</td>
</tr>
<tr>
<td>msACE</td>
<td>migration shift affinity capillary electrophoresis</td>
</tr>
<tr>
<td>PBS</td>
<td>phosphate buffered saline</td>
</tr>
<tr>
<td>PC</td>
<td>phosphatidylcholine</td>
</tr>
<tr>
<td>PF-CE</td>
<td>partial filling capillary electrophoresis</td>
</tr>
<tr>
<td>pI</td>
<td>isoelectric point</td>
</tr>
<tr>
<td>PNGase F</td>
<td>peptide-N-glycosidase F</td>
</tr>
<tr>
<td>POPC</td>
<td>1-palmitoyl-2-oleoyl-sn-glycero-3-phosphatidylcholine</td>
</tr>
<tr>
<td>PS</td>
<td>phosphatidylserine</td>
</tr>
<tr>
<td>RnA</td>
<td>ribonuclease A</td>
</tr>
<tr>
<td>SDS</td>
<td>sodium dodecyl sulphate</td>
</tr>
<tr>
<td>SDS-PAGE</td>
<td>sodium dodecyl sulphate polyacrylamide gel electrophoresis</td>
</tr>
<tr>
<td>SPR</td>
<td>surface plasmon resonance</td>
</tr>
<tr>
<td>SUV</td>
<td>small unilamellar vesicles</td>
</tr>
<tr>
<td>tITP</td>
<td>transient isotachophoretic sample stacking</td>
</tr>
<tr>
<td>Trg</td>
<td>trypsinogen</td>
</tr>
</tbody>
</table>
wt  wild type

Annotations

\( \zeta \)  zeta potential
\( \varepsilon \)  dielectric constant
\( \eta \)  viscosity
\( \Delta \mu \)  change in electrophoretic mobility
\( \mu_{\text{EOF}} \)  electroosmotic mobility
\( \mu_{\text{ep}} \)  electrophoretic mobility
\( \Delta \mu_{\text{max}} \)  change in electrophoretic mobility at saturating ligand concentration
\( \mu_{\text{net}} \)  net mobility
\( \nu_{\text{EOF}} \)  electroosmotic velocity
\( \nu_{\text{ep}} \)  electrophoretic velocity
\( E \)  electric field strength
\( I \)  current
\( K_D \)  equilibrium dissociation constant
\( k_{\text{off}} \)  rate constant for dissociation
\( k_{\text{on}} \)  rate constant for association
\( L \)  ligand
\( l_d \)  length to detector
\( l_t \)  total length
\( P \)  power
\( q \)  charge
\( r \)  radius
\( R \)  receptor
\( RL \)  receptor-ligand complex
\( t \)  migration time
\( t_A \)  migration time of analyte
\( t_M \)  migration time of marker
\( t_{\text{meas}} \)  experimentally measured mean temperature
\( t_{\text{set}} \)  set temperature of capillary cassette
\( U \)  voltage
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1. Introduction

To find efficient drugs and treatments against human diseases it is important to understand the underlying biological mechanisms behind the diseases. To do this, we need efficient tools that allow systematic studies of how different species interact with each other. There are a number of different techniques available today, all possessing different benefits and drawbacks. The ongoing development of analytical tools strives to develop new and to improve the performance of already existing techniques.

Patients suffering from antiphospholipid syndrome (APS) run an increased risk of thrombotic events, such as thrombosis, recurrent fetal loss and preeclampsia [1-4]. The underlying biological mechanism behind this disease is not known. However, it has been shown that autoantibodies against the human plasma protein β2-glycoprotein I (β2gpI) are associated with this increased risk [1-2]. β2gpI is known to be involved in the blood coagulation cascade, where both procoagulant and anticoagulant functions have been proposed [5-6]. It also binds to a number of different species: DNA [7], mitochondria [8], lipoproteins [9], platelets [10], heparin [11] and anionic phospholipids [12-13]. Even though all these different actions of β2gpI have been shown, the precise function of β2gpI in health and disease is still unknown. Therefore, it is important to understand quantitative and mechanistic aspects of this binding under conditions that mimic in vivo conditions as much as possible.

For physiologically relevant studies of β2gpI it is desirable to use a solution based technique, because it is known that binding in one end of the β2gpI-molecule affects the binding in the other end [1, 11]. Many of the existing techniques for interaction studies require immobilization to a surface. Capillary electrophoresis (CE) is an analytical technique that facilitates simultaneous analysis of low- and high molecular weight compounds in solution under non-denaturing conditions. These properties are important for molecular interaction studies where active species are required. CE also offers high efficiency, high resolution and it is easy to automate with on-line detection. The sample consumption is very low and the speed of analysis is high. Binding that leads to changes in analyte peak areas and peak appearance times gives both qualitative and quantitative information on molecular interactions [14-16]. All these properties make CE a promising technique for studying molecular interactions [15]. One problem though, especially at the neutral pH required for physiologically relevant functional studies, is that protein analytes in many cases exhibit recovery problems due to interactions with the inner surface of the fused-silica capillaries.
Suppression of such interactions is therefore a general requirement when performing protein functional studies with CE.
2. Aim of study

The final aim of this study is to establish a microscale, solution phase quantitative analysis method that allows characterization of interactions between β₂gpI and some of its ligands. This will help elucidate the mechanisms involved in the increased thrombosis risk associated with the presence of circulating autoantibodies against β₂gpI.

More specifically, the purposes of the subprojects have been the following:

- Develop a method for suppression of solute-wall interactions.
- Investigate and optimize a method for suppression of solute-wall interactions.
- Establish experimental parameters for studying the β₂gpI-heparin interaction.
- Study the characteristics of the β₂gpI-heparin interaction, with respect to the effect of ionic strength, temperature and protein conformation.
- Establish experimental parameters for studying the β₂gpI-anionic phospholipid interaction.
- Study the role of glycosylation of β₂gpI on its ligand binding affinities.
3. Biomolecular interactions

Biomolecular interactions are the essence in communication between cells, transport, metabolism, regulation, DNA replication, protein synthesis and immunoreactions, just to name a few. Associations and dissociations between different species are necessary for all living organisms to function [17]. In each biological cell, numerous non-covalent interactions occur at any given time. Cellular reactions often occur in cascades and via different types of interaction between the reacting species. Defects in these reactions may have severe biological consequences. Because molecular interactions play such a central role in cellular processes, detailed knowledge of such interactions are required in order to develop new and better drugs and treatments against different diseases.

The aim of interaction studies is to qualitatively determine whether or not interaction takes place between species being studied and to quantitatively gain information about stoichiometry and strength of the interactions. Combined with knowledge of concentrations of the proteins in vivo this sheds lights on the physiological relevance of the interactions studied in vitro. If a ligand L binds to a receptor R, a complex RL will be formed. The term ligand means any molecule that interacts with a given molecule [18]. The equilibrium for a reversible 1:1 molecular binding interaction can be described by reaction I:

\[ R + L \leftrightarrow RL \]  

A measure of the affinity between the two species can be obtained by estimating the equilibrium dissociation constant, \( K_D \) [19-20]. The \( K_D \) is an apparent equilibrium constant in terms of concentrations, rather than the true equilibrium constant that requires activities [21]. \( K_D \) is sometimes referred to as binding constant [15, 21-22]. \( K_D \) depends on the kinetics of dissociation/association between R and L as described in equation 1:

\[ K_D = \frac{k_{off}}{k_{on}} \]  

The rate constant for the forward reaction I is designated \( k_{on} \), and the rate constant for the backward reaction is designated \( k_{off} \). High affinity interactions are characterized by slowly dissociating complexes, i.e. low values of \( k_{off} \) and hence \( K_D \), while low affinity interactions are characterized by relatively fast dissociations, i.e. high values of \( k_{off} \) and hence \( K_D \) [22-23]. Equations for the determination of equilibrium dissociation constants have been described.
and used by several researchers [15, 21, 24-28]. The general rectangular hyperbolic binding isotherm for the simple 1:1 binding [29] as in reaction 1 is:

\[ y = \frac{dx}{f + ex} \]  

(2)

where \( y \) is the dependent variable, \( x \) is the independent variable (free ligand concentration), and \( d, e \) and \( f \) are constants or parameters [21]. Any change in an appropriate analytical parameter (\( y \) in equation 2) that results from binding of a receptor to a ligand may be used to estimate \( K_D \) [21, 30]. This may be a change in size, charge, shape, conformation or structure, or a change in a physicochemical property such as optical properties or heat uptake/release [15, 17-18, 21, 26, 31-35].
4. \( \beta_2 \)-glycoprotein I

\( \beta_2 \)-glycoprotein I (\( \beta_2 \)-gpI) is a human plasma protein which was first isolated in 1961 [36]. It consists of a single polypeptide chain folded in five domains, mainly consisting of \( \beta \)-sheets, into a fish-hook like shape [2, 37-38]. Its structure was determined in 1999 by both Bouma et al. [39] and Schwarzenbacher et al. [2]. The structure of \( \beta_2 \)-gpI is shown in figure 1 and some data about \( \beta_2 \)-gpI are given in table I.

![Ribbon structure of human \( \beta_2 \)-gpI based on crystallography](image)

Isoelectric focusing has revealed microheterogeneity in the carbohydrate content of \( \beta_2 \)-gpI. Several bands with isoelectric points (pI) of 5.1-6.2 were obtained of \( \beta_2 \)-gpI (wild type, wt-\( \beta_2 \)-gpI) [40-43], whereas deglycosylation of \( \beta_2 \)-gpI with neuraminidase (asialo-\( \beta_2 \)-gpI) decreased the number of bands to two with pI’s of 8.0 and 8.2 [41]. The heterogeneity is not entirely due to variations in carbohydrate content but also to genetic variations resulting in polymorphism in the polypeptide chain [40, 44]. Given the pI values, one might assume that wt-\( \beta_2 \)-gpI is an acidic protein. Only the asialo-forms of \( \beta_2 \)-gpI are basic in absolute terms [41]. However, the protein moves in the \( \beta_2 \)-zone upon agarose gel electrophoresis – hence
compared with many other plasma proteins it is relatively basic. Also, many binding studies suggest that wt-\(\beta_2\)gpI behaves as a basic protein [7, 11, 45-46].

**Table I. Some data on \(\beta_2\)gpI.**

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Data</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>No of amino acids</td>
<td>326</td>
<td>[2, 37-38]</td>
</tr>
<tr>
<td>Molecular weight</td>
<td>32.70 kDa</td>
<td>[1-2, 8-9, 37-38]</td>
</tr>
<tr>
<td>(pI)</td>
<td>5.1-6.2 (wt-(\beta_2)gpI)</td>
<td>[40-43]</td>
</tr>
<tr>
<td></td>
<td>8.0-8.2 (asialo-(\beta_2)gpI)</td>
<td></td>
</tr>
<tr>
<td>Carbohydrate content</td>
<td>15-20 (w/w) %</td>
<td>[37-39, 47]</td>
</tr>
<tr>
<td>Plasma concentration</td>
<td>0.15-0.20 mg/mL</td>
<td>[1, 9]</td>
</tr>
</tbody>
</table>

The interactions of \(\beta_2\)gpI with various ligands have been studied for more than three decades. A number of different species that bind to \(\beta_2\)gpI are known: DNA [7], mitochondria [8], lipoproteins [9], platelets [10], heparin [11] and anionic phospholipids [12-13]. \(\beta_2\)gpI has been suggested to play a primary role in mediating the clearance of liposomes and foreign particles [48] and is known to be involved in the blood coagulation cascade, where both procoagulant and anticoagulant functions have been proposed [5-6].

**4.1 Antiphospholipid syndrome**

The protein is involved in the serious autoimmune condition antiphospholipid syndrome (APS). Circulating autoantibodies against \(\beta_2\)gpI are associated with an increased risk of thrombotic events, such as thrombosis, recurrent fetal loss and preeclampsia [1-4]. Autoantibodies leading to the prothrombotic state in APS have been shown only to recognize \(\beta_2\)gpI bound to phospholipid membranes [1-2, 11-13, 37, 39, 49-52]. The autoantibodies bind a cryptic epitope on domain I of \(\beta_2\)gpI, which only is accessible for binding after a conformational change of \(\beta_2\)gpI [1]. This conformational change is induced by docking of \(\beta_2\)gpI to the phospholipid membrane. The docking mechanism is suggested to involve two steps [2, 37, 39, 49-50]. The first step is an electrostatic interaction between positive charges on domain V of \(\beta_2\)gpI (the positive region marked in figure 1) and negatively charged head groups of anionic phospholipids. This is followed by the second step, an insertion of the surface exposed hydrophobic loop of \(\beta_2\)gpI (see figure 1) into the hydrophobic part of the phospholipid membrane. When the \(\beta_2\)gpI molecule is bound to the phospholipid membrane and has undergone the conformational change, domain I and II are positioned far away into the solution and the epitope is exposed. This enables interactions of domain I and II with antibodies [37]. Domains III and IV of \(\beta_2\)gpI may be regarded as linker or “bridging” domains because these are shielded from protein-protein interaction by glycans [39]. The glycans are of minor importance for the phospholipid binding [53], but difference in glycosylation may have recognition relevance for antibodies [1]. In spite of all
existing knowledge about $\beta_{\text{gpI}}$ and its interactions, the precise function of the protein in health and disease is still unknown. Therefore, it is important to understand quantitative and mechanistic aspects of this binding under conditions that mimic in vivo conditions as much as possible.
5. Heparin

Heparin is a glucosaminoglycan, a family of linear, polyanionic polysaccharides [54]. Figure 2 shows a representative chemical structure of heparin. There are a number of structural variants of heparin, making it microheterogenous. Heparin is a repeating unit of linearly linked glucosamine and uronic acid residues. As a result of the high content of sulpho and carboxyl groups, heparin is highly negatively charged. Heparin is also polydisperse, the length of the glucosaminoglycan chains of commercial heparins can vary between 5 000 to 40 000, but typically have an average weight of 13 000 to 15 000 g/mol.

![Typical chemical structure of heparin.](image)

The microheterogeneity and the high content of sulpho groups make heparin able to bind a wide range of proteins and to regulate a number of biological activities [54]. Pharmaceutically, heparin is most commonly used as anticoagulant and antithrombotic agent. Heparin is a competitive inhibitor of the binding of β<sup>2</sup>gpl to anionic phospholipids, and binds via the positively charged region located in domain V [45].
6. Phospholipid membranes

The basic architectural backbone of biological membranes is a phospholipid bilayer [55]. Different proteins and molecules are attached to and incorporated into the bilayer and determine the unique function of each membrane. Phospholipids are amphiphilic molecules composed of a polar lipid head group and a double hydrophobic tail consisting of acyl chains, see figure 3 A. The structure of the acyl chains as well as the head group varies between different phospholipids, giving them different properties and functions.

When phospholipids are dispersed in an aqueous phase, the hydrophobic tails spontaneously align against each other as shown in figure 3 B and the polar head groups are exposed to the aqueous solution. A bilayer with a hydrophobic interior and polar exterior is created. Because phospholipids have two acyl chains, the shape is relatively cylindrical [56]. This can be compared to a surfactant with one acyl chain and a conical shape, figure 3 D. When dispersed in an aqueous phase in concentrations above the critical micellar concentration (CMC) or critical aggregation concentration (CAC), the surfactants form micelles (figure 3 E) and phospholipids form spherical vesicles (liposomes) encapsulating aqueous phase (figure 3 C). Formation of vesicles such as micelles or liposomes is energetically favorable [57].

![Figure 3. Schematic structure of (A) a phospholipid; (B) a part of a phospholipid bilayer; (C) a cross section of a phospholipid liposome; (D) a surfactant; (E) a micelle.](image-url)
Liposomes can carry many types of solutes and are therefore used commercially for controlled delivery of e.g. drugs, enzymes, hormones and DNA [58]. They are also used as sensitive reagents for analytical detection [57]. For this thesis, the most interesting aspect is the structural resemblance to natural membranes. Liposomes are often used as biological membrane models [57, 59].

In electrophoretic analyses, liposomes are implemented as carriers dispersed in the background electrolyte (BGE) or for the coating of fused silica capillaries [60-62]. The vesicles are composed of multilamellar lipid bilayers, so called multilamellar vesicles (MLV). MLVs give noisy background and low sensitivity in CE and are therefore not suitable as carriers in the BGE [63]. Instead, the MLVs can be downsized to small unilamellar vesicles (SUV) by sonication or to large unilamellar vesicles (LUV) by extrusion through a polycarbonate membrane [64]. SUVs have a large curvature which makes them unstable and they will spontaneously form larger vesicles upon storage [63-64]. Hence, LUVs are considered the most suitable carrier in CE [63] and were therefore the vesicles used in this thesis. The phospholipids used in our liposomes were zwitterionic phosphatidylcholine (PC) and anionic phosphatidylserine (PS) and the structures of these phospholipids are shown in figure 4.

(A) Phosphatidylcholine, PC

(B) Phosphatidylserine, PS

Figure 4. Structural formula of (A) phosphatidylcholine and (B) phosphatidylserine at neutral pH (R1 and R2 corresponds to acyl chains).
7. Methods to study biomolecular interactions

There are many techniques available that can be used for studying biomolecular interactions. Table II lists some common techniques for this purpose, as well as their benefits and drawbacks. The techniques can be divided into two categories, separative and non-separative methods [65]. Separative methods separate bound and free species from each other and determine the concentrations of these. Non-separative methods detect a change in a physicochemical property of the receptor or ligand.

Using a separative method, $K_D$ can be determined by measuring the equilibrium concentrations of all interacting species and then calculate $K_D$ according to equation 3:

$$K_D = \frac{[R][L]}{[RL]}$$

(3)

The concentrations of the species are measured after first establishing equilibrium by incubating the receptor with various concentrations of ligand, and then separate the bound from the free species. Quantification of the species can be performed online and offline. Common methods using this setup are filtration, electrospray mass spectrometry and certain modes of affinity chromatography and affinity CE (ACE). Some methods, such as equilibrium dialysis and certain modes of affinity chromatography and ACE, separate bound and free species during the course of interaction. $K_D$ can also be determined, as mentioned above, by detecting the change in a physicochemical property using a non-separative method. Examples of this are fluorescence, calorimetry, surface plasmon resonance (SPR) and circular dichroism. The degree of the change of the physicochemical property is a measure of the affinity between the receptor and the ligand. Depending on which of the non-separative methods that is used, different equations are used to calculate the $K_D$ [21].

The compatibility of a method with a given interaction is determined by the off-rate of the ligand in the interaction [66]. Filtration, gel filtration and ligand adsorption assays all reduce the concentration of free ligand in the vicinity of the receptor-ligand binding site during the separation process, and will therefore initiate ligand dissociation. Whether or not the amount of dissociation that ensues is acceptable depends on the separation time and the dissociation rate constant. Most techniques are compatible for studying either high and intermediate affinity interactions or low and intermediate affinity interactions (see table II). Equilibrium dialysis and ACE facilitate high, intermediate and low affinity interactions to be studied.
A widely used technique for high and intermediate affinity interaction studies is SPR, where one of the species is immobilized on a solid support and the other species is added in a flowing solution to initiate interaction. In SPR, very low concentrations can be detected. The drawback with this technique is that immobilization of the receptor or ligand is required, and this may change the binding properties [32]. Calorimetry and some spectroscopy techniques are rapid solution techniques also suitable for high and intermediate affinity interactions. These do not require complicated and expensive equipment, but they require relatively high sample amounts [15, 65].

For low affinity interaction studies, ultrafiltration, equilibrium dialysis and ACE are suitable techniques. Ultrafiltration and equilibrium dialysis are simple and relatively cheap techniques, but they often require long equilibration times and relatively large sample amounts [18, 65].

Using ACE for studying molecular interactions is beneficial in many ways. First, it is one of the few techniques that do not require highly purified samples. Second, there is no need for immobilization or labeling of any of the interacting species, as the interaction takes place in solution. Third, a large benefit is the high resolution that facilitates the detection of very small changes due to complexation between the interacting species. Fourth, the sample

<table>
<thead>
<tr>
<th>Technique</th>
<th>High affinity</th>
<th>Intermediate affinity</th>
<th>Low affinity</th>
<th>Low sample amount (&lt;10 µg)</th>
<th>Online detection based</th>
<th>Solution analysis</th>
<th>Fast analysis (min-h)</th>
<th>Label free</th>
<th>Insensitive to contaminants</th>
</tr>
</thead>
<tbody>
<tr>
<td>Surface plasmon resonance</td>
<td>✔️</td>
<td>✔️</td>
<td>✔️</td>
<td>✔️</td>
<td>✔️</td>
<td>✔️</td>
<td>✔️</td>
<td>✔️</td>
<td>✗</td>
</tr>
<tr>
<td>Calorimetry</td>
<td>✔️</td>
<td>✔️</td>
<td>✗</td>
<td>✔️</td>
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Red cross: not applicable  
Green tick: applicable  
Yellow tick: possible in modified version of the application
consumption is very low, only a few nanoliters are injected into the instrument and the same sample preparation (which can be as low as 5 µL) can be used for multiple injections. Fifth, CE is in principle compatible with both low- and high molecular weight compounds. So, CE should be considered for affinity interaction studies when highly purified sample is not available and/or there are only scarce sample amounts available [15].
8. Capillary electrophoresis

Electrophoresis is a separation technique with Swedish roots. In the 1930’s, the Swedish scientist Arne Tiselius constructed the moving-boundary method to study the electrophoresis of proteins [16, 67]. The moving-boundary electrophoresis took place in a U-tube filled with liquid (Tiselius tube). The principles of moving-boundary electrophoresis are still employed today, but take place in different types of gels, to separate proteins, DNA and RNA [16]. During the 1960’s Tiselius student Stellan Hjertén developed the Tiselius tube concept and performed zone electrophoresis in a rotating tube with the internal diameter of 3 mm [68]. During the 1970’s, the Finnish scientist Rauno Virtanen executed his electrophoresis in narrow-bore tubes with internal diameters reaching down to 0.2 mm [69]. In the early 1980’s, Jorgenson and Lukacs improved the technique by performing the electrophoretic separations in fused silica capillaries with an internal diameter of 75 µm [70]. Fused silica capillaries are further described in section 8.1.

Electrophoresis is the movement of ions in an electric field. Separation of the ions is based on the ability of charged molecules to migrate at different velocities in an electric field. When this takes place in a capillary it is called capillary electrophoresis (CE). A scheme of a CE instrument is shown in figure 5.

![Figure 5. Scheme of a capillary electrophoresis instrument.](image)

The basic parts of a CE instrument are the separation capillary, buffer reservoirs, a high voltage supply and a detector. The capillary is typically made of fused-silica with an internal diameter of 20-100 µm and a length of 40-100 cm. This is filled with a BGE connected to a high voltage supply. The BGE makes it possible to pass current through the capillary and the electrolyte is buffered to maintain constant pH throughout the analysis. When an electric field is applied, ions migrate towards the electrode of opposite charge unless the electroosmotic flow (EOF) pulls the ions in the opposite direction (cf. section 8.1). The ions...
are commonly detected by UV absorption or laser-induced fluorescence online. The detector is normally placed near the cathodic end of the capillary. The use of more information-rich detectors such as mass spectrometry is of great value and various schemes for this have been developed [71].

8.1 The electrical double layer and electroosmotic flow

The inner surface of the silica capillary wall contains ionizable silanol groups, which becomes deprotonated at pH above approximately 2 [72]. A simplification of this process is shown in reaction II:

\[
\text{SiOH} \rightleftharpoons \text{SiO}^- + \text{H}^+
\]

II

This creates negative charges at the capillary wall which attract positively charged BGE ions, see figure 6. An electrical double layer is created, where a rigid layer (also called Stern layer) of tightly adsorbed positively charged ions, and a mobile diffuse layer with an excess of positive charges are described by the Stern-model [73]. When an electric field is applied, the diffuse part of the double layer migrates towards the cathode. Because the diffuse layer contains an excess of positive BGE ions, a net flow is formed which drags the BGE solution towards the cathode. This flow, called the electroosmotic flow (EOF), has a plug-like profile and this leads to very high separation efficiencies, i.e. narrow peaks. Due to the EOF, both positively and negatively charged analytes can be analyzed in a single run.

The velocity of the EOF, \( \nu_{\text{EOF}} \), depends on the electric field strength, \( E \), and the mobility of the EOF, \( \mu_{\text{EOF}} \) [74]:

\[
\nu_{\text{EOF}} = \mu_{\text{EOF}} E
\]

(4)

The \( \mu_{\text{EOF}} \) in turn is dependent on the zeta potential, \( \zeta \), the dielectric constant of the BGE, \( \varepsilon \), and inversely dependent on the viscosity of the buffer solution, \( \eta \):

\[
\mu_{\text{EOF}} = \frac{\zeta \varepsilon}{\eta}
\]

(5)
The zeta potential is the potential difference between the outer boundary of the Stern layer and the free solution at an infinite distance [75]. As the pH is increased, more silanol groups become deprotonated and hence the surface becomes more negatively charged, attracting even more positively charged buffer ions and a higher zeta potential is obtained. Thus, a higher EOF is obtained.

8.2 Electrophoretic mobility

The velocity of a migrating molecule, \( v_{\text{ep}} \), depends on the electrophoretic mobility, \( \mu_{\text{ep}} \), of the molecule and of the applied electric field strength [74, 76]:

\[
v_{\text{ep}} = \mu_{\text{ep}} E
\]  

(6)

The \( \mu_{\text{ep}} \) in turn is dependent on the charge of the molecule, \( q \), and inversely dependent on \( \eta \) and the hydrodynamic radius, \( r \), of the molecule:

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

(7)

Under constant buffer conditions and electric field strength, the electrophoretic mobility is approximately dependent on the charge to mass ratio of the analyte. In a CE separation, the \( \mu_{\text{EOF}} \) and the \( \mu_{\text{ep}} \) both act at the same time, which gives the molecule a net mobility, \( \mu_{\text{net}} \):
Under standard conditions with the cathode at the detector end, positively charged molecules will have two positive contributions to the $\mu_{\text{net}}$ and will therefore have the shortest migration times, while negatively charged molecules have longer migration times. However, negatively charged species only pass the detector if $\mu_{\text{EOF}}$ exceeds $\mu_{\text{ep}}$. This is usually the case under neutral electrophoresis buffer conditions.

Charged molecules can be separated using the separation technique described above. Neutral molecules have no electrophoretic mobility and will therefore move only with the EOF. However, by addition of a surfactant in a concentration above its CMC to the electrophoresis buffer and at a temperature above the Krafft temperature, a pseudostationary phase is formed [77]. This micellar phase provides a mechanism for retention of neutral analytes. Neutral molecules can partition between the micelles and the BGE and thereby achieve an apparent mobility which will be determined by the electrophoretic mobility of the micelles, $\mu_{\text{EOF}}$ and the degree of partitioning. The approach, named micellar electrokinetic chromatography, was introduced by Terabe et al. [78] in 1984. Additives such as organic solvents, urea, salts, cyclodextrins and other chiral selectors can be added both alone and in different combinations. With this approach very high selectivities can be achieved.

8.3 Joule heating

When current passes through a conductive medium, heat is generated [79]. This is called Joule heating, but is also named ohmic or resistive heating. In CE, Joule heating causes an increase in the overall temperature of the BGE as well as a radial non-uniformity of the temperature [79]. A radial temperature profile over the capillary induces variations in electrolyte conductivity and viscosity. The effects of these variations include peak broadening [80], changes in migration times [81-82] and boiling or superheating of the sample zone [83]. To add to the problem, Gobie and Ivory [83] described a positive feedback between conductivity and temperature known as the “autothermal effect”: An increase in temperature gives an increase in electrolyte conductivity, which in turn gives a further increase in temperature.

The temperature increase of the BGE depends on the power, $P$:

$$P = IU$$  \hspace{1cm} (9)
where $I$ is the current in the capillary and $U$ is the applied voltage. The current in turn depends on the conductivity of the BGE. A certain amount of heat can be dissipated in CE instruments, by thermostating of the capillary. Liquid cooling of the capillary is more efficient than air cooling. Also, narrow-bore capillaries gives much more efficient heat dissipation because of the high surface-to-volume ratio [84]. The amount of heat that can be dissipated limits the voltage that can be applied.

8.4 Sample adsorption onto fused silica surfaces

Ideally, the only contribution to band broadening in CE would be diffusion. Analytes of high molecular weight, such as proteins, have a low diffusion coefficient and should therefore be separated as narrow zones. However, in practice, other factors often contribute to band broadening in CE. The inner surface of fused silica capillaries is highly negatively charged at the neutral pH that is required for physiologically relevant functional studies. High molecular weight species, such as proteins, have a tendency to adsorb onto this negatively charged surface. Basic proteins and proteins that contain positively charged patches have a large preference to adsorb onto the negatively charged inner silica surface. This leads to absent, irreproducible, or tailing peaks that preclude reliable estimates of e.g. dissociation constants. Solute adsorption also has a negative effect on separation efficiency. Because of the increased surface-to-volume ratio narrow-bore capillaries makes this effect more pronounced. The tendency to adsorb onto silica surfaces depends on the primary structure, conformation, structural stability, charge and size of the protein as well as the hydrophilic or hydrophobic properties of the surface [85-86].

Numerous charge suppression strategies have been proposed to enable general protein analysis in CE (see e.g. refs [87-98]). There are three main approaches that are used to minimize sample adsorption to the silica surface [98]. The first strategy is quenching of wall interactions via BGE pH and ionic strength. At low pH the silanols on the inside wall of the silica capillary becomes protonated and thereby uncharged. Hence, charge-dependent sample adsorption is prevented [99-100]. Performing separation at high pH (approximately 10) also prevents charge-dependent sample adsorption [101]. At this pH the proteins will most likely be negatively charged, because the pH exceeds the pI’s of most proteins. The silanols on the silica surface will also be negatively charged (cf. section 8.1), thus, electrostatic attractions are ruled out. The second strategy is the addition of ions that compete with the silanols for receptor binding sites on the proteins [98]. These competing ions can be alkali metal salts [102], surfactants [101] or a ligand that confers a suitable charge to the protein [28]. However, additives may influence analytes, e.g. denature proteins and can therefore be
difficult to use for interaction studies where an active protein is desired during the separation. The third strategy is coating of capillaries [88-90, 94, 103-110]. The coating can be permanent (or static), e.g. by synthesizing a polymer film on the capillary wall, or dynamic, i.e., an additive in the electrophoresis buffer covers the wall reversibly and/or shields the proteins in solution from the wall by additive-protein interactions [108, 111]. A pioneering work within this field was made by Hjertén, who stated that in ideal electrophoresis in free solution neither electroosmosis nor adsorption of solutes onto the capillary wall should occur [68, 106]. He described a procedure to permanently coat capillaries and could thereby diminish both of these phenomena. Today numerous types of coatings are commercially available, but no universal remedy has been found [98]. The preparation of permanently coated capillaries is somewhat cumbersome and prone to variability, but for a given capillary, coating usually results in stable performance for quite a long time [107, 112-113]. Dynamic capillary modification is simpler but may complicate the detection, for example when mass spectrometry is used.

8.5 Sample stacking

One disadvantage with CE for biomolecular analysis may be that a relatively high concentration of the sample is required when using UV detection. The cuvette length in CE is very short, typically 20-100 µm (the inner diameter of the capillary). To increase length and, hence, decrease the detection limit, different types of detection cells have been developed, e.g. extended light path using a bubble cell or Z-cell. Also, different pre-concentration techniques have been developed, which are discussed in section 8.5. One disadvantage with CE for studying biomolecules is the poor sensitivity due to the short cuvette length for UV detection. Several techniques have been developed that will concentrate, or stack, the sample after injection; see e.g. refs [114-116]. Almost all stacking techniques are based on changing the $\nu_{ep}$ of the sample components during their migration process [114]. The most common ones are based on discontinuous electric field throughout the separation capillary. Other techniques involve change of pH, formation of complexes and interaction with micelles. These are not compatible with interaction studies and are therefore not discussed here.

In field amplified sample stacking (FASS) the sample is dissolved in 1/10 BGE. This will give the injection zone a lower ionic strength and thereby lower electric conductivity and higher electric resistance [76]. The ions in the injection zone will experience a higher field strength than the ions in the rest of the capillary. As seen in equation 6, higher field strength will increase the $\nu_{ep}$. Hence, the sample ions will have a higher $\nu_{ep}$ in the injection zone.
When they reach the BGE zone the sample ions will slow down, because the electric field strength over the BGE zone is lower (due to the higher ionic strength and electric conductivity of the BGE). The sample ions will therefore be stacked at the boundary between the two zones and the concentration will be increased [115]. If a very large volume of sample dissolved in water or a low concentration of BGE is injected, high sample concentration enhancement can be achieved [115, 117]. This stacking technique is called large volume sample stacking and is based on the same discontinuity principle as FASS.

Low conductivity sample matrix is not always valid for biological samples [116, 118]. These samples can contain huge amounts of other charged components and thus have a high ionic strength. In these cases, transient isotachophoretic sample stacking (tITP) is a more suitable stacking method. Here, the sample contains a macroion that acts as either leading (higher electrophoretic velocity than the analyte) or terminating (lower electrophoretic velocity than the analyte) stacker. If the macroion acts as a leading stacker, the BGE should contain a co-ion that acts as terminating stacker, and vice versa. When applying the electric field strength, the analyte and the stacker ions experience different electric field strengths, as in FASS, and the analytes are therefore stacked into narrow zones with sharp boundaries [118].
9. Affinity capillary electrophoresis

The dissociation kinetics of an interaction determine which mode of ACE that can be used. Figure 7 shows the experimental principles of the ACE modes used in this thesis. The basics for the different modes are discussed in sections 9.1 and 9.2.

<table>
<thead>
<tr>
<th>Mode and experimental setup</th>
<th>Resulting electropherogram</th>
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<tr>
<td>Partial filling affinity CE</td>
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<td>Peak areas $\frac{[R]}{[R]}$, $\frac{[RL]}{[R]}$ and $\frac{[L]}{[L]}$</td>
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<td>Peak plateau heights $\frac{[R]}{[R]}$, $\frac{[RL]}{[R]}$ and $\frac{[L]}{[L]}$</td>
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<td>Frontal analysis CE</td>
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Figure 7. Schematic illustration of four ACE experiments.

9.1 Migration shift affinity capillary electrophoresis

In migration shift affinity capillary electrophoresis (msACE) one of the species, normally the ligand, is added to the electrophoresis buffer and the other, the receptor, is injected as a narrow sample zone [21-22]. During electrophoresis, if interaction of the species occurs, the complex is likely to experience a different electrophoretic mobility than the receptor molecule, due to a change in charge and/or size. Complexation is detected as a change in peak appearance time in msACE, thus the concentration of the receptor must not be known [22].

The $\mu_r$ is inversely proportional to the migration time of the analyte [15, 76] according to:
\[ \mu_{ep} = \frac{l_d}{t_A} \]  

(10)

where \( l_d \) is the capillary length to the detector and \( t_A \) the migration time of the analyte. A shift in electrophoretic mobility due to complexation will thus lead to a shift in migration time as compared to an electrophoresis run without ligand added. The observed migration time of the peak will be a weighted average of the ongoing associations and dissociations. The time spent as a complex will depend on the concentration of the ligand. The more ligand added, the more time the receptor will be in the complexed form and a greater change in migration time will be observed.

There are some requirements that must be fulfilled for the application of this approach. First, there must be a change in electrophoretic mobility upon complexation in order to get a response [21] and the mobility change must be due specifically to the association of receptor and ligand [15]. Second, the analysis time must be long enough to achieve equilibrium conditions [15, 21-22, 119-120]. This is practical only for low-to-intermediate affinity interactions with fast on-off-kinetcis [22]. Slow on-off-kinetcis are observed as peak broadening. Therefore more stable complexes are normally analyzed after pre-equilibration (cf. section 9.2). Third, the ligand concentration must be at least 10 times higher than the receptor concentration. Finally, for detection purpose sufficient concentrations of receptor molecules should be present to provide a signal [21].

Equation 2 describes the general rectangular hyperbolic binding isotherm for the simple 1:1 binding [29]. In msACE, the dependent variable is the change in electrophoretic mobility \( \Delta \mu \), and equation 2 can be rearranged to equation 11 [16, 26]:

\[ \Delta \mu = \Delta \mu_{\text{max}} - K_D \frac{\Delta \mu}{[L]} \]  

(11)

where \( \Delta \mu_{\text{max}} \) is the change in electrophoretic mobility at saturating ligand concentration. A non-interacting marker should be added to the sample, to ensure that shifts in migration time are due specifically to binding of ligand and to correct for possible changes in EOF [15, 24, 121]. \( \Delta \mu \) is calculated according to:

\[ \Delta \mu = \mu_{ep+L} - \mu_{ep0} = (\mu_{net} - \mu_{EOF})_{+L} - (\mu_{net} - \mu_{EOF})_{0} \]  

(12)
where $\mu_{\text{net}}$ and $\mu_{\text{EOF}}$ is calculated according to equation 10, substituting $t_A$ with $t_M$ (the migration time of the marker) to obtain $\mu_{\text{EOF}}$. The subscripts denote experiments with, $+L$, and without 0, ligand added. A plot of $\Delta\mu$ against the free ligand concentration $[L]$ should show a saturated dependence according to equation 11. The binding constant can be calculated using a linearization method (there are several to choose from), but these come from a time where no computers were available and are now obsolete. Also, estimation using non-linear curve fitting is less biased [66].

Because electrophoretic mobility is inversely dependent on the migration time (cf. equation 10), the difference in the inverse peak appearance time can be used as a measure of $\Delta\mu$ when capillary dimensions, buffer conditions (except for ligand addition), current, temperature and electric field strength are kept constant [16]. The difference in corrected peak appearance times, $\Delta(1/t)$ can be calculated according to:

$$\Delta \frac{1}{t} = \left( \frac{1}{t_M} - \frac{1}{t_A} \right)_{+L} - \left( \frac{1}{t_M} - \frac{1}{t_A} \right)_0$$

(13)

A direct plot of $\Delta(1/t)$ against $[L]$ should show a saturable dependence (according to Eq. 11) [15]. Non-linear curve fitting of equations 12 and 13 yield the same $K_D$. However, if values of the migration shifts that are comparable to other researchers work are desired, equation 12 should be used.

9.2 Other modes of affinity capillary electrophoresis

Some ligands are very expensive or only available in small amounts [122]. The ligand may also have high UV absorbance, which interferes with the UV detection. In such cases, the ligand can be injected in the BGE filled capillary as a large sample zone before or after the receptor [123-124]. This mode of ACE is called partial filling CE (PF-CE). The $K_D$ is determined in the same manner as in msACE.

In high affinity interactions, the complex dissociation rate is very slow in relation to the analysis time [19]. When the value of the inverse of $k_{\text{off}}$ for an interaction is approaching the analysis time, the complexes will not dissociate during separation and are therefore analyzed after pre-equilibration (see figure 7) [15, 22]. The receptor is pre-equilibrated with the ligand and then complex and free receptor are separated in the BGE filled capillary. Equation 3 is used to determine $K_D$ and the concentrations of free and complexed receptor and ligand are
derived from the peak areas after calibration [19]. Pre-equilibration ACE requires a known receptor concentration.

In frontal analysis CE, pre-equilibrated receptor and ligand are injected as one large sample zone into a BGE filled capillary [19, 125]. Because a large zone is injected, the equilibrium is maintained during the separation step. Separation of the interacting species results in peak plateaus rather than peaks. $K_D$ is determined as in pre-equilibration ACE using equation 3, with the substitution of the height of the peak plateaus for the peak areas. The benefit of frontal analysis CE compared to pre-equilibration ACE is that frontal analysis CE can be used independent of on-off kinetics [125].

Other modes of ACE involve injections of the ligand into receptor filled capillary or injections of the BGE into a capillary filled with ligand and receptor. The peaks obtained can be positive and/or negative and the areas of the peaks are obtained by internal or external calibration and are used to determine the $K_D$. These approaches are not further discussed in this thesis.
10. Electrophoresis of $\beta_2$glycoprotein I

$\beta_2$gpI adsorbs strongly onto fused silica capillaries during electrophoresis at physiological pH. A simple way to overcome capillary adsorption problems in the analysis of specific proteins is to perform a running buffer pH scan. This may show that CE analysis is feasible at a pH value that is acceptable for subsequent binding studies. We experienced in early experiments that $\beta_2$gpI is not recoverable using plain fused-silica capillaries and physiological or near-physiological pH electrophoresis. $\beta_2$gpI is most likely adsorbed onto the silica wall.

10.1 Coated capillaries

To suppress the wall interactions for $\beta_2$gpI, permanently dimethylacrylamide (DMA) coated capillaries were prepared and used for electrophoresis of $\beta_2$gpI at physiological pH. Our laboratory has shown that bonded DMA as coating provides good performance for the separation of a selection of basic proteins [107]. However, in paper I, poor recovery and reproducibility was obtained when analyzing $\beta$2gpI. A more hydrophilic polymer, acrylamide (AA) was implemented instead and the method described by Hjertén (cf. section 8.4) was used [106]. With AA-coated capillaries at physiological pH we found partial but inconsistent recovery of $\beta_2$gpI. The reason for the poor recovery is not known, but $\beta_2$gpI has a hydrophobic region exposed to the solvent (see figure 1) and has well-known lipid binding capabilities and thus likely affinity for hydrophobic surfaces. The higher adsorptivity of $\beta_2$gpI on the DMA-coating compared to the AA-coating may be due to the somewhat more hydrophobic character of DMA. Another explanation could be that the adsorption problems are due to incomplete coverage of the surface and to the presence of precipitated proteins that originate from previous runs [112].

10.2 Mobilization with ligand

Another approach to overcome wall interactions is to add a known ligand to the running buffer and thus confer a suitable charge to the analyte, e.g. a negative charge upon complexation with basic analytes. The CE analysis of human lactoferrin has been shown to require the addition of heparin [28]. The protein $\beta_2$gpI is, like lactoferrin, a heparin-binding protein. In paper I, $\beta_2$gpI was recovered using complex formation with heparin in both uncoated and coated capillaries. This is shown in figure 8. However, the presence of interactions with a soluble ligand as well as with the wall makes ligand binding analyses complicated and another approach to suppress wall interactions had to be found.
Figure 8. Mobilization of $\beta_{2}$-gpI with bovine lung heparin in paper I. (A) Uncoated fused silica capillary; (B) Acrylamide coated capillary. Conditions: (A), 15 kV; 20 °C. BGE: 0.13 M Tris base, 0.5 M glycine pH 8.6 with added heparin at concentrations given in the figure; (B), constant current conditions -120 µA; 22 °C. BGE: 0.1 M phosphate pH 7.4 with added heparin at concentrations given in the figure.
11. The pH hysteresis effect

In 1990, Lambert and Middleton measured the $\mu_{\text{EOF}}$ as a function of pH with both alkaline and acidic pretreatments of the fused silica capillary [126] and found that the values of $\mu_{\text{EOF}}$ were consistently lower after an acidic pretreatment than the values of $\mu_{\text{EOF}}$ obtained after an alkaline pretreatment. They also discovered that the equilibration of the surface charge on the fused silica surface appears to be a relatively slow phenomenon, especially at intermediate pH. Theoretically, this could be used to suppress charge dependent wall adsorption. After an acidic pretreatment of the capillary fewer deprotonated silanol groups are available. Due to the slow deprotonation process of the silanols the surface would remain relatively uncharged at subsequent electrophoresis at neutral pH.

The results in paper I indicate that this theory is correct. The acidic pretreatment approach was successful in eliminating recovery problems in the CE analysis of $\beta_{2}$-gpI (figure 1C-D in paper I). The pretreatment approach was also implemented in binding studies of $\beta_{2}$-gpI to heparin and anionic liposomes (cf. section 14.2). With this approach it was possible to avoid analyte-wall interactions at the same time as physiological binding experiments were feasible and meaningful.

The physicochemical basis of this phenomenon is not entirely clear, but a porous gel model of the silica-solution interface has been suggested as explanation [72, 127]. The model suggests that a gel layer is formed close to the silica surface due to hydrolysis of SiO$_2$. When a porous gel layer is formed at the silica-solution interface, the magnitude of the zeta potential is reduced by counterions that are trapped in the gel layer. This would change the electrokinetic behavior of the capillary and reduce the EOF. Acidic and alkaline pH influence the rate of dissolution of silica [128-129]. An alkaline pretreatment would dissolve the gel layer and maintain a constant value of the zeta potential, and such a pretreatment is a general recommendation when performing CE. Acidic pretreatment, and thus the pH hysteresis effect, has earlier been used to manipulate the migration time in order to control the reaction time in kinetic studies of metal complexes [130] and to suppress the EOF during stacking of anionic molecules [131].

11.1 Optimization of the acidic pretreatment

In paper II, the pretreatment approach was further characterized to investigate how to achieve as much suppression as possible and to estimate the time for deprotonation of the silanols in our buffer system. The $\mu_{\text{EOF}}$ was used as a measure of the surface charge density and was measured after different pretreatments of the capillary, using the method described
by Williams and Vigh [132]. A low $\mu_{EOF}$ means less surface charge and hence, higher suppression of protein-wall interaction would be expected.

The type of acid had a small impact on the degree of silanol protonation, however there was an indication that a stronger acid offered a larger magnitude of suppression. The molarity of the acid showed a direct influence on the degree of suppression. The values of $\mu_{EOF}$ obtained at neutral pH after different pretreatments of the silica capillary are presented in figure 9.

![Figure 9. Electrophoretic mobility of the EOF after different pretreatments in fused-silica capillaries in paper II. (A) capillary pretreated for 1 h with 1 M HCl and with intermediary 1 min 1 M HCl-washes between each measurement; (B) intermediary 1 min 1 M HCl-washes between each measurement; (C) pretreatment with 1 h wash with 1 M HCl but no intermediary washes between measurements; (D) capillary left for 15 h after being filled with 1 M HCl, no intermediary washes; (E) prolonged prewash with NaOH and intermediary washes with 1 M NaOH between each measurement.](image-url)

The acidic pretreatments were compared to the generally recommended alkaline pretreatment with respect to the value of $\mu_{EOF}$ (stars in figure 9). Opposed to other researchers who state that omission of an alkaline pretreatment leads to loss in reproducibility of the migration times [126-127, 133], high reproducibility of the $\mu_{EOF}$ was obtained with an acidic pretreatment. Acidic pretreatment offered lower values of $\mu_{EOF}$ than alkaline pretreatment. The rinse time had only a minor impact on the suppression of $\mu_{EOF}$. A 1 min pretreatment with 1 M HCl was found to be sufficient to effectively suppress the $\mu_{EOF}$. 

39
It is important to preserve the protonated surface for as long time as the analysis is going on, i.e. the conditions should be kept relatively stable. Consecutive measurements of $\mu_{\text{EOF}}$ at pH 7.4 without intermediary washes after acidic pretreatment of the capillary (filled squares in figure 9) showed that the surface remains relatively uncharged during the time of a routine CE separation of 10-30 min. Analysis times over 30 min after acidic pretreatment should therefore be avoided, because wall adsorption of the protein then becomes an increasing problem.

Figure 10. Reproducibility of $\beta_{2}\text{gpI}$ analysis in paper II. Ten consecutive analyses of $\beta_{2}\text{gpI}$ on (A) HCl- and (B) NaOH-pretreated fused-silica capillary. Pretreatment: (A) 1 min 1 M HCl, 2 min electrophoresis buffer; (B) 1 min 1 M NaOH, 2 min electrophoresis buffer. Conditions: 20 kV; 22 °C BGE: 50 mM phosphate pH 7.4.

The established pretreatment procedure needed for efficient pretreatment of the capillary was tested again for $\beta_{2}\text{gpI}$ (figure 10 A). A higher reproducibility for the migration time was obtained after optimization of the acidic pretreatment (RSD=2.0 %, n=10 for the $\beta_{2}\text{gpI}$ peak in figure 10 A) compared to the results in paper I (RSD=5.7 %, n=9). A comparison with an alkaline preconditioned capillary was also performed (figure 10 B) and with this capillary poor recovery of $\beta_{2}\text{gpI}$ was in accordance with our earlier studies.
11.2 Separation of basic proteins

Because the approach with acidic pretreatment was promising for the CE analysis of βgpI, the procedure could be useful for CE analysis of other proteins as well. A mixture of basic protein standards was subjected to analysis in differently pretreated capillaries. Figure 11 shows the electropherograms from these experiments. The acidic pretreatment offered considerably better peak shapes of these proteins compared to the alkaline pretreatment where the recovery problem is obvious.

![Figure 11. Electropherograms of basic protein standards on differently pretreated fused-silica capillaries in paper II. Pretreatment: (A) 1 min 1 M HCl, 2 min electrophoresis buffer, (B) 1 min HCl, 1 min 1 M NaOH, 2 min electrophoresis buffer, (C) 1 min 1 M NaOH, 2 min electrophoresis buffer. Sample: lysozyme (lys), cytochrome c (cyt c), ribonuclease A (RnA), α-chymotrypsinogen (α-C Trg), trypsinogen (Trg). Conditions: 12 kV; 22 °C. BGE: 50 mM acetate/Tris pH 4.8.](image)

Hjertén and coworkers discussed that the accumulation of proteins at the capillary wall can be due to precipitation of proteins caused by moving non-cross-linked polyacrylamide chains at the wall of AA coated capillaries [112]. These precipitates were efficiently removed by HCl rinsing between runs. The possibility that the acidic pretreatment effect in our case may be due to better rinsing of the silica surface is addressed in figure 11 B, where the capillary is rinsed with HCl prior to an alkaline preconditioning. The results show that for the protein
test mixture this is not the case. In paper IV it was shown that NaOH was more efficient in rinsing off previously adsorbed proteins from the silica surface (cf. section 11.3). The acidic pretreatment approach efficiently prevents proteins from adsorbing to the fused silica capillary if used prior to electrophoresis and thus improves the recovery of proteins such as β2gpI.

### 11.3 Probing the amount of adsorbed protein on the silica surface

The results in paper II showed that the approach with acidic pretreatment of the capillary is a simple and effective way to suppress wall interactions. The positive results in papers I-II encouraged us to in more detail probe the amount of protein remaining on the silica surface after electrophoresis of a protein sample. In paper IV, β2gpI was used as a sample when three capillary pretreatments were compared: acidic (HCl), neutral (BGE) and alkaline (NaOH). The adsorbed amount of protein was estimated with three independent techniques to gain complementary information. To ascertain that the adsorbed β2gpI could be detected, neutral pretreatment with only BGE was included to obtain a capillary known to have β2gpI adsorbed to the inner wall.

First, desorption of attached proteins from the inner surface of silica capillaries was performed by forcing SDS micelles through the capillary [89, 134-136]. This technique is called SDS displacement CE and is successful in detecting proteins recently attached to the silica surface. Proteins that have been attached more than 24 h undergo an aging process that impedes desorption with SDS [89, 137].

Second, the change in electroosmotic flow (EOF) during multiple CE analyses of a protein was used as a measure of protein adsorption on the silica wall, because adsorption alters the zeta potential at the capillary wall and, hence, the EOF (cf. section 8.1) [136]. By including a neutral EOF marker in the protein sample, the EOF was easily monitored during electrophoresis.

Third, a fluorescence microscopy based method was devised to facilitate an estimation of the adsorbed amount of β2gpI directly on the surface. In this measurement, fluorescein isothiocyanate-labeled antibody against β2gpI (FITC-anti-β2gpI mAb) was allowed to bind to adsorbed β2gpI in differently pretreated capillaries and then the fluorescence was measured in a fluorescence microscope.
Figure 12. Estimation of the amount of protein adsorbed on the inner surface of fused silica capillaries after different pretreatments in paper IV. The results for the \( \beta_{2}gpI \)-containing capillaries are shown for SDS displacement electrophoresis and change in EOF and for the \( \beta_{2}gpI \)-containing capillaries (samples) and only marker-containing (controls) capillaries are shown for the fluorescence microscopy experiments.

The results from the three methods offered complementary information and are summarized in figure 12. All methods were shown to detect \( \beta_{2}gpI \) adsorbed to the silica surface, provided of course that the protein occurs above the detection limit. It is clearly shown that HCl-pretreatment is the only pretreatment that offers low amount of adsorbed \( \beta_{2}gpI \), low change in EOF and a low intensity in the fluorescence measurements. Figure 13 shows electropherograms of \( \beta_{2}gpI \) obtained on the differently pretreated capillaries. Clearly, \( \beta_{2}gpI \) was recovered on HCl-pretreated capillary, but not on NaOH- and BGE-pretreated capillaries. This is evident also from the results in paper II (cf. figure 10).

The change in EOF in NaOH-pretreated capillaries is the lowest of all tested pretreatments. This capillary also showed low intensity in the immunofluorescence measurement. However, SDS displacement CE experiments showed an 80 % loss of protein, and no electrophoretic recovery was obtained on NaOH-pretreated capillaries. These results in combination contributed to the conclusion that NaOH efficiently rinses adsorbed proteins off the silica surface, which was shown by Righetti and coworkers [135]. Pretreating the capillary with NaOH directly followed by an HCl-pretreatment, or vice versa, might leave a surface beyond control and is not recommended [126, 135].

These results are significant because they show that acidic pretreatment offers a simple remedy to the adsorption problems experienced with proteins such as \( \beta_{2}gpI \). They verify our previous findings in both paper I and II, that acidic pretreatment does diminish \( \beta_{2}gpI \) adsorption on fused silica capillaries by decreasing the charges on the inner wall.
Figure 13. Recovery of $\beta_2$gpI after different capillary pretreatments. Sample: $\beta_2$gpI, 0.01 % (v/v) DMSO (marker, M). Conditions: 15 kV; 55 µA; 22 °C. Pretreatment: as stated in the figure. BGE: 50 mM phosphate pH 7.4.

11.4 Change in pH due to deprotonation of silanol groups

Even though the deprotonation of the silanol groups is a slow process, a certain number of protons is continuously released from the wall and these may lower the pH of the BGE. A theoretical calculation of the maximum pH change was performed in paper IV to address this aspect. Table III summarizes this calculation. The number of silanol groups on fused silica surface is determined to be 4.6 per nm$^2$ [138]. With the capillary dimensions used, $2.93 \times 10^{14}$ (4.86 $\times 10^{-10}$ moles) surface silanol groups are present. Two assumptions are made: first, all silanol groups become protonated during the acidic pretreatment and all silanol groups are deprotonated during electrophoresis (this is of course not true, because the $\mu_{EOF}$ during electrophoresis would be the same after acidic pretreatment as after alkaline pretreatment and also because $\beta_2$gpI would not be recovered, however, a maximum change is sought, hence the assumption); second, the BGE is not moving (this is not true either, but it is a reasonable assumption that greatly simplifies the process).
The pH of the BGE after electrophoresis is calculated to be 7.38, using the Henderson-Hasselbalch equation. Hence, the deprotonation of the silanol groups during electrophoresis in a worst case scenario only lowers the pH by a maximum of 0.02 pH-units.

| Table III. Theoretical calculation of pH change due to deprotonation of silanols and electrolysis. | Deprotonation | Electrolysis |
| Vessel volume (mL) | 7.85×10⁻⁴ | 1.5 |
| Maximum amount of H⁺ released (moles) | 4.86×10⁻¹⁰️ a | 1.03×10⁻⁶  b |
| [H₃PO₄]_{initial} (mM) | 19.34 | 19.34 |
| [HPO₄²⁻]_{initial} (mM) | 30.66 | 30.65 |
| [H₂PO₄⁻]_{initial} (mM) | 19.96 | 26.18 |
| [HPO₄²⁻]_{final} (mM) | 30.04 | 23.82 |
| pH_{final} | 7.38 | 7.16 |
| ΔpH | -0.02 | -0.24  d |

a Calculated from the amount of silanol groups in fused silica
b Calculated using Faraday’s law
c Calculated using Henderson-Hasselbalch equation.
d After 10 electrophoresis runs of each 30 min.

The same calculation can be performed to calculate the decrease in pH due to electrolysis, which generates protons in the anodic compartment and hydroxide ions in the cathodic compartment. Faraday’s law can be used to calculate the number of protons generated in the anodic compartment during 10 electrophoresis runs [139]. The estimated pH change in the inlet vial due to electrolysis after 10 runs is approximately 0.2 pH units. Hence, electrolysis affects the pH more than deprotonation of silanol groups. Luckily, this effect is easily remedied by changing to fresh inlet buffer vial before each run.
12. Stacking

Electrophoresis of $\beta_2$gpI at physiological pH resulted in relatively broad $\beta_2$gpI peaks (see e.g. figure 10A). Different techniques for stacking analytes to increase sample concentration after injection was described in section 8.5. These techniques can also be used to sharpen analyte zones, hence improve overall separation efficiency. Sample enrichment of biological samples is preferably accomplished by tITP because of the complex sample matrix. In this stacking mode, transition from isotachophoretic mode to zone electrophoretic mode must be complete prior detection to obtain useful results [118]. In interaction studies, the ligand type and concentration in the sample or BGE are varied from run to run. These additions may act competitively with the leading and/or trailing ions and thus affect the stacking process in a non-reproducible manner. The presence of leading and/or trailing ions may also affect the interaction. Stacking with tITP was therefore not implemented.

Attempts to sharpen the $\beta_2$gpI peak using different modes of FASS were unsuccessful (unpublished data). In FASS, the samples should be in a low-conductivity solution to achieve efficient sharpening of the analyte zones [140]. The $\beta_2$gpI sample is dissolved in PBS, which is a relatively high ionic strength solution (i.e. high conductivity). High ionic strength solutions in the injected sample lead to destacking or anti-stacking [141]. Increasing the BGE ionic strength to overcome that of the sample would in theory permit stacking of the analyte, but in reality lead to substantial Joule heating. Dilution of the sample to obtain a lower ionic strength than that of the BGE, often results in a non-detectable analyte concentration even after sample enrichment [116]. This was the case for $\beta_2$gpI (unpublished data). Desalting of the $\beta_2$gpI sample with different desalting tips and columns resulted in lost sample. Thus, no attempt to sharpen the $\beta_2$gpI zone was successful.
13. Joule heating

As described in section 8.3, heat is generated during electrophoresis because current is passing through the BGE. Commercial CE instruments are equipped with either liquid or air cooling to dissipate the generated heat. However, there is a limit in the amount of heat that can be dissipated. The amount of heat must therefore not be too large; otherwise consequences such as irreproducible migration times and superheating of the samples can harm the analysis. To ensure that the actual temperatures in our system are not too high to harm the interaction studies, the temperature inside the capillary was experimentally determined.

Kok described a method where the conductivity was used to determine the temperature of the BGE inside the capillary [139]. Evenhuis et al. used the conductance to measure the temperature [142]. The conductivity method takes the dimensions of the capillary into consideration, but Evenhuis propose that their method is more straightforward to use [142]. In both methods, different voltages are applied and the current at each applied voltage is measured. Both methods were used to determine the mean temperature of the BGE inside the capillary (paper III and unpublished data) in our system. The current was measured five minutes after the voltage was applied to allow equilibration of the temperature within the capillary. The resulting temperature values are shown in table IV. The estimated temperatures were slightly higher using Kok’s method as compared to Evenhuis, however the difference does not appear to be significant for our purpose. The temperatures deviate significantly from the set temperatures of the capillary cassette. The instrument used in this thesis is equipped with air cooling of the capillary cassette, which is less efficient than liquid cooling [139]. However, the protein samples will presumably not boil or denature at any of the set temperatures.

Table IV. Experimental determination of the temperature.  

<table>
<thead>
<tr>
<th>BGE (mM)</th>
<th>( T_{\text{set}} ) (°C)</th>
<th>( T_{\text{meas}} ) (°C)</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Evenhuis</td>
<td>Kok</td>
<td></td>
<td></td>
</tr>
<tr>
<td>50</td>
<td>15.0</td>
<td>21.2</td>
<td>21.4</td>
<td></td>
</tr>
<tr>
<td>50</td>
<td>22.0</td>
<td>30.0</td>
<td>30.1</td>
<td></td>
</tr>
<tr>
<td>50</td>
<td>37.0</td>
<td>43.9</td>
<td>44.0</td>
<td></td>
</tr>
<tr>
<td>100</td>
<td>22.0</td>
<td>38.3</td>
<td>38.4</td>
<td></td>
</tr>
<tr>
<td>150</td>
<td>22.0</td>
<td>47.4</td>
<td>47.5</td>
<td></td>
</tr>
</tbody>
</table>

\( T_{\text{set}} \): Set temperature of the air cooled capillary cassette  
\( T_{\text{meas}} \): Experimentally measured mean temperature of BGE in capillary
14. **Structure-activity studies of β₂-glycoprotein I**

To understand the function of β₂gpI in vivo, it is important to use experimental parameters (such as pH and ionic strength) that mimic the physiological conditions as much as possible. To simplify development and facilitate verification of the experimental platform, heparin was used as a ligand for β₂gpI. Heparin is a relatively well-defined, commercially available compound that binds to the same binding site on β₂gpI as anionic phospholipids. As mentioned above, heparin acts as a competitive inhibitor to the β₂gpI-anionic phospholipid interaction.

A number of different buffers at physiological pH were tested during the establishment of the separation conditions. Phosphate buffered saline (PBS) is commonly used to mimic physiological ionic strength and pH. However, the use of PBS as BGE resulted in very high currents, frequent loss of current and irreproducible peak appearance times. The relatively high concentration of NaCl gives a high conductivity and, hence, a very high current. Therefore CE based binding experiments in PBS were not possible. Plain sodium phosphate buffer at pH 7.4 offered the best peak shape and reproducibility (data not shown) of all buffers tested. Sodium phosphate is a multivalent electrolyte which gives a relatively high ionic strength compared to its concentration. A concentration of 50 mM phosphate buffer at pH 7.4 corresponds to an ionic strength of 131 mM. This may be considered near-physiological ionic strength conditions (corresponding to 154 mM NaCl in the blood) and therefore 50 mM phosphate was used as a BGE in all binding experiments. The suitability of phosphate as BGE has been shown by Tran et al. [99] and phosphate buffers are frequently used in CE for separation of proteins [89]. The higher performance of phosphate compared to other buffers is related to the formation of complexes with silanol groups, thereby reducing the capillary net charge [89,99-100], but also to the low absorbance down to 200 nm [89].

14.1 **Binding to heparin**

Binding between β₂gpI and heparin was shown in papers I and III using msACE. In paper I we were able to fractionate β₂gpI into several peaks (figure 14A) but in paper III only one peak of β₂gpI was resolved (figure 14B) when including heparin in the BGE. In the two papers different batches of β₂gpI purified from plasma were used and they may contain different numbers of contaminating proteins or different compositions of structural variants. It has been shown that β₂gpI exhibits microheterogeneity in its glycosylation [40-41, 43, 143], as well as a polymorphism in the polypeptide chain [40-41]. These different glycoforms and/or isoforms may have different binding characteristics. In paper I equation 13 was used...
to extract the binding constants for the resolved $\beta_2$gpI peaks. The resulting binding constants are presented in table V. Different $K_D$'s were obtained for peaks II and III, indicating that the $\beta_2$gpI glycoforms and/or isoforms do have different affinity for heparin.

Figure 14. MSACE of $\beta_2$gpI and heparin in (A) paper I and (B) paper III. Conditions: (A) constant current conditions 120 µA; (B) 15 kV; 22 °C. BGE: (A) 0.1 M and (B) 50 mM phosphate pH 7.4 with added heparin as stated in the figure.
Table V. Binding constants and experimental parameters for the β₂glycoprotein I-heparin interaction.

<table>
<thead>
<tr>
<th>T&lt;sub&gt;set&lt;/sub&gt; (°C)</th>
<th>[phosphate] (mM)</th>
<th>K&lt;sub&gt;D&lt;/sub&gt; (µM)</th>
<th>Cl (µM)</th>
<th>R&lt;sup&gt;2&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>15.0</td>
<td>50</td>
<td>2.98</td>
<td>1.9</td>
<td>0.36</td>
</tr>
<tr>
<td>22.0</td>
<td>50</td>
<td>3.86</td>
<td>1.3</td>
<td>0.62</td>
</tr>
<tr>
<td>37.0</td>
<td>50</td>
<td>2.51</td>
<td>1.4</td>
<td>0.51</td>
</tr>
<tr>
<td>22.0</td>
<td>100</td>
<td>39.8</td>
<td>3.5</td>
<td>0.99</td>
</tr>
<tr>
<td>22.0</td>
<td>150</td>
<td>233</td>
<td>62</td>
<td>0.98</td>
</tr>
<tr>
<td>22.0</td>
<td>50</td>
<td>39.3&lt;sup&gt;a&lt;/sup&gt;</td>
<td>15</td>
<td>0.83</td>
</tr>
<tr>
<td>22.0</td>
<td>100</td>
<td>48.8&lt;sup&gt;II&lt;/sup&gt;</td>
<td>11.3</td>
<td>0.98</td>
</tr>
<tr>
<td>22.0</td>
<td>100</td>
<td>15.4&lt;sup&gt;III&lt;/sup&gt;</td>
<td>3.4</td>
<td>0.96</td>
</tr>
</tbody>
</table>

<sup>a</sup> 4 M urea in BGE.
<sup>II</sup> peak II in figure 14A; <sup>III</sup> peak III in figure 14A.

T<sub>set</sub>: Set temperature of capillary cassette

All estimations were performed with three replicate measurements.

14.1.1 Influence of temperature on β₂-glycoprotein I-heparin interaction

In paper III, the influence of the temperature on the binding between β₂glycoprotein I and heparin was investigated simply by applying different capillary temperatures during electrophoresis. Equation 12 was used to extract the binding constants at the different temperatures investigated (see table V for K<sub>D</sub>'s). The correlations of the fit of the mobility data to the 1:1 binding site model obtained with 50 mM BGE at the three temperatures investigated was low (R<sup>2</sup> ≤ 0.62). Except for the 37 °C data, we found no more complex models using higher order polynomials that fitted better than a one-site model. Even though the mobility data at a set temperature of 37 °C can be fitted to a two site binding hyperbola, the experimental data yield confidence intervals of the K<sub>D</sub>'s that are extremely wide. Therefore it does not allow exact estimates of K<sub>D</sub>'s.

A 1:1 binding stoichiometry between β₂glycoprotein I and heparin may be a simplification. As mentioned above, heparin acts as a competitive inhibitor to the β₂glycoprotein I-anionic phospholipid interaction, using the same binding site on the β₂glycoprotein I molecule. Other researchers have calculated that β₂glycoprotein I occupies about 70-200 lipid molecules [144-145]. However, this calculation means that there are about 70-200 lipid molecules in the membrane for each binding site on a phospholipid vesicle, not that each β₂glycoprotein I molecule can bind so many lipid molecules. Experimentally, if β₂glycoprotein I could bind more than one heparin molecule (which in turn could bind more than one β₂glycoprotein I molecule) then we would expect precipitates to form at β₂glycoprotein I-heparin ratios where lattice formation would be facilitated. This, however, has never been observed. Also, from other work it is well established that the binding is
electrostatically mediated by a surface-exposed patch [45]. For the estimation of the binding constants we therefore assume only one ligand binding site on β₂gpI and independent binding of each β₂gpI to putative multiple sites on heparin.

14.1.2 Influence of ionic strength on β₂-glycoprotein I-heparin interaction

The influence of the ionic strength on the β₂gpI-heparin binding was investigated in paper III by performing msACE at three different BGE concentrations. The K_D’s obtained at the three different BGE concentrations (i.e. different ionic strengths) tested were significantly different as can be seen in table V. Thus, the ionic strength has a major impact on the binding between β₂gpI and heparin. This is an indication that the interaction with heparin is highly dependent on electrostatic interactions and that their contribution to the binding energy means that the interaction will be quite weak (K_D estimated to be 3.86 ± 1.3 µM) under near-physiological ionic strength conditions.

14.1.3 Influence of protein conformation on β₂-glycoprotein I-heparin interaction

The results in both paper I and paper III suggested an electrostatic nature of the β₂gpI-heparin interaction, but do not account for presumed conformational contributions to binding. Unfolding of proteins can be monitored in CE by the change in the electrophoretic mobility of the protein. As can be seen in equation 7, the electrophoretic mobility depends among other factors, on the hydrodynamic radius of the protein. This radius is larger for the unfolded polypeptide chain than for the folded protein. Thus, unfolding of a protein yields a larger hydrodynamic radius and, hence, a decrease in the µₑₑ [146-150].

In paper III, different denaturing agents were added to the BGE to induce unfolding of β₂gpI. The results show that neither ACN nor sodium dodecyl sulphate (SDS) lead to unfolding of β₂gpI. Actually, our results suggested that β₂gpI is highly resistant to unfolding with SDS. The migration shift observed in figure 4 in paper III may instead be a consequence of an interaction between β₂gpI and SDS micellar vesicles. β₂gpI is known to bind to negatively charged species and surfaces (cf. section 4). Also, denaturation with SDS is inhibited by the hydrophilic oligosaccharide units in glycoproteins and by electrostatic binding to the sulphate groups on SDS by strongly basic proteins [98]. The use of urea to induce unfolding of β₂gpI was more successful. The unfolding of the proteins β₂gpI and cytochrome C can be followed in figure 15. As the urea concentration increases, the electrophoretic mobility of the proteins decreases.
Total unfolding of β2gpI was not accomplished in our system. The reason for the resistance of β2gpI to completely unfold may be found in its three-dimensional structure. β2gpI is a protein rich in cysteine residues [38-39] which form disulfide bridges and stabilize the protein conformation. Therefore, β2gpI may be less prone to unfold. However, the purpose of these experiments was to find means to achieve a controlled and limited unfolding of β2gpI. We take advantage of the resistance to unfold by simply adding urea in a quantity high enough to unfold whatever the 3D structure of the protein allows. Migration shift ACE of partially unfolded β2gpI and heparin indicated binding and the K_D for this interaction is presented in table V. The dissociation constant for the β2gpI-heparin interaction was 10 times higher when urea was present, compared to when it was absent (plain BGE). Thus, the interaction between partially unfolded β2gpI and heparin was weaker than between fully folded β2gpI and heparin. Accordingly, the conformation of the protein is of importance for the β2gpI-heparin interaction. Heparin has been shown to bind to a positively charged region on domain V of β2gpI [45]. In the folded state of β2gpI, this positively charged region has the shape of a loop [2, 39] which may adopt a more linear form upon unfolding and thereby be less accessible for heparin.

### Influence of protein glycosylation on β2-glycoprotein I-heparin interaction

It has been suggested that the glycosylation of β2gpI plays a role for antibody recognition [1, 151]. As stated in section 4.1, binding of β2gpI to negatively charged surfaces is thought to induce a conformational change that leads to exposure of an epitope, and thereby enabling binding of a certain type of autoantibodies [1, 151]. To contribute to the elucidation of the
role of glycosylation, interaction studies of deglycosylated \( \beta_{2} \text{gpI} \) were performed (unpublished results) using the CE approach described in sections 11 and 14.1.1-14.1.3. Heparin was used as ligand to establish the experimental parameters. Later on, this method could be used to characterize the interaction between deglycosylated \( \beta_{2} \text{gpI} \) and autoantibodies.

Treatment of \( \beta_{2} \text{gpI} \) with PNGase F was performed to remove all carbohydrates. Figure 16 shows the samples after 24 h incubation. After treatment of \( \beta_{2} \text{gpI} \) with PNGase F, four bands were resolved with molecular weights ranging from 51-32 kDa (lane 1 in figure 16), plus one band corresponding to the enzyme. The original \( \beta_{2} \text{gpI} \) sample contained two fractions of proteins, probably reflecting the inherent microheterogeneity.

Figure 16. SDS-PAGE of PNGase F treated \( \beta_{2} \text{gpI} \). Lanes: 1, \( \beta_{2} \text{gpI} \) treated with PNGaseF; 2, PNGase F; 3, \( \beta_{2} \text{gpI} \).
Electrophoresis of deglycosylated β₂gpI was somewhat difficult to perform because of frequent loss of current. In the runs where the current was constant, no peak corresponding to deglycosylated β₂gpI could be observed. Therefore, we suspected that the protein precipitates in the absence of carbohydrates. Thus, the carbohydrates may help keeping the conformation of β₂gpI in plasma. To withhold deglycosylated β₂gpI in solution, urea was added to the BGE (cf. the experiments in section 14.1.3). A selection of electropherograms of deglycosylated β₂gpI and β₂gpI is shown in figure 17. The addition of urea improved the electrophoretic performance, but current was still lost during a number of runs. Without heparin in the urea-containing BGE, one peak (denoted with an asterisk and open ring) was resolved in figure 17A. Upon the addition of as low concentration of heparin a 0.05 mg/mL to the urea-containing BGE, two peaks were resolved. The second peak (open ring) shifts towards the anode upon increased heparin concentration, but is not observed at all heparin concentrations. At 2 mg/mL heparin, the migration time exceeded the 30 min detection time.
of the analysis, longer analysis times are not desirable after acidic pretreatment, because wall
adsorption of the protein then becomes an increasing problem (cf. section 11.1). The 3D
spectrum indicated that both peaks in figure 17A correspond to proteins. Therefore, it was
suspected that two fractions of deglycosylated β2gpI were resolved and that these fractions
have different affinity to heparin. Thus, the carbohydrates may affect binding to heparin.
However, because so many of the electrophoresis runs failed, the migration data is
incomplete and reliable estimates of $K_D$’s were not possible to extract. The method needs
further development to facilitate relevant interaction studies msACE of deglycosylated β2gpI
and heparin, and further on, autoantibodies. These developments could consist of an
increase in the urea concentration to improve the solubility of deglycosylated β2gpI, and the
use of shorter capillaries to overcome the long migration time.

14.2 Phospholipid binding

The binding of β2gpI to phospholipid membranes plays an important role in APS. The
protein β2gpI has affinity for specific anionic phospholipids, e.g. phosphatidyserine (PS),
phosphatidic acid and cardiolipin, but less affinity for other phospholipids [12, 37, 39, 48,
152]. The possibility of using CE for detecting and characterizing these interactions by β2gpI
was examined in paper I, and in unpublished work.

Dynamic light scattering (DLS) was used to determine the average diameter and the CAC of
the liposomes in our system (unpublished results). The average size was 110 nm and
indicated independency of the anionic phospholipid content. The CAC was < 100 µM and if
stored at 4 °C, the liposomes were stable for about one week.

14.2.1 Migration shift affinity capillary electrophoresis

In the studies of the β2gpI-heparin interaction, we found msACE very useful. Therefore, the
same approach was utilized in the study of the β2gpI-anionic phospholipid interaction. The
results in paper I showed that when liposomes with a total lipid concentration of 4 mM were
added to the BGE, the baseline became very unstable and the analyte peak could not be
distinguished from the background. Liposomes are colloidal particles that scatter light and
thus interfere with the UV detection. Experiments using lower lipid concentrations showed
that liposomes with lipid concentrations above 0.5 mM caused noisy baselines, but with
concentrations below this value electropherograms such as in figure 18 could be obtained.
However, no shift in peak appearance time for β2gpI was observed (unpublished results).
Unfortunately, in this experiment, the detection time was set too short in the electrophoresis
runs without liposomes present.
Even though no migration shift was observed for β2gpI when liposomes were present in the electrophoresis buffer, a small peak appeared (denoted S in figure 18) between the marker peak and the β2gpI peak upon addition of liposomes. The control experiments (grey traces in figure 18) and the 3D spectrum indicates that the S peak contains proteinaceous material. Thus, it appears as if two β2gpI peaks are partially resolved when liposomes are present. However, binding of β2gpI to negatively charged liposomes would be expected to lead to a shift towards the anode, because of the negative charge of the liposome and thus to a longer migration time. Also, while affinity between β2gpI and PC/PS-liposomes is anticipated, affinity between β2gpI and PC-liposomes is unlikely (cf. sections 4 and 14.2). For now, the reason for the resolved S peak in figure 18 remains unknown.

Figure 18. msACE of β2gpI and PC/PS liposomes. Sample, black trace: 0.2 mg/mL β2gpI, 0.01 % (v/v) DMSO (marker, M); grey trace: 0.01 % (v/v) DMSO (marker, M); S: unknown species. Conditions: 13 kV, 90 µA, 22 °C. BGE: 0.1 M phosphate pH 7.4 with 100 µM liposomes added with PC/PS composition stated in the figure.
In paper I, PF-CE was used to avoid that the liposomes would interfere with the UV detection. At the same time higher lipid concentrations were allowed, because the liposomes were detected as a single peak (cf. section 9.2). At neutral pH the net charge of the zwitterionic phospholipids PC and POPC is neutral and neutral liposomes are therefore expected. However, liposomes composed of zwitterionic phospholipids have been found to exhibit non-zero electrophoretic mobilities (paper I and [58, 63, 153-154]). It has been suggested that the negative net charge of liposomes constituted by zwitterionic phospholipids depend on the orientation of the lipid head groups [154] or originate from liposomal deformation and field-induced polarization during electrophoresis [153]. All liposomes in the study had a negative net charge and an electrophoretic mobility towards the anode that exceeded the electrophoretic mobility of β2gpI. Therefore the liposomes were injected as separate sample zones in front of the β2gpI sample to ensure mixing of sample and ligand zones.

After a few runs with liposomes only partially filling the capillary, all peaks disappeared. They reappeared after a couple of runs without liposomes present. As in the msACE experiments, the phospholipids may adhere to the silica wall. Hautala et al. described a method for dynamically coating capillaries with phospholipids using the buffer ions as linkers (Hepes in their case) [155]. Before coating, the silica capillaries were preconditioned with HCl. Acidic pretreatment using HCl in our system may facilitate phospholipid adsorption to the capillary wall. Therefore, no or only a small EOF will be present and the neutral marker will not be detected. Washing the capillary at high pressure with an acid ensured the detachment of the liposomes stuck at the wall and enabled subsequent analyses.
Figure 19 (A) PF-CE and (B) phospholipid coated capillaries. Sample, (A) black trace: β2gpI and EOF marker, M; blue trace: EOF marker, M. Liposomes, with compositions stated in the figure, injected as a zone before β2gpI. BGE: 0.1 M phosphate pH 7.4. (B) Sample: β2gpI and EOF marker, M. Capillaries coated with phospholipids with compositions stated in the figure. BGE: 0.1 M phosphate pH 7.4 (black trace) with added 5 mg/mL heparin (red trace). (A and B) conditions: constant current conditions 120 µA; 22 °C.

Figure 19A shows electropherograms obtained from the binding studies of β2gpI and liposomes when the PF-CE technique was used with 0.5 M HCl pre-wash. The migration patterns are too complicated to reliably extract quantitative binding data, but they indicate affinity of β2gpI for PS. A more thorough optimization of the method is necessary before a general use in protein analysis.

**14.2.2 Pre-equilibration of β2gpI and liposomes**

No clear shift in migration time was observed with msACE or PF-CE. A possible reason for this could be that the β2gpI-phospholipid interaction has slow on-off rates and msACE and PF-CE are not compatible with such kinetics (cf. section 9.2). Strongly binding complexes with slow dissociation rates, are more suitably analyzed after pre-equilibration of the sample.
with the ligand and the complexed and free forms are separated before dissociation occurs. The peak areas of the separated components are used to determine the concentrations of free and complexed \( \beta_2 \text{gpI} \) and liposomes. However, electrophoresis of pre-equilibrated \( \beta_2 \text{gpI} \) and liposomes of different PS-content did not result in any change in the peak area for \( \beta_2 \text{gpI} \) (unpublished data).

Frontal analysis CE can be used independent of the rates for association and dissociation [125]. However, injections of large zones of \( \beta_2 \text{gpI} \) only resulted in peaks (unpublished data). Different zone lengths and BGEs were employed but did not obtain any plateau of \( \beta_2 \text{gpI} \). The microheterogeneity of the \( \beta_2 \text{gpI} \) sample may impede the formation of a plateau.

### 14.2.3 Phospholipid coated capillary

Electrokinetic chromatography (EKC) with capillaries coated with phospholipids was investigated as a possible method to detect the interaction between \( \beta_2 \text{gpI} \) and PS-containing liposomes. Capillaries were dynamically coated with phospholipids using the procedure described by Hautala et al. [155]. Figure 19B shows electropherograms of \( \beta_2 \text{gpI} \) performed on these capillaries and table VI shows the electrophoretic mobility of \( \beta_2 \text{gpI} \) (unpublished data). The value of the electrophoretic mobility in table IV increases at increasing PS-content in the liposomes used in the coating procedure. Upon addition of heparin to the BGE, the electrophoretic mobility increases further. However, in figure 19B, the migration time of \( \beta_2 \text{gpI} \) diminishes upon increasing PS-content. This is explained by an increased EOF due to more negative charges on the phospholipid bilayer when more anionic PS is present. The net mobility of \( \beta_2 \text{gpI} \) increases, because of an increased mobility of the EOF, hence the diminished migration time.

The results in figure 19B indicate that binding between PS-containing phospholipid bilayer can be detected with this approach and that competitive interaction studies could be performed as well. However, the \( \beta_2 \text{gpI} \) peaks are broad and difficult to integrate. The method needs further optimization before used in general protein analysis.

**Table VI. Electrophoretic mobility of \( \beta_2 \text{gpI} \) on phospholipid coated capillaries.**

<table>
<thead>
<tr>
<th>PS</th>
<th>Heparin</th>
<th>( \mu_e ) of ( \beta_2 \text{gpI} ) ( \times 10^{-8} \text{ m}^2 \text{ s}^{-1} \text{ V}^{-1} )</th>
</tr>
</thead>
<tbody>
<tr>
<td>0 %</td>
<td>No data</td>
<td>No data</td>
</tr>
<tr>
<td>0 %</td>
<td>5 mg/mL heparin</td>
<td>0.61</td>
</tr>
<tr>
<td>10 %</td>
<td>No data</td>
<td>0.55</td>
</tr>
<tr>
<td>20 %</td>
<td>No data</td>
<td>0.59</td>
</tr>
<tr>
<td>0 %</td>
<td>5 mg/mL heparin</td>
<td>0.78</td>
</tr>
</tbody>
</table>

* Bovine lung heparin was added to the BGE in figure 19B.
15. Concluding remarks and future perspective

Protein analysis with CE is powerful, but often problematic because of protein adsorption phenomena. Today, there is no generally applicable strategy suited for suppressing the adsorption of proteins to capillary walls in CE. This thesis shows that acidic pretreatment of fused silica capillaries prevents adsorption of β₂gpI and a number of other basic proteins. Acidic pretreatment should be considered in the selection of suppression strategies, for several reasons. First, it is simple and non-expensive to use and it can be implemented in any analytical laboratory because it does not require any special reagents. Second, it is a rapid procedure. Third, it is less laborious than the preparation of coated capillaries. Fourth, it should be compatible with MS detection because it does not require any additives that suppress the MS signal. The main limitation with this procedure is that analysis time is usually restricted to not more than 30 min. However, analysis times over 30 min are rarely applied.

The developed methods for interaction studies of the interaction between β₂gpI and its biological ligands are considered as physiologically relevant. Quantitative binding data were extracted and these confirmed the already known electrostatic character of the β₂gpI-heparin interaction. It was also shown that the β₂gpI-heparin binding depends on the conformation of the protein.

Every protein is unique in its functions and ability to interact with its surroundings. The CE analysis of β₂gpI was the ultimate challenge: it is a large, microheterogeneous, polymorphic protein with hydrophobic and basic patches and it binds a number of negatively charged biomolecules and thus has exquisite affinity for negatively charged surfaces such as the capillary surface at neutral pH. Nevertheless, it was accomplished. The work also contributed with important information regarding the functions of β₂gpI.

Screening of different proteins and buffer systems would reveal if acidic pretreatment has a more general use for preventing protein adsorption to the silica surface. I would also encourage to the elucidation of the physicochemical basics behind the acidic pretreatment and the pH hysteresis effect. Knowledge is a mighty tool: it is the outcome of a series of consecutive advances and improvements that successively generates new ideas and new successful applications.

And so it goes on.
16. Acknowledgements

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17. References


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18. Addendum för icke-kemister

Det slutliga målet med denna avhandling har varit att utveckla analysmetoder som ska användas för att karaktärisera samspelet mellan ett humant protein, β2-glykoprotein I (β2gpI), antikroppar och cellmembran. β2gpI är ett protein som finns i blodplasma och är involverat i blodkoagulationen, men den fysiologiska funktionen är inte helt klarlagd. Både pro- och antikoagulativa egenskaper har tillskrivits β2gpI. Proteinet är också involverat i vissa autoimmuna sjukdomar, t.ex. antifosfolipidsyndrom (APS), som har allvarliga komplikationer i form av blodproppar (trombos), havandeskapsförgiftning och återkommande missfall. Man har funnit en stark korrelation mellan förekomsten av vissa autoantikroppar mot β2gpI och dessa komplikationer.

**Biomolekylära interaktioner**

Biomolekylära interaktioner spelar en central del i cellulära processer. Detaljerad kunskap behövs för de biologiska mekanismer som ligger bakom mänskliga sjukdomar som APS för att finna nya effektiva läkemedel och behandlingar mot sjukdomarna. För att få fram denna förståelse behöver vi ha tillgång till bra verktyg som hjälper oss att studera hur olika komponenter förhåller sig till varandra. Ny kunskap kräver nya och förbättrade verktyg. De krav som har ställts på metoderna i denna avhandling har varit att

- bindningsstudierna ska kunna utföras i lösning, eftersom bindning till en yta kan förändra proteinets egenskaper
- de vara mikroskaliga, eftersom endast små mängder av proteinet finns tillgängligt och för att liganderna är dyra att köpa
- de ska kunna utföras under betingelser som efterliknar den naturliga miljön för β2gpI, annars kan proteinets egenskaper påverkas
- de ska kunna användas till att få fram kvantitativa bindningsdata


I cellerna pågår ständigt processer där proteiner och andra molekyler påverkar varandra. Vissa molekyler dras till varandra och släsus ihop. På så sätt kan olika funktioner i cellen sättas igång eller stängas av. Att dessa interaktioner mellan proteinerna sker på rätt sätt är ytterst

![Diagram of a protein (oval) and a ligand (round) binding together and forming a complex.](image.png)

Figur 1. Ett protein (oval) och en ligand (rund) binder till varandra och bildar ett komplex.

När ett protein och en godtycklig molekyl – ligand – binder till varandra, se figur 1, bildas ett komplex som kommer att ha andra egenskaper än proteinerna var för sig. Denna förändring kan mätas med en mängd olika metoder.

**Kapillärelektrofores**

Svensken Arne Tiselius fann att olika proteiner rör sig i elektriska fält med olika hastighet. Han utvecklade därför en metod som kom att kallas elektrofores och fick för detta Nobelpris i kemi 1948. Tiselius metod har vidareutvecklats och idag används många olika varianter av elektrofores till olika ändamål inom forskning.


Varför ska man välja kapillärelektrofores?


**Vad har denna avhandling bidragit till?**


procedur som kan utnyttjas i praktiskt taget vilket analyslab som helst eftersom den inte kräver några specialreagens; för det andra så är det en snabb procedur; för det tredje så är syratvätten mindre arbetskrävande än att tillverka polymerbelagda kapillärer. Den största begränsningen med syratvätten är att analystiden är begränsad till vanligtvis ca 30 min. Å andra sidan så appliceras sällan analytider över 30 min.

I avhandlingsarbetet har metoder för interaktionsstudier av β₂gpl och dess ligander utvecklats och använts. Resultaten av interaktionsstudierna visade att interaktionen mellan β₂gpl och den antikoagulerande substansen heparin sker med elektrostatiska krafter och att den påverkas av proteinets 3D-struktur. Kvantitativa data i studierna stämmer väl överens med tidigare publicerade data, och därför anses metoderna vara fysiologiskt relevanta. Resultaten har bidragit med ny kunskap om β₂gpl’s funktion, som kan bidra till att kartlägga de biologiska mekanismerna bakom t ex APS och därmed i förlängningen leda till nya behandlingsmetoder.
Method development for affinity capillary electrophoresis of \( \beta_2 \)-glycoprotein I and biological ligands

The final goal of this study is to establish a microscale analysis method that allows solution phase characterization of interactions between \( \beta_2 \)-glycoprotein I (\( \beta_2 \text{gpI} \)) and some of its ligands. Human \( \beta_2 \text{gpI} \) is a phospholipid- and heparin-binding plasma glycoprotein. The physiological role of the protein in normal blood coagulation is not entirely known, nor is its role in autoimmune diseases characterized by blood clotting disturbances (thrombosis). Quantitative binding data of \( \beta_2 \text{gpI} \) interactions with some of its ligands may help elucidating the mechanisms behind these diseases and in the development of new approaches for diagnostics, prevention, and therapy.

In this thesis, capillary electrophoresis (CE) was used as methodological platform for the interaction studies. The analysis of peptides and proteins by CE is desirable due to low sample consumption, possibilities for non-denaturing and highly effective separations. The first objective of this thesis was to find an approach to prevent charge dependent adsorption of \( \beta_2 \text{gpI} \) to the inner surface of the capillaries. Analyte adsorption at the negatively charged inner surface of fused silica capillaries is detrimental to interaction analyses. This phenomenon is especially pronounced in the analysis of basic proteins and proteins containing exposed positively charged domains, such as \( \beta_2 \text{gpI} \). A new strategy to suppress these solute-wall interactions was devised, investigated and optimized. This strategy exploits the pH hysteresis behavior of fused silica surfaces, by simply performing an acidic pretreatment of the capillary. The results in this thesis show that the acidic pretreatment efficiently prevents protein adsorption.