Title
Capillary electrophoresis for blood doping analysis: modification of the electroosmotic flow for the separation and detection of blood doping agents in human whole blood

Permalink
https://escholarship.org/uc/item/8mh3r9nd

Author
Vydha, Srilatha

Publication Date
2015

Peer reviewed|Thesis/dissertation
Capillary electrophoresis for blood doping analysis: modification of the electroosmotic flow for the separation and detection of blood doping agents in human whole blood.

A dissertation submitted in partial satisfaction of the requirements for the degree of

Doctor of Philosophy

in

Chemistry

by

Srilatha Vydha

Committee in charge:

University of California, San Diego

Professor John Crowell
Professor Ulrich Muller

San Diego State University

Professor Christopher R Harrison, Chair
Professor Forest Rohwer
Professor Diane K Smith

2015
The Dissertation of Srilatha Vydha is approved, and it is acceptable in quality and form for publication on microfilm and electronically:

Chair

University of California, San Diego
San Diego State University
2015
DEDICATION

To my family. Thank you for your patience and support.
TABLE OF CONTENTS

Signature page .......................................................................................................................... iii

Dedication ................................................................................................................................ iv

Table of contents ...................................................................................................................... v

List of Abbreviations ................................................................................................................. x

List of symbols ........................................................................................................................ xii

List of Figures .......................................................................................................................... xiii

List of Tables ............................................................................................................................ xvii

Acknowledgements ................................................................................................................. xviii

Vita ........................................................................................................................................... xxi

Abstract of the Dissertation ...................................................................................................... xxiii

Chapter 1 .................................................................................................................................. 1

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

History ..................................................................................................................................... 3

Fundamentals of CE ................................................................................................................. 6

Electroosmotic flow ................................................................................................................... 8

Electrophoretic mobility of the ions ......................................................................................... 10

EOF control and modification of the capillary surface ......................................................... 13

UV-VIS detection in CE ....................................................................................................... 18

Online sample stacking in CZE ............................................................................................. 20

References ................................................................................................................................. 27

Chapter 2 ................................................................................................................................ 31

Abstract .................................................................................................................................. 31
Introduction .................................................................................................................................................. 32
Experimental .................................................................................................................................................. 37
Apparatus ....................................................................................................................................................... 37
Chemicals ....................................................................................................................................................... 38
HBOCs preparation and optimization ........................................................................................................ 39
Polymerization of lyophilized hemoglobin .............................................................................................. 39
Polymerization of hemoglobin from lysed RBCs ...................................................................................... 40
Bradford protein assay .................................................................................................................................. 42
Standard and test solutions .......................................................................................................................... 44
Dye reagent ..................................................................................................................................................... 44
Assay ............................................................................................................................................................... 44
Phospholipid solution preparation .............................................................................................................. 45
Capillary coating and stability .................................................................................................................... 47
Results and Discussion ............................................................................................................................. 52
Sample preparation ....................................................................................................................................... 52
Separation optimization .............................................................................................................................. 57
Hp interference .............................................................................................................................................. 60
Sample volume influence ........................................................................................................................... 63
Limit of detection .......................................................................................................................................... 65
Animal testing (In Vivo testing of HBOCs) .................................................................................................. 68
Is the CE separation necessary for the detection of ............................................................................... 71
perfluorocarbon emulsions in the whole blood sample? .......................................................................... 71
Acknowledgements ...................................................................................................................................... 73
Further improvements of CZE HBOCs separation .............................................. 152
Further improvements of CZE-LVSS separation ................................................. 153
Further improvements of autologous blood doping test...................................... 153
# LIST OF ABBREVIATIONS

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>AOCs</td>
<td>Artificial oxygen carriers</td>
</tr>
<tr>
<td>CE</td>
<td>Capillary Electrophoresis</td>
</tr>
<tr>
<td>CZE</td>
<td>Capillary Zone Electrophoresis</td>
</tr>
<tr>
<td>CGE</td>
<td>Capillary Gel Electrophoresis</td>
</tr>
<tr>
<td>CIEF</td>
<td>Capillary Isoelectric Focusing</td>
</tr>
<tr>
<td>CITP</td>
<td>Capillary Isotachophoresis</td>
</tr>
<tr>
<td>CPD</td>
<td>Citrate phosphate dextrose</td>
</tr>
<tr>
<td>CAPS</td>
<td>3-(Cyclohexylamino)-1-propanesulfonic acid</td>
</tr>
<tr>
<td>DAD</td>
<td>Photodiode array detector</td>
</tr>
<tr>
<td>DDAB</td>
<td>Didodecyldimethylammonium bromide</td>
</tr>
<tr>
<td>DLPC</td>
<td>1, 2-dilauroyl-sn-phosphatidylcholine</td>
</tr>
<tr>
<td>EOF</td>
<td>Electroosmotic flow</td>
</tr>
<tr>
<td>GC</td>
<td>Gas chromatography</td>
</tr>
<tr>
<td>FASS</td>
<td>Field amplified sample stacking</td>
</tr>
<tr>
<td>FASI</td>
<td>Field amplified sample injection</td>
</tr>
<tr>
<td>Hb</td>
<td>Hemoglobin</td>
</tr>
<tr>
<td>HBOCs</td>
<td>Hemoglobin based oxygen carriers</td>
</tr>
<tr>
<td>ID</td>
<td>Inner diameter</td>
</tr>
<tr>
<td>LIF</td>
<td>Laser induced fluorescence</td>
</tr>
<tr>
<td>LVSS</td>
<td>Large Volume Sample Stacking</td>
</tr>
<tr>
<td>MWCO</td>
<td>Molecular weight cut off</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
</tr>
<tr>
<td>--------------</td>
<td>-------------</td>
</tr>
<tr>
<td>MEKC</td>
<td>Micellar electrokinetic chromatography</td>
</tr>
<tr>
<td>MS</td>
<td>Mass Spectrometry</td>
</tr>
<tr>
<td>MEEKC</td>
<td>Microemulsion electrokinetic chromatography</td>
</tr>
<tr>
<td>OD</td>
<td>Outer diameter</td>
</tr>
<tr>
<td>PFCEs</td>
<td>Perfluorocarbon emulsions</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate Buffered Saline</td>
</tr>
<tr>
<td>PIPES</td>
<td>Piperazine-N-N'-bis (2-ethanesulfonic acid)</td>
</tr>
<tr>
<td>pI</td>
<td>Isoelectric point</td>
</tr>
<tr>
<td>pH</td>
<td>Negative logarithm of the hydrogen ion concentration</td>
</tr>
<tr>
<td>pKa</td>
<td>Negative logarithm of the dissociation constant</td>
</tr>
<tr>
<td>Rcf</td>
<td>Relative centrifugal force</td>
</tr>
<tr>
<td>RPLC</td>
<td>Reverse phase liquid Chromatography</td>
</tr>
<tr>
<td>RBCs</td>
<td>Red blood cells</td>
</tr>
<tr>
<td>Tris</td>
<td>Tris (hydroxymethyl) aminomethane</td>
</tr>
<tr>
<td>UV-Vis</td>
<td>Ultraviolet-Visible</td>
</tr>
<tr>
<td>WADA</td>
<td>World anti-doping agency</td>
</tr>
</tbody>
</table>
LIST OF SYMBOLS

AU        Ampere units
µA       microamperes
mM       millimolar
M        Molar
kDa      Kilodaltons
µm      Micrometers
µL      Microliters
kV      Kilovolts
nL      nanoliters
nm      nanometers
mΩ      megaohms
Hz      Hertz
°C       degree Celsius
LIST OF FIGURES

Figure 1. Capillary electrophoresis block diagram................................................... 7
Figure 2. EOF in a fused silica capillary filled with aqueous buffer pH >3. .......... 9
Figure 3. Comparison of the flow profile: EOF vs pressure driven flow.......... 10
Figure 4. Influence of the EOF on the net mobility of the ions......................... 13
Figure 5. Scheme for the interaction of proteins with the silica surface.......... 15
Figure 6. Hb crosslinking with the glutaraldehyde at the neutral pH.............. 42
Figure 7. Bradford assay: determination of the amount of HBOCs................. 45
Figure 8. DLPC solution preparation: wand sonication is the modified step.... 46
Figure 9. Determination of the magnitude of the suppressed EOF [35]........... 49
Figure 10. Electropherogram showing the detection of neutral marker peaks... 50
Figure 11. Separation of Bovine poly Hb in bovine serum............................... 52
Figure 12. Separation of Poly Hb from sheep’s blood in bovine serum........... 55
Figure 13. CE-UV electropherograms of 10 μL whole blood samples doped with
220 μg of HBOCs separated with various pH buffers................................. 60
Figure 14. CE-UV electropherograms of Hp interference............................... 62
Figure 15. Electropherograms were obtained at wavelength 214 nm from the in
vitro doped polymerized sheep’s Hb sample............................................. 63
Figure 16. CE-UV electropherograms of increasing volumes of whole blood
sample (10–50 μL) each doped with 220 μg of HBOCs............................ 65
Figure 17. CE-UV electropherograms of increasing amounts of HBOC added to
10 μL of whole blood.............................................................................. 67
Figure 18. CE-UV electropherograms of In vivo doping with the HBOCs.

Figure 19. LVSS in DLPC coating with reversed EOF.

Figure 20. Comparison of separation and detection of proteins in regular versus LVSS.

Figure 21. Repeatability of separation and detection of proteins in LVSS.

Figure 22. Comparison of protein mixture separation with change in the applied voltage.

Figure 23. Comparison of protein mixture separation in various run buffer concentrations.

Figure 24. Comparison of protein mixture separation in various sample buffer concentrations.

Figure 25. Effect of capillary length on the stacking.

Figure 26. Comparison of separation and detection of standard myoglobin peptides.

Figure 27. Comparison of output current generated during the separation and detection of myoglobin peptides.

Figure 28. Single step LVSS without sample matrix in DLPC coating with reversed EOF.

Figure 29. Comparison of separation and detection of proteins in single step stacking.

Figure 30. Repeatability of single step stacking: separation and detection of proteins.
Figure 31. Double step LVSS without sample matrix in DLPC coating with reversed EOF. ................................................................. 108

Figure 32. Comparison of separation and detection of proteins in double step stacking versus the normal CZE injection................................. 109

Figure 33. Repeatability of double step stacking.................................................. 110

Figure 34. Non-enzymatic protein glycation reaction........................................... 120

Figure 35. Electropherogram shows the separation of the unglycated and the glycated Lysozyme. ................................................................. 127

Figure 36. Electropherogram shows the repeatability of the separation of unglycated (blank) and the glycated Lysozyme (sample) ................. 128

Figure 37. Protein glycation reaction and the cis-diol interactions...................... 130

Figure 38. Electropherogram shows the separation of the unglycated and the glycated Ribonuclease A................................................................. 131

Figure 39. Electropherogram shows reproducible separation of the unglycated (blank) and the glycated Ribonuclease A. .............................................. 132

Figure 40. Electropherogram shows the separation of the unglycated and the glycated Cytochrome c................................................................. 133

Figure 41. Electropherogram shows the reproducible separation of the unglycated (blank) and the glycated Cytochrome c (sample) ................. 134

Figure 42. Electropherograms shows the separation of the 3-hour trypsin digested solution of intact fresh RBCs...................................................... 135

Figure 43. Electropherograms shows the separation of the Ribonuclease A glycated and native protein......................................................... 137
Figure 44. Electropherograms shows the separation of the overnight trypsin digested solution of RBCs membrane ghosts.................................................. 138

Figure 45. Electropherograms shows the separation of the overnight trypsin digested solution of RBCs membrane ghosts.................................................. 140

Figure 46. Electropherograms shows the separation of the overnight trypsin digested solution of RBCs membrane ghosts.................................................. 141

Figure 47. Electropherograms shows the separation of the overnight trypsin digested solution of RBCs membrane ghosts with different injection times...... 142

Figure 48. Electropherograms shows the separation of the in-vitro glycated human hemoglobin. .......................................................... 143

Figure 49. Electropherograms shows the reproducibility of separation of the in-vitro glycated human hemoglobin. .................................................. 144
LIST OF TABLES

Table 1. Comparison of electrokinetic and hydrodynamic injections: .................. 22
Table 2. Effect of pH: .......................................................................................... 59
Table 3. LVSS signal enhancement comparison: ...................................................... 92
ACKNOWLEDGEMENTS

First and foremost, I would like to thank Professor Christopher R. Harrison for being a great mentor throughout my graduate studies at San Diego State University. When I was ambitious to continue my doctoral studies, he kept his confidence in my capabilities, and gave me an opportunity to do what I liked most. His continuous encouragement and support has been a never-ending source of inspiration during the course of my work. I am grateful to say Dr. Harrison has had a significant impact on my life and career. I am also grateful for his thoughtful guidance and assistance especially whenever the experiments were not at all successful and whenever the instruments needed a repair. I would like to thank my doctoral committee members, Professors John Crowell, Diane Smith, Forest Rohwer and Ulrich Muller for their help over the years. I would like to thank them for discussing the research progress and filling the year end evaluation, I am grateful to know and to learn about science from so many great scientists.

I would like to thank Harrison’s lab collaborator Dr. Gregory Kalyuzhny for providing several batches of Cadmium selenide nanoparticles samples with which I started my research using Capillary Electrophoresis. I would like to thank Professor Dale Chatfield and Dr. Kalyuzhny for providing me the recommendation letters required for the enrollment in the JDP program. I would also like to thank amazing other SDSU faculty and fellow graduate students, without their help I wouldn’t have come this far. I would like to thank Harrison’s lab group members past and present graduate and undergraduate students for convenient CE
scheduling and making my time at SDSU a more enjoyable experience. When I helped them improve their conceptual or technical skill, I learned something new which will be of great help in my future scientific career. It has been a privilege to work and learn from great scientists and I truly appreciate all your help.

I would like to thank undergraduate teaching lab coordinator Kathleen McNamara, Gayle Anderson (SDSU Chemistry administration), other teaching coordinators and teaching faculty for their help in making my teaching assistant (TA) assignments a success. I would like to thank the chemistry undergraduate lab storeroom personnel Mark Gelle and Marcia Godinez for helping me with the lab checkout when I was not able to make it. I thank you all for your support and friendship over the years.

I would like to thank my husband, Arugadoss, for encouraging me to pursue doctoral education and taking care of our children Akhilesh and newborn Harini in my absence without failing patience, I cannot thank him enough for being such a wonderful husband. I feel incredibly grateful for my parents and grandparents, who raised me with love and I would like to thank my siblings for helping me whenever I needed them the most.

I would like to thank SDSU International student center for helping me with my immigration documentation and for helping me purchase student health insurance for the family.

I would like to acknowledge the co-author of the published paper in which I was the primary researcher and contributing author. Chapter 2, contains material
that has been published in Electrophoresis: Christopher R Harrison and Srilatha Vydha, volume 33, issue 7, and pages 1087-1094, April 2012.

The contents in Chapter 3 and Chapter 4 are being prepared for the publication, I am the primary researcher for the data presented and Dr. Christopher Harrison is the contributing author.
VITA

1996 Bachelor of Science, Chemistry and Biology, Osmania University, India.

2000 Master of Science, Medicinal chemistry, Osmania University, India.

2000-2004 Research associate, Analytical division, Aurobindo Pharmaceuticals, India.

2006-2008 Teaching assistant, Indiana University, Bloomington Indiana.

2008-2014 Teaching assistant, Graduate Research associate, San Diego State University.

2015 Doctor of Philosophy, Chemistry, University of California, San Diego, San Diego State University

Publications

Christopher R Harrison and Srilatha Vydha, “Capillary electrophoretic analysis of whole blood samples for hemoglobin-based oxygen carriers without the use of immunoprecipitation”. *Electrophoresis* volume 33, issue 7, and pages 1087-1094, April 2012.

Srilatha Vydha, Christopher R Harrison; “Large volume sample stacking in the zwitterionic phospholipid coated capillary for the separation of proteins”. *In Preparation*.

Srilatha Vydha, Christopher R Harrison; “Autologous blood doping test method development for the analysis of the erythrocyte surface proteins by capillary electrophoresis”. *In Preparation*.

Srilatha Vydha, Ashley Morris, Christopher R Harrison; “Novel capillary coatings for EOF control for protein and ion separations”. Review paper, *In Preparation*.

Oral Presentations

Srilatha Vydha and Christopher R Harrison, “Doping Test by Capillary Zone Electrophoresis” 4th Annual Student Research Symposium, San Diego State University, March 5-6, 2011.

Srilatha Vydha and Christopher R Harrison, “Large volume sample stacking (LVSS) in the zwitterionic phospholipid coated capillary for the separation of proteins”. 7th Annual Student Research Symposium, San Diego State University, March 7-8, 2014.

Poster Presentations


Srilatha Vydha and Christopher R Harrison, “Rapid CZE method to detect HBOCs in the human whole blood”. ACS spring national meeting, March 25, 2012.

FIELDS OF STUDY

Major Field: Chemistry (Bioanalytical)

Studies in Electrophoretic separations
Professor Christopher R Harrison
ABSTRACT OF THE DISSERTATION

Capillary electrophoresis for blood doping analysis: modification of the electroosmotic flow for the separation and detection of blood doping agents in human whole blood.

by

Srilatha Vydha

Doctor of Philosophy in Chemistry

University of California San Diego, 2015
San Diego State University, 2015

Professor Christopher R Harrison, Chair

Blood doping is an illegal method, prohibited by World anti-doping agency to prevent the athletes increase their oxygen carrying capacity during the professional sports. The rapid detection of the dopants in the complex human whole blood sample is a difficult task and requires high throughput doping detection methods. The main focus of our work includes the separation and detection of artificial oxygen carriers such as hemoglobin based oxygen carriers (HBOCs) and perfluorocarbon emulsions; autologous blood transfusion markers present in the whole blood by using the capillary electrophoresis (CE).
Chapter 2 describes the preparation of HBOCs, sample pretreatment and capillary zone electrophoresis (CZE) separation method development. The phospholipid bilayer capillary coating is important in this method to suppress the electroosmotic flow and prevent the protein adsorption. The molecular weight cut-off filter purified and concentrated the HBOCs which were detected with a sensitivity below the doping amount. This method is much more simplified and rapid than the methods reported before.

The CE UV-Vis on-column detection is associated with the difficulty of poor sensitivity because of the small path length. To overcome this difficulty, an online sample stacking method has been developed. Chapter 3 describes the development and optimization of large volume sample stacking (LVSS) method with the same conditions as in HBOCs-CZE method. Simultaneous application of voltage and back pressure to remove the sample matrix; double step stacking are the crucial steps in this method. The detection sensitivity significantly improved compared to CZE (more than 100 fold) when a standard protein mixture was tested.

Autologous blood transfusion is a potential blood doping method carried out by transfusing the athlete’s own stored blood. Because there is no direct detection method, we investigated the applicability of CZE for the detection of the transfused blood marker glycated proteins present in the doped blood which is described in chapter 4. The standard glycated proteins were successfully separated and detected. A significant signal enhancement has been observed with the trypsin digests of RBCs membrane ghosts in the normal CZE-LVSS. However, because
of the presence of intrinsic glycated proteins in normal individuals this method will not be a direct doping test.
CHAPTER 1

Introduction

The world anti-doping agency (WADA) is an independent international agency established on November 10, 1999 to combat doping in sports after the report of several cases in which variety of doping materials were used. WADA sets the unified standards for anti-doping work and coordinates the effort of sports organizations and public authorities [1]. Blood doping effects were seen in the sports for several decades, first one was reported in 1945 by Pace group [2]. Blood doping as defined by WADA is a prohibited process of administration of blood substitutes or employing transfusion methods for non-medical reasons through which the athletes increase their oxygen carrying capacity. Because blood doping increases the amount of oxygen delivering substances in the blood, it allows efficient transport of oxygen from the lungs to the peripheral muscles and improves the athletic performance in endurance sports [3-5].

Detection of these substances in the athlete’s blood sample prior to a sport not only ensures the fairness of the competition but also protects the athletes from the adverse side effects of the doping substances. With this goal WADA set out detecting the blood manipulations by a test known as Athletes’ biological passport, which is based on the personalized monitoring of biomarkers indicative of doping [6]. It is an indirect test and wouldn’t conclude the presence of any single substance and it necessitates the establishment of blood profile of each athlete and compare
it with the profile during sport. This approach may be useful for famous athletes whose blood profile has been tested already but it is a difficult process in a big sport event such as international football where high throughput analysis is necessary and direct methods of detection will be beneficial [4, 7]. Therefore, the goal of this dissertation is to develop direct methods to detect the blood doping substances which has the potential for the high throughput analysis.

Because the blood sample is complex, detection of doping substances is a challenging task [8], therefore, prior to the detection it is necessary to separate the blood doping substances from the other interfering blood components present in the sample. The main interfering blood components are plasma, plasma proteins, white blood cells, membranes and the red blood cell proteins which needs to be separated from the doping substance. The extracellular blood component and the red blood cell membranes present in the sample can be separated by simple centrifugation process but the intracellular proteins requires a protein separation method. Furthermore, the success of detection and confirmation of the presence of the dopant in a given sample depends on the development of appropriate sample preparation procedure and a suitable separation method. Reversed phase high performance liquid chromatography (RPLC) with mass spectrometry (MS) detection is a popular choice for the intact protein separation and analysis (top-down proteomics), however, RPLC MS is more successful for peptide analysis than the proteins. In RPLC the proteins with molecular weights more than 35 kDa are broad peaks, with longer elution time and their detection requires expensive high resolution mass spectrometers [9, 10].
Capillary electrophoresis (CE) [9] has become an efficient method of separation because of its simple instrumentation, ease of handling it, lower sample volume requirement and shorter analysis time when compared to other separation methods such as high performance liquid chromatography (RPLC for proteins) and slab gel electrophoresis. Even though MS is a highly sensitive detection system, the instrument and its maintenance is expensive therefore mostly not available in the academic labs. If they are available the coupling of the CE and the MS instruments is a complicated process. CE equipped with the ultraviolet (UV) detection is the simplest alternative method for the blood doping analysis [11], successful methods with the UV can be applied to the sensitive detection should the necessity arise. The main aim of the work presented in this dissertation is solving two problems associated with the detection of blood doping using the whole blood, the first is the blood sample preparation and the next is the CE-UV separation method development. Development of the CE-UV method involves prevention of the protein adsorption onto the capillary surface, modification of the electroosmotic flow and optimization of the separation.

History

The process of movement of the charged molecules towards the opposite electrode in the influence of the uniform electric field was discovered and characterized by Kohlrauch through the Kohlrauch’s law of independent migration of ions. The applicability of this independent migration of ions for the separation of compounds of interest became evident when it was initially developed by Arne
Tiselius [12] in 1937 as the moving boundary electrophoresis, his experiments on the separation of serum proteins in the free solution has led to the observation that the proteins in a sample migrated in different boundaries and direction based on their charge and electrophoretic mobility. The main problem that Tiselius encountered with his U- tube experimental setup was the band broadening because of diffusion and thermal convection, convection is the movement of parts of solution relative to other parts due to the heat generated. In 1940s and 1950s, zone electrophoresis was done on anti-convective gels and filter papers and became useful for separating the biological compounds. In 1966 agar gels were first used to separate virus DNA from host cell DNA by Thorne group [13], however, there are limitations associated such as operates at relatively low applied voltage, therefore, the separations are not fast when compared to CE. In gel electrophoresis, the gel preparation and analysis is labor intensive, the samples needs to be stained and it is not quantitative because the detection depends on the extent at which the sample associates with the staining dye.

To minimize the band broadening effects, Hjertan in 1967 described a free zone electrophoresis and separated the biological compounds and inorganic ions in a horizontal, 1- 3 mm rotating quartz tube using the UV detection [14] [15, 16]. In this the rotation of the tube minimized the heating effects. The electrophoresis in the tubes became popular when Jorgensen and Lucaks developed free zone electrophoresis in 1981 in the glass capillaries to reduce the convective flow [17]. After the introduction of fused silica capillaries for the gas chromatography, capillary zone electrophoresis (CZE) method was described by Jorgensen and
Lucaks [18]. They demonstrated that the use of smaller diameter capillaries efficiently transferred the heat generated inside the capillary and facilitated the use of high voltage, which gave them the separations with high resolution in rapid analysis time. In 1984 Jorgensen and Walbroehl [19] developed a fixed wavelength on column UV absorbance detector for the capillary zone electrophoresis with small illumination slit. This development provided accurate migration time of the analytes and the concentration of the zone, furthermore, detection with the on-column UV solved the problem of sample derivatization with fluorescent tags associated with the on column fluorescent detectors. In the meantime, fully automated commercial CE instruments were available in the market in late 1980s, and led to the development of wide range of applications and progress in various fields of science. The main advantage of CZE is the separation of charged molecules, however, the limitation of CZE is not separating the neutral species. This limitation was overcome by the micellar electrokinetic chromatography (MEKC) introduced by Terabe group in 1984 [20, 21] to separate the neutral species, which is another mode of CE that became popular with several applications in various fields, for example separation of enantiomers using MEKC [21, 22]. The main limitation of MEKC is that it is applicable only to the small molecules or the ions that can partition into the micelles, it is not applicable to the macromolecules like the proteins which bind to the single micelles like sodium dodecyl sulphate.

The other main advantage of CE over other separation methods is the use of different modes of CE other than CZE that could be used to improve the
sensitivity and selectivity of the separations especially for the proteins. The other common CE modes that were developed and used are the capillary gel electrophoresis (CGE) initially introduced by Hjerten in 1980s and later developed in 1999 [23], capillary isoeletric focusing (CIEF) [24] also developed in 1980s and capillary isotachophoresis (CITP) developed 1970-1990 [25-27]. Moreover, significant advances in the CE applications have been achieved in the last three decades in combination with the development of capillary surface modification methods, online sample stacking techniques and CE equipped with sensitive detection systems such as laser induced fluorescence and mass spectrometer. In the recent years, electrophoresis in microfluidic devices is the major area of research, which could be the future of electrophoretic separations. In the present studies, we set out to explore the applicability of CE-UV in its simplest mode CZE, for the blood doping analysis with the hope of developing straightforward and simplistic methods to detect the dopants in a microliter volume of blood sample.

Fundamentals of CE

The separations in CE takes place when the solutes move with different velocities based on their charge to size ratios in an electric field. The separations are carried out typically in the 25-75 µm inner diameter (id), 30 - 60 cm length fused silica capillaries commonly filled with the run buffer. Because of the high electrical resistance of the capillaries, the separations can be performed with high applied voltage (upto 30 kV). The capillaries efficiently dissipates the heat because of its large surface area-to-volume ratio, therefore, the detrimental effects of heat generation are minimized and performing the separations with high applied voltage
reduces the run time. Figure 1 is the block diagram of typical CE apparatus, both the ends of the capillary are submerged in the run buffer along with platinum electrodes which are connected to a high voltage power supply. In majority of CE separations, the sample is injected at the anode end of the column and when a voltage is applied the analytes migrate toward the cathode end and elute after their detection. When the voltage is applied across the capillary it generates the electroosmotic flow (EOF) inside the capillary. It is important to have an understanding of the EOF and electrophoretic mobility of the ions in the CE because these are the important related electrokinetic processes responsible for the ion transport toward the detector and separation inside the capillary.
Electroosmotic flow

The phenomenon of EOF is the bulk flow of the liquid and takes place as a consequence of the surface charge on the capillary interior wall. The most commonly used fused silica capillaries have ionizable acidic silanol (SiO-H) groups with the isoelectric point of about 2, these silanols deprotonate (SiO⁻) and acquire the negative charge when the capillary is filled with the run buffers pH greater than 3. Hence, the degree of ionization of these silanols is controlled by the run buffer pH. As shown in figure 2, the counterions (cations) present in the run buffer build up near the surface to maintain charge balance, form the double-layer and create a potential difference very close to the capillary wall which is known as the zeta potential, the zeta potential is directly proportional to the EOF. The double layer is made up of the fixed layer, which consists of stationary cations held tightly to the negatively charged capillary wall, and the diffused layer has mobile cations. When a voltage is applied across the capillary, the hydrated mobile cations in the diffused layer move towards the cathode dragging the bulk liquid with equal velocity. This bulk liquid movement is the responsible factor for the net migration velocity of the neutral species and the ions regardless of their charge through the capillary toward the cathode. Thus, the generation of the EOF takes place and allows the separation of cations, anions and neutral species in a single run when the EOF exceeds the individual mobilities of the ions. The EOF depends on the buffer composition, pH and any additives as explained by the Smoluchoski’s equation (1):
\[ \mu_{eof} = \frac{\varepsilon \zeta}{\eta} \]  

Where, \( \mu_{eof} \) is the electroosmotic flow (electroosmotic mobility), \( \varepsilon \) is the dielectric constant, \( \zeta \) is the zeta potential or the charge on the capillary surface and \( \eta \) is the viscosity of the solution filling the capillary. The magnitude of the EOF or the bulk liquid movement is given in terms of its velocity as described by Jorgensen and Lucaks [28] in the following equation 2:

\[ v_{eof} = \mu_{eof} \times E \]  

Where \( E \) electric field, it is equal to the voltage applied across the length of the capillary (\( E=V/L \)), \( \mu_{eof} \) is the EOF mobility.

Figure 2. EOF in a fused silica capillary filled with aqueous buffer pH >3.
The unique and important aspect of the EOF inside the narrow inner diameter capillaries is the generation of the flat liquid flow profile also known as laminar flow. Because there is uniform drag of the bulk solution along the entire diameter of the capillary and a very small drop in the velocity of the EOF at the capillary walls as shown in figure 3a, the CE separations are more efficient with relatively less band broadening and high peak capacity than the pressure driven systems like HPLC. In HPLC, the parabolic flow is observed where there is a decrease in the pressure along the walls of the column with decrease in the flow velocity and there is highest flow velocity in the center as shown in figure 3b. As a result, in the parabolic flow, the band broadening is increased and the peak capacity reduced.

Figure 3. Comparison of the flow profile: EOF vs pressure driven flow.

**Electrophoretic mobility of the ions**

The separation of the analyte ions in the CE takes place because of the difference in their electrophoretic mobilities in an electric field. The migration
velocity $v_{ep}$ of the ions through the bulk solution is given in the equation 3, where $\mu_{ep}$ the electrophoretic mobility of the analyte ions in the electric field $E$.

$$v_{ep} = \mu_{ep} \times E \quad (3)$$

The uniform motion of an ion is a result of the balanced forces experienced by the ion inside the solution. The force due to the electric field ($qE$) accelerates the movement of the ion toward the opposite electrode and the other force that balances this electric force is the frictional force ($6\pi\eta r v_{ep}$) that retards the movement of the ion, therefore a steady state is achieved. The balance of the forces at steady state for a spherical ion is given by the following equation:

$$qE = 6\pi\eta r v_{ep} \quad (4)$$

Where $q$ is the charge of the ion, $E$ is the electric field, $\eta$ is the viscosity, $r$ is the Stoke’s radius of the ion and $v_{ep}$ is the migration velocity of the ion. The electrophoretic mobility can be calculated by substituting the equation 3 in equation 4 and solving for $\mu_{ep}$:

$$\mu_{ep} = \frac{q}{6\pi\eta r} \quad (5)$$
In equation 5 the charge of the ion, viscosity and radius of the ion are not directly measurable properties, therefore the electrophoretic mobility of an ion is measured using the experimental data by using the following conversion:

\[
\mu_{ep} = \frac{v_{ep}}{E} = \frac{L_{Det}}{t_m} \times \frac{1}{E} = \frac{L_{Det}}{t_m} \times \frac{1}{V/L_{tot}} = \frac{L_{Det} \times L_{tot}}{V \times t_m}
\]  

(6)

Where \( L_{Det} \) and \( L_{tot} \) are length to the detector and the total length of the capillary, \( V \) is the applied voltage and \( t_m \) is the migration time. This equation allows the determination of the migration time of the analyte ion when the applied voltage is constant, \( L_{Det} \) and \( L_{tot} \) are known.

It is beneficial to predict the net mobility (apparent mobility) of the ions before the method development [29]. As shown in the figure 4a, in the normal EOF (cathodal) the cations move faster than the neutral molecules followed by the anions. The migration of the anions toward the detector takes place only when the EOF is faster than the mobility of the anions. Figure 4b shows the net mobility of the ions with the effect of magnitude and direction of the EOF on the mobility. It can be seen that the net mobility of the cations is greater because these are migrating in the same direction as the EOF, the neutral molecules migrate with the EOF and anions migration is slow because their electrophoretic mobility is opposite in direction of the EOF. Thus rapid separations are obtained when the ions move with EOF.
In an unmodified capillary, the EOF is faster giving rapid separations and some of the bigger anions move toward the detector in the cathodic EOF. However, the small fast moving anions with the net mobility greater than or close to the EOF migrate in opposite direction to the EOF, therefore, the small ions go undetected. In these conditions, reversal of EOF will be necessary which is done by using the capillary coatings. In the reversed EOF the anions migrate in the same direction as the EOF and as a result detected rapidly. This is an example where the modification of capillary surface to alter the EOF is necessary, in addition, it is necessary to modify the EOF when rapid analysis time is required or where reproducibility and resolution needs to be improved. The resolution in CZE is given
by the following equation, where $R$ is the resolution, $N$ is the efficiency (theoretical plates), $\Delta \mu$ is the difference in the mobility of the ions, $\bar{\mu}$ is the mean apparent mobility of the ions. Thus the resolution improves when there is decrease in the mean apparent mobility of the ions which is achieved by the control of the EOF.

$$R = \frac{1}{4} \sqrt[N]{\frac{\Delta \mu}{\bar{\mu}}} \quad (7)$$

Another important factor that necessitates the modification of the capillary surface is the analyte and capillary wall interaction. In this dissertation, it is important to introduce different types of the capillary coatings because most of the analytes are proteins and prevention of protein adsorption onto the capillary surface is necessary along with the EOF control to develop the separation method and improve the separations. Protein adsorption is a major problem in CE separations, the polycationic proteins adsorb onto the negatively charged silica surface [30], as described in Lucy group review [31] electrostatic, hydrophobic interactions and protein structural changes are responsible for the protein adsorption as shown in figure 5 [32].
Figure 5. Scheme for the interaction of proteins with the silica surface.

The longer the proteins stays at the surface of the capillary, the adsorption of the protein changes from loosely bound to reversibly bound or permanently bound to the surface wall. The most important reason for the protein-capillary wall electrostatic interactions is the overall charge of the protein based on its isoelectric point ($p_I$). Therefore, prevention of the protein adsorption is being studied extensively and is an active area of research in the past three decades, it has been demonstrated previously that peak broadening, poor separation efficiency and analyte loss occurs as a result of capillary wall and protein interactions [33, 34].

The protein $p_I$ is the pH at which the overall charge on the protein is zero. The isoelectric point of proteins depends on the charged aminoacids such as the glutamate (δ-carboxyl group), aspartate (ß-carboxyl group), cysteine (thiol group), tyrosine (phenol group), histidine (imidazole side chains), lysine (ε-ammonium group) and arginine (guanidinium group) and the protein amino or carboxy terminal
groups. Moreover, the pI is dependent on the pH of the buffer solution, therefore by adjusting the pH of the buffer solution the net charge on the protein can be changed and the protein pI at a particular pH can be determined by using the Henderson–Hasselbach equation [35]. In addition to this simple method, several online methods have been reported to determine the pI of complex and unknown proteins [35]. The knowledge of protein pI is of great importance in CE techniques because it allows proper device of experimental environment before the experiment starts. Generally, the proteins are positively charged if the buffer pH is below the pI and on the other hand, negatively charged if the pH is above the pI of the proteins. During electrophoresis, the direction of the proteins migration depends on their charge. If buffer pH is higher than protein pI, the proteins will migrate to the anode and if the buffer pH is lower than the pI, the proteins are positively charged they will go to the cathode. In CE, the capillary and positive proteins electrostatic interactions alters capillary surface permanently and results in failed separations. Thus prevention of protein adsorption became an important area of research.

To this end, a number of run buffer modifications have been introduced before in order to reduce protein adsorption onto the capillary surface. Some of the most common approaches are adjusting the run buffer pH [36] and using high salt concentrations [37]. When extremely basic or acidic run buffers are used these buffers may denature the proteins losing the native protein structure which could result in protein-protein interactions and become detrimental for the separations. On the other hand, the main drawback of using high salt concentration in the run
buffer is the high conductivity of the run buffer which results in Joule heating in turn raises the capillary temperature and alters the EOF. To overcome these effects capillary coatings have been used to modify the capillary surface, the surface modification can be permanent, semi-permanent or dynamic. Formation of dynamic, permanent or semi-permanent capillary coating depends on the type of attachment of the coating material to the capillary surface. It is preferable to use a coating that is inexpensive, easily regenerated, be functional in the broad pH range with variety of buffers and not interfere with the detection of the molecules of interest. Furthermore, hydrophilic coatings are preferred over the hydrophobic because hydrophilic coating prevents the capillary wall protein interaction and there is no hydrophobic interactions of the coating material and the proteins [38].

In the permanent coatings, the coating material is usually attached covalently to the capillary wall to shield the bulk solution from the surface. Several polymerized permanent coatings are used and reported for the prevention of protein adsorption and tuning the EOF successfully [39], however, the coating process is usually lengthy with several steps involved with poor column to column reproducibility and may be functional with small pH range [38]. In dynamic wall coatings, the coating material is present in the run buffer as the buffer additive [29] and the coating is formed as a result of the rinsing the capillary with the run buffer containing the coating material. The coating material adsorbs to the capillary wall, equilibrates with the capillary surface and alters the surface charge [29]. The coating procedure is simple and requires less time, it is regenerated by rinsing the capillary in between the runs, however, the presence of coating ions in the run
buffer result in the interaction with the analytes, or interfere with the detection of the analytes. The semi-permanent coating overcomes these drawbacks, and several studies have reported repeatable separations with this type of coatings [31, 40]. The capillary coatings used in the present studies are the semi-permanent coatings, and the coating is regenerated by rinsing the capillary before the sample injection.

UV-VIS detection in CE:

UV-Vis detection is most frequently used detection in the CE analysis showing an absorbance in the ultraviolet or visible region (200-600 nm). Proteins usually show the absorption maxima between 240-300 nm because of the presence of the aromatic amino acids tryptophan, tyrosine, phenylalanine and to a small extent, by the absorbance of cysteine disulfide bonds. Moreover, the peptide bonds in the protein chain absorbs between 180-230 nm; the protein cofactors such as copper-containing cofactors and heme groups absorb in the visible region.

The commercial CE instruments are equipped with Photodiode array detectors (DAD) which offers the advantage of detecting the entire spectrum of absorbance in the UV-Vis range instead of conventional UV-Vis detection which detects only one wavelength. CE separations are done in the narrow inner diameter (25-75 µm) capillary which efficiently reduces the thermal effects inside the capillary and analysis can be done with small volume of analytes. The main limitation of using these narrow diameter capillaries with on column UV-Vis detection is the small path length which decreases the sensitivity, nanoliter sample
volume is another factor that adds to it. Injection of large volume can increase the
detection sensitivity, however, causes band broadening and decrease in the
resolution as shown in equation 6. The efficiency in CE is given by the following
equation, where \( N \) is the number of theoretical plates, \( V \) is the applied voltage, \( \mu \)
is the apparent or the net mobility of the ion, \( D_m \) is the diffusion coefficient of the
analyte.

\[
N = \frac{\mu V}{2D_m}
\]  

(8)

UV-VIS detection is performed directly on column by burning the polyimide coating
to give a small window where the light passes. The absorbance of the analyte is
given by the following equation:

\[
A = \varepsilon cl
\]  

(9)

Where \( A \) is the absorbance, \( \varepsilon \) is the molar absorptivity, \( c \) is the concentration of
the analyte and \( l \) is the path length. Thus the absorbance is proportional to the
pathlength, therefore, the sensitivity reached with the pathlength in 25-75 µm inner
diameter capillary in CE is several fold less than that reached with the detection
cell in HPLC.

To overcome this difficulty, other popular high sensitivity detection systems
such as LIF, MS has been used but these are equipped with highly expensive
instrumentation. When compared to the UV-Vis absorbance/detection, the main
limitation of the LIF detection is that it requires sample derivatization because
relatively small number of compounds possess natural fluorescence. The main
limitation of MS detection includes incompatible capillary wall coatings and surfactants used for the CE protein/peptide separation, difficulty to interface CE and MS, and dilution of CE eluent before detection. A more cost effective way to improve the absorbance in CE is by increasing the c term in the equation 9, to that end, several offline preconcentration methods were developed and used but these methods are tedious and requires additional sample preparation steps and may result in sample loss which is critical for biological samples [41]. Alternately, hundreds of online preconcentration techniques in CE have been developed in the past two decades [42, 43] for different types of analytes.

Online sample stacking in CZE

The nanoliter sample injection volume is an advantage and a disadvantage in the CE analysis, the advantage is being able to analyse and obtain reproducible data with a low amount of given sample and not waste the precious material. The disadvantage is the poor detection sensitivity in combination with the on-column UV-Vis detection. Sample stacking is a sample preconcentration technique which improves the detection sensitivity without the loss in resolution and time, thus became a very well-known and extensively used method among the CE researchers. Sample stacking comprises of several methods in CE which are used for on-line preconcentration of diluted analytes [44-48]. During the stacking process, a large sample zone with the analytes present at low concentrations is injected then stacked into a concentrated narrow zone. The stacked analytes are then separated and individual zones are detected, thus the sample stacking provides better separation efficiency and detection sensitivity. The success of
online sample stacking methods in CE depends on two factors with which the analyte signal enhancement takes place. Firstly, the success of the stacking method depends on the resultant zone width, the narrower zones increases the peak height and the detection sensitivity of the analytes, and the second is the amount of sample that can be loaded in the given method without the band broadening.

Two types of injection methods are commonly used for the routine analysis and sample stacking in CE, hydrodynamic and electrokinetic injection. In hydrodynamic, the injection of the sample is accomplished by the application of pressure at the injection end of the capillary. The volume of sample loaded in the hydrodynamic injection is a function of the capillary dimensions, the viscosity of the buffer in the capillary, the applied pressure, and the time. This volume can be calculated using the Hagen-Poiseuille equation in which \( \Delta P \) is the pressure difference across the capillary, \( d \) is the inside diameter of the capillary, \( t \) is the time, \( \eta \) is the buffer viscosity, \( L \) is the total capillary length.

\[
\text{Volume} = \frac{\Delta P d^4 \pi t}{128 \eta c L} \tag{10}
\]

In electrokinetic injection, the injection-end reservoir is replaced with the sample vial and the voltage is applied. Usually a field strength 3 to 5 times lower than that used for separation is applied. Because the amount of the sample loaded is dependent on the electrophoretic mobility of the individual solutes, electrokinetic injection bias [49] occurs for the ionic species since the more mobile ions are loaded to a greater extent than those that are less mobile ions. When both the
injections types are compared as shown in the table 1 [50], the sensitivity is better with the electrokinetic, but other parameters such as repeatability is better in hydrodynamic. Therefore, hydrodynamic sample injection is routinely used in CE.

Table 1. Comparison of electrokinetic and hydrodynamic injections:

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Electrokinetic injection</th>
<th>Hydrodynamic injection</th>
</tr>
</thead>
<tbody>
<tr>
<td>Instrumental requirements</td>
<td>No additional apparatus required</td>
<td>Additional apparatus to produce pressure difference is needed</td>
</tr>
<tr>
<td>Selectivity of injection</td>
<td>Selective for ions</td>
<td>Non-selective</td>
</tr>
<tr>
<td>Repeatability</td>
<td>0.2–2% for migration time</td>
<td>0.1–0.5% for migration time</td>
</tr>
<tr>
<td></td>
<td>2–5% for peak area</td>
<td>0.5–3% for peak area</td>
</tr>
<tr>
<td>Dynamic range</td>
<td>1–2 orders of magnitude</td>
<td>2–3 orders of magnitude</td>
</tr>
<tr>
<td>Sensitivity</td>
<td>Relatively high, but ion-dependent</td>
<td>Relatively low</td>
</tr>
<tr>
<td>Matrix effect</td>
<td>Significant</td>
<td>Negligible</td>
</tr>
<tr>
<td>Main application area(s)</td>
<td>Sample preconcentration, gel electrophoresis</td>
<td>Routine quantitative analysis</td>
</tr>
</tbody>
</table>

In the simplest sample stacking with hydrodynamic injection, the samples are dissolved in a low conductivity matrix (e.g., water) and the resulting solution introduced using pressure into the capillary. With electrokinetic injection, on the other hand, sample stacking works whether the matrix is or is not a low conductivity solution [51].

In recent reviews on sample stacking, the stacking methods have been divided into two categories based on their mechanism involved, proportional stacking and boundary stacking [52]. In the proportional stacking, the migrating analyte crosses a boundary and enters a zone where its migration velocity is
decreased. In boundary stacking, the effective velocities of an analyte in two zones forming a sharp moving boundary force the analyte to focus at this boundary. The boundary stacking has better signal enhancement when compared to the proportional stacking [52]. Large-volume sample stacking (LVSS) which is also known as Field amplified sample stacking (FASS) is presented in chapter 3 of this dissertation comes under the proportional stacking method.

There are important physiochemical effects responsible for the stacking, in LVSS or FASS, the Kohlraush adjustment of concentration is the principle involved. In this, the capillary is filled with a high conductivity run buffer containing high concentrations of ions formed by strong electrolytes or strongly ionized weak electrolytes prior to analysis, and a long sample zone with low concentration of analytes and low conductivity is injected hydrodynamically, and voltage is applied, sample ions migrate rapidly in the injection zone as a result of high electric field strength in this zone. Then, when sample ions pass through the boundary of the sample zone and enter the run buffer, they are slowed down due to lower electric field strength and concentrated into a short zone [52-57]. Based on the nature of the analytes, the method development and the optimization involves creating a suitable stacking zone with appropriate sample and run buffers inside the capillary. When the sample is injected electrokinetically and involves the Kohlraush adjustment of concentration, it is Field amplified sample injection (FASI) or Large volume sample injection [58, 59].

The pH-step is another effect used for the stacking, dynamic pH junction and pH mediated stacking are the two types in this method [52, 53, 56, 57, 60]. In
the dynamic pH junction, weak electrolytes are selectively focused, their net charge and mobilities are a function of pH [61]. The sample has a pH significantly different from that of the run buffer and is injected into the capillary hydrodynamically. When a highly protonated base from the sample zone enters a run buffer region of higher pH, its ionization degree is decreased and this results in a lower migration velocity and a stacking effect. For weak acids, the principle is similar except that stacking is reached by migration of weak acids from a high-pH zone into a zone with lower pH. In the pH mediated stacking, the sample or its matrix forms a highly conductive solution. The sample is electrokinetically injected into the capillary and counterions of the run buffer migrate into the sample zone. Then, a plug of strong acid or base is electrokinetically introduced into the capillary to titrate the run buffer counterions in the sample zone and create a low-conductivity sample zone and conditions for Kohlrausch-type stacking [62].

Isotachophoretic stacking is another effective stacking technique [25, 27, 54]; this type of stacking is characterized by the process where the analyte migrates and meets a sharp moving boundary between two neighboring zones migrating in the same direction, usually called the leading and terminating zone. When the velocity of an analyte in the leading zone which is in front of the analyte boundary is lower than that of the boundary, and the velocity of the analyte in the terminating zone which is behind the boundary is higher than that of the boundary, then the analyte is stacked in the boundary region.

The other effective stacking techniques are chromatographic methods such as micellar electrokinetic chromatography (MEKC), in this stacking is reached
when the migrating analyte meets a region containing micelles, interacts with them, and the velocity of its zone is reduced. Microemulsion electrokinetic chromatography (MEEKC) stacking is similar to MEKC, analytes are partitioned between an aqueous phase and oil droplets of a microemulsion, which act as pseudostationary phase [52, 63]. Formation of complex inside the capillary is another stacking phenomenon, complexation of metal ions with ethylene diamine tetraacetate and sweeping has been reported as an effective preconcentration method [64, 65]. Although MEKC, MEEKC and complexation combined with sweeping methods are widely used methods, these cannot be used for the macromolecules such as proteins which wouldn’t partition inside the micelles or microemulsion droplets. In addition to these methods, several combined stacking procedures using more than one stacking method are reported recently: FASI with 18-crown-6-tetracarboxylic acid [66], LVSS with the EOF pump [67, 68], LVSI-sweeping-MEKC [69].

In addition to the basic stacking principle, a successful sample stacking method is achieved with the optimization of the operating steps. Some of main steps involved are i) Capillary surface modification to prevent protein adsorption and EOF control ii) optimizing the concentration and pH of the sample, run buffers and the buffer additives based on the application iii) optimizing the amount of the sample injected into the capillary without losing the resolution between the analytes; iv) optimization of stacking variables such as applied voltage or pressure in appropriate direction; v) monitoring the output current to minimize the sample loss and minimize Joule heating; vi) optimization of the separation voltage. These
steps allows the electrophoretic migration of the stacked sample zone, the analytes will be separated and detected with improved sensitivity. Based on the sequence of steps involved in the sample stacking inside the capillary there are several names given to the stacking procedures in the recent reviews [52, 55-57, 60].
References:


[34] Towns, J. K., Regnier, F. E., Analytical Chemistry 1992, 64, 2473-2478.


CHAPTER 2

CZE-UV separation and detection of artificial oxygen carriers in the whole blood which are potential blood doping agents.

Abstract:

Hemoglobin-based oxygen carriers (HBOCs) are blood substitutes, synthesized by polymerizing hemoglobin, which are being developed and investigated as alternatives to blood for medical purposes. However, due to their ability to increase the oxygen carrying capacity when taken by healthy individuals, HBOCs have been used as a doping agent among endurance athletes and are included in the World Anti-Doping Agency's Prohibited List. To maintain the fairness of competitions and continue the battle against doping, it is essential to be able to detect HBOCs if present in an athlete's blood. To achieve this goal, it is necessary to differentiate HBOCs from the native hemoglobin and to do so in a cost and time effective manner. We have developed a rapid capillary zone electrophoresis (CZE), UV absorbance, method capable of detecting HBOCs, in whole blood samples, at levels below those considered necessary to provide a performance enhancement. Our approach to the analysis for HBOCs utilizes the whole blood sample, not just the plasma, and does not require the use of immunoprecipitants to ensure accurate analysis. By lysing the red blood cells and using centrifugal filtration, followed by our CZE separation, we are able to effectively distinguish between native hemoglobin and HBOCs. Through this method, we have been able to reliably detect concentrations of HBOCs at the...
equivalent of 5.5 g/L, the equivalent to a 3.5% increase in blood hemoglobin concentration for an athlete.

Introduction:

This chapter describes the separation and detection of artificial oxygen carriers which are listed as potential blood doping agents in WADA’s prohibited substances. The main reason that the research groups are inclined toward the development of the artificial oxygen carriers (AOCs) is the blood typing and the unavailability of the desired type of donor’s blood when it is extremely necessary. AOCs are considered as therapeutics and formulated as solutions, in addition to their role as supplements in emergencies, they offer the possibility of temporary oxygen supply to the hypoxic tissues in the patients with circulatory problems [1]. These AOCs are sometimes known as the blood substitutes which is a misnomer because these do not contain the blood components such as the blood group antigens and coagulation factors [2]. The other important factors that interests the synthesis of these AOCs is that these could be used as plasma expanders in place of solutions such as saline which adds to the benefit of their oxygen carrying capacity. Blood transfusions are considered safe and majority of them are performed during the surgical procedures, however, these carry a low risk of infectious diseases because of the storage effects. Noninfectious complications such as sepsis, renal failure related to the transfusion of the more than two weeks old stored blood were reported before. During the storage the RBCs undergo several time dependent structural and functional changes progressively which are responsible for the reduced function of the RBCs and the adverse effects [2-4].
AOCs are being developed to overcome the shortage and improve their long term stability by lyophilizing them into powders, minimize the infection risks associated with the blood transfusion with some efforts such as sterilization of AOCs solutions [5]. Several AOCs are being investigated currently as an alternative to the blood transfusion [3], among them modified hemoglobin based solutions and fluorocarbon emulsions are mostly studied and under trials in an effort to be marketed for human purposes [1, 6, 7].

Because the motivation behind the blood doping is to increase the oxygen carrying capacity of the blood in one way or the other, administration of AOCs such as hemoglobin based oxygen carriers (HBOCs) and perfluorocarbon emulsions (PFCEs) could be an easier way to improve the performance in sports when compared to the blood transfusion. HBOCs [8] and PFCEs [7] are being developed and evaluated for their use as blood substitutes for the temporary supply of oxygen. Recently, cobalt chloride which is an erythropoietin stimulating agent is hypothesized to be emerging as a potential blood dopant to boost the oxygen carrying capacity [9, 10], however, it is not included in the WADA’s prohibited list [11]. As the use of new substances increase there is an increasing necessity for the detection methods to be developed. In the present work we developed and optimized a CZE method to detect the HBOCs in the blood sample which is described in this chapter. Because the centrifugation separated the PFCEs from the whole blood it did not require a CE separation method, which is also described here.
The HBOCs are intra/intermolecular cross linked forms of Bovine or human hemoglobin (Hb) such as Polyheme, Hemospan [8]. From the biological studies, it has been shown that the main purpose of Hb crosslinking is to prevent the renal toxicity caused by RBC free Hb which disassociates to form dimers from its native tetrameric structure and aggregates in the kidneys. Because of the polymerization of the Hb, the intravascular half-life of HBOCs increases eliminating the risk of renal toxicity [12] which also indicates that these products won’t be present in the urine. Additionally, HBOCs have higher p50 values than Hb, as a result they have decreased oxygen affinity than Hb which facilitates efficient delivery of oxygen into the muscles[13]. These advantages provide a safer alternative to blood transfusion, thus the athletes could misuse these HBOCs for their benefit in sports, hence WADA included these in their prohibited list of doping substances/method [11]. Despite these advantages, there are adverse effects of these HBOCs, such as stroke, multi organ failure and mortality in general and some effects are specific to some of the products. As a result the clinical trials of the HBOCs were terminated and the pursuit of these substances is not approved for the humans in the US [14] but one product with name Oxyglobin is approved for the canine use [15]. Because these products are sold in the other parts of the world for humans and could be misused, effective screening of these HBOCs in the human blood is necessary to maintain the fairness of the competitions and prevent the athletes from abusing themselves. Therefore, several approaches have been developed previously to detect these HBOCs.
The perfluorocarbon emulsions (PFCEs) [16] are prepared using chemical compounds that are low molecular weight (450-500 Da) linear or cyclic hydrocarbons and the hydrogen atoms of the carbon chain have been replaced by fluorine. They are highly hydrophobic and denser than water; however, they can become soluble if emulsified with a surfactant. PFCEs dissolve oxygen rather than binding to it, then transport and deliver it to the target tissue, therefore these are being developed as AOCs [17, 18]. Because of their high oxygen delivering capacity when compared to Hb and the biochemical inertness they could become a potential doping agent for endurance athletes. PFCEs have been included in the WADA’s prohibited list to ensure the safety of athletes and maintain the fairness of the competitions, thus requires a method of determination of presence of these PFCEs in the human blood. Previously, Mathurin et al. reported a solid phase microextraction gas chromatography (GC)-MS method to detect the PFCEs present in the whole blood [19]. Because PFCE are excreted by lungs and exhaled this approach will be more useful to detect the PFCEs by GC-MS. However MS detection is complex for a doping test and becomes expensive for high throughput studies, therefore, we investigated the usefulness of CE.

In order for effective screening for the presence of HBOCs in a blood sample, it is necessary to separate Hb from HBOCs present in the lysed RBCs as a contaminant and be able to detect these below or equivalent to 5% increase which is approximately 8 g/L Hb or HBOCs above the average blood hb concentration (~156g/L) [20]. A number of studies detecting the HBOCs below the doping concentration have been reported. Varlet-Marie et al. developed a size
exclusion/ high performance liquid chromatography method to detect HBOCs under development, at clinical trials stage in *in vivo* doped human serum [21], Lasne *et al.* developed a slab gel electrophoresis method to screen *in vivo* doped human serum samples [22]. Thevis *et al.* [23] and Simistek *et al.* [24] developed Liquid Chromatography–Mass Spectrometry based techniques to detect HBOCs, however, in all these methods sample preparation is time consuming. Thevis group used overnight trypsin digested samples and other groups used immunoprecipitation of the plasma protein haptoglobin. These analysis methods are complex when compared to CE methods and their lengthy sample preparation methods requires more sample.

Recently, Staub *et al.* [25] developed a CE-UV/electrospray ionization-time of flight MS based method for detecting HBOCs; later, relatively less complex CZE-UV method was developed by Donati *et al.* [26] group. These methods are rapid compared to the other methods however, these methods required immunodepletion (immunoprecipitation) which is a lengthy process to remove interfering proteins, this adds time and cost to their procedure. Although this CE method and previously reported methods are direct methods which detected HBOCs and confirmed its presence in the sample, either the sample preparation procedures are time consuming or the analysis methods are complex and both in some of the methods. Therefore, our goal was set to develop a simple CE method that will be cost effective for high throughput analysis with a less complex and efficient sample preparation. Thus, to make the method a straightforward approach we used centrifugal filtration with a molecular weight cut off filter to
remove the haptoglobin instead of lengthy immunoprecipitation and other unwanted proteins. This process allows easy removal of unwanted proteins and preconcentrates the HBOCs present in the blood sample. Moreover, because the analytes are proteins and possess close charge to size ratios a capillary coating is required to prevent the protein adsorption on the capillary surface and achieve their separation rapidly. It has been demonstrated in the previous studies that a zwitterionic phospholipid DLPC bilayer capillary coating prevented protein adsorption efficiently [27, 28]. In the present studies, the phospholipid solution preparation procedure has been modified to make it an easier process and used as capillary coating to get a suppressed reversed electroosmotic flow.

Experimental:

**Apparatus:**

All separations were performed on a P/ACE™ MDQ CE system from Beckman-Coulter, Inc. (Fullerton, CA, USA) equipped with a photo diode array detection system and using P/ACE™ MDQ 32 Karat™ software (version 8.0). The detection was performed at 415 nm and with a data acquisition rate of 4 Hz; all separations were temperature controlled at 25°C. Fused-silica capillaries, 360 μm od, and 50 μm id were purchased from Polymicro Technologies (Phoenix, AZ, USA); individual capillaries were cut to 42 cm in length, with a detection window burnt at 32 cm from the inlet. The selection of capillary dimensions as well as the separation voltage of −15 kV were made in order to maintain the separation current below 50 μA while providing adequate separation speed and sensitivity. All injections were performed by applying a pressure to the sample vial at the inlet
end of the capillary; the injection pressure of 5 psi (34.5 kPa) was applied for 5 s. When needed, solutions were centrifuged using a Minispin centrifuge from Eppendorf (Hauppauge, NY, USA) or an accuSpin Micro 17 centrifuge from Fisher Scientific (Pittsburgh, PA, USA); centrifugal filtration was performed using Amicon Ultra 0.5 mL MWCO 100 kDa filters from Millipore (Billerica, MA, USA). Fingertip lancing was performed with Unistik 3 Lancets from Owen Mumford (Woodstock, England). The lysis of red blood cells was performed using water bath sonicator model FS20 from Fisher Scientific. Phospholipid solutions were prepared using a Misonix XL-2000 wand sonicator from Qsonica (Newton, CT, USA). The Bradford assay of the HBOCs was performed using a Genesis 10 UV spectrometer from Thermo Electron Corporation (Waltham, MA, USA).

Chemicals:

All solutions, unless specified otherwise, were prepared using 18.2 MΩ water from a Milli-Q Academic water filtration system (Millipore). Buffer solutions were prepared with either Tris (hydroxymethyl) aminomethane (Tris) purchase from Alfa Aesar (Ward Hill, MA, USA), Piperazine-N-N′-bis (2-ethanesulfonic acid) (PIPES) (Fisher Scientific), or 3-(Cyclohexylamino)-1-propanesulfonic acid (CAPS) from Acros Organics (Fair Lawn, NJ, USA). Glutaraldehyde (25% w/v) (Fisher Scientific) was used as a cross-linking agent for the sheep's Hb, which was obtained from sheep's blood samples purchased from Colorado Veterinary Products (Denver, CO, USA). The phospholipid, DLPC was purchased from Avanti Polar Lipids (Alabaster, AL, USA). Additional chemicals used include phosphate-
buffered saline tablets, purchased from MP Biomedicals (Solon, OH, USA); tricine (Acros Organics); calcium chloride (Alfa Aesar); mesityl oxide (Acros Organics); Gamma globulin (MP Biomedicals); phosphoric acid (85% w/v) from BDH (Radnor, PA, USA); Ethanol (95%) (Fisher Scientific) and Brilliant Blue G (Acros Organics); methanol certified ACS (Fisher Scientific); sodium hydroxide (EMD Chemicals); and hydrochloric acid (BDH).

**HBOCs preparation and optimization:**

As polymerized Hb solutions are not commercially available in the US, we undertook the synthesis of our own HBOCs for this project. The procedures outlined below was developed based upon established techniques for Hb polymerization [29]. In the present HBOCs preparation we used 100 kDa MWCO ultra centrifugal filter to purify the HBOCs, the main objective is to develop a simple and straight forward procedure by eliminating lengthy steps using gel filtration, wet heat treatment, use of dialysis cartridges and chromatography involved in the product purification such as removal of excess glutaraldehyde or unreacted Hb [29-31]. Although the final product may not be as pure and sterile as obtained with the other methods, it is suitable for the CE separation method development.

**Polymerization of lyophilized hemoglobin:**

In the initial studies, lyophilized bovine hemoglobin was crosslinked with glutaraldehyde. 0.5 mM bovine hemoglobin was prepared by weighing 30 mg into a 1.5 mL plastic vial and dissolved in Phosphate Buffered Saline (PBS) solution by
vortexing it for 5 minutes. This solution was centrifuged for 5 minutes at 2790 rcf to remove any undissolved solid hemoglobin. The supernatant was collected into a new 1.5 mL plastic vial and 300 µL of 2.5% glutaraldehyde in 25 mM phosphate buffer (pH 7) was added to it and allowed to react in the refrigerator for 24 hours at 4°C. The reaction was quenched by adding 200 µL of 0.1 mM Glycine in Tris buffer pH 7. The glutaraldehyde cross linked hemoglobin solution was filtered using Millipore- Amicon 100 kDa MWCO (molecular weight cut off) ultracentrifugal filters. The filtration/purification of cross linked hemoglobin solutions was done by adding 500 µL of it to the filtration device and centrifuging it for 10 minutes at 11860 rcf to remove excess glutaraldehyde and unreacted hemoglobin following the manufacturer’s protocol. This solution washed with 60 µL PBS and centrifuged for 5 minutes. To collect the crosslinked hemoglobin solution retained in the filtration device, it was turned upside down into a new vial and centrifuged it for 2 minutes at 2 rcf. The final solution collected was stored at 4°C without further dilution. The lyophilized powder was not completely soluble in water, retained in the MWCO filter with HBOCs and became detrimental to the CE separations. To overcome this difficulty, hemoglobin from lysed sheep’s RBCs was polymerized.

**Polymerization of hemoglobin from lysed RBCs:**

Hb from sheep blood cells was obtained by mixing 1.5 mL of the purchased sheep's blood solution (50% sheep's whole blood, 50% Alsever's solution) with 5.5 mL of water in a 15 mL centrifuge tube. This solution was sonicated in the Fisher FS20 sonicator water bath for about 30 s to ensure the complete lysis of the red
blood cells. This cell lysate was transferred in 1 mL volume fractions to several 1.5 mL plastic centrifuge tubes and centrifuged for 5 minutes at 2790 rcf to sediment the cell remnants. The supernatant from each vial was collected and transferred to a new 15 mL centrifuge tube and mixed with 2 mL of 2.5% glutaraldehyde in 25 mM phosphate buffer (pH 7) and allowed to stand for 24 h at 4°C. The reaction took place as shown in figure 6, which was quenched with the addition of 2 mL of 0.1 M glycine in 50 mM Tris buffer at pH 7.4. Following quenching, the solution was separated into several 500 μL fractions, each was filtered using a 100 kDa MWCO centrifugation filter in order to remove the low molecular weight proteins and reagents, while retaining the polymerized Hb (MW ≥ ~120 kDa). Due to the high concentration of proteins, in each fraction of the solution was filtered several times. First, each fraction was filtered at 14 000 rcf for 10 minutes; the retained protein solution was then mixed with 250 μL of water and filtered by centrifuging for another 5 minutes at 14 000 rcf; this process was repeated two more times before the proteins were finally collected. The fully purified HBOC solution was collected from each filter, yielding approximately 2 mL of total solution, and stored in a new centrifuge tube at 4°C.
Figure 6. Hb crosslinking with the glutaraldehyde at the neutral pH.

a) shows the formation of HBOCs from the Hb. b) Reaction of glutaraldehyde with the Hb. c) Centrifugal filtration to purify the polymerize Hb/HBOCs.

**Bradford protein assay:**

To ascertain a quantitative measure of the concentration of Hb in the HBOC solutions, a Bradford protein assay was performed [32]. Following a protocol published by Stoscheck [32, 33], a series of stock gamma globulin protein solutions were prepared and stained with a solution of Brilliant Blue G. Similarly, a range of solution concentrations of our HBOCs solution were prepared and stained.
Extrapolation of the absorbance measurements made for the HBOCs and gamma globulin solutions at 595 nm revealed a protein concentration of 22 mg/mL for the stock HBOCs solution (figure 7).

This assay is a colorimetric protein assay and it is based on the absorbance shift of the dye Coomassie Brilliant blue G. Under acidic conditions, the red form of the dye is converted into its blue form which then binds to the protein that is being quantitated. The protein-dye complex formation involves the non-polar and ionic bond interactions. The red form of the dye donates its free electron to the ionizable groups on the protein, as a result the native protein structure is disrupted and its hydrophobic pockets are exposed. These hydrophobic pockets of the protein binds to the non-polar region of the dye via vanderwaals forces, which brings the positive amine groups of the protein closer to the negative charge of the dye. The protein-dye complex is therefore strengthened further by the ionic interaction between the two. The protein binding stabilizes the blue form of the dye thus the amount of the complex present in the solution is a measure of the protein concentration which is measured by using the UV absorbance reading at 595 nm. The extinction coefficient is attained from the known protein and using this the unknown protein concentration is calculated. The procedure involves the following steps which are described briefly.
Standard and test solutions

2 mg/mL gamma globulin stock solution was prepared by weighing appropriate amount of protein into a 1.5mL plastic vial and dissolved in 1 mL of deionized water. The stock solution was diluted further to obtain 100-190 µg/mL protein standard solutions. 5-6 µL aliquots of HBOCs stock solution were diluted to 1 mL using a micropipette.

Dye reagent

25 mg of Brilliant Blue G was dissolved in a mixture of 12.5 mL of ethanol and 25 mL of 85% (w/v) phosphoric acid, diluted to 250 mL when the dye was completely dissolved. 1 volume of this solution was diluted with 4 volumes of water and filtered with Whatmann #1 filter paper.

Assay

To 5 mL of dye reagent 100 µL of standard /sample solution was added, then the solutions were incubated for 10 minutes and the absorbance was measured with a Spectrophotometer at 595 nm (UV-VIS) including the solution with 0 protein. Water was measured as blank.
Figure 7. Bradford assay: determination of the amount of HBOCs.

**Phospholipid solution preparation:**

Initially, 0.1 mM solution was prepared by weighing appropriate amount of solid 1, 2-dilauroyl-sn-phosphatidylcholine (DLPC) phospholipid in a 50 mL eppendorf plastic vial and 50 mL of 20mM Tris-CaCl\(_2\) buffer pH 7.2 was added to it. This solution was then sonicated with sonication probe tip in the hot water bath while maintaining the temperature at 60°C for 15 minutes. A clear solution was obtained which was then used to coat the capillary. The measured EOF values were not in the expected range so the solution preparation was modified and the concentration of DLPC was increased.
Figure 8. DLPC solution preparation: wand sonication is the modified step.

A 50 mM solution of DLPC was prepared in a 1.5 mL Eppendorf centrifuge vial by first weighing the appropriate amount of solid DLPC to yield the desired concentration in a 1 mL solution. The solid was then dissolved in 1 mL of a 20 mM Tris, 20 mM CaCl$_2$, buffer, previously adjusted to pH 7.2; complete dissolution was achieved by vortexing the solution for 2 minutes. This produced a solution wherein the DLPC had adopted a multilamellar vesicle conformation; the multilamellar vesicles were converted to small unilamellar vesicles by sonication with a Qsonic probe sonicator. The sonication was conducted at a power setting of 10, for 20 minutes, the solution was kept in an ice bath for the duration of the sonication process. This process was adapted from a protocol for the reconstitution of lipid membranes [34] and was found to be more reliable in our lab than previously published methods for the preparation of DLPC solutions [27]. The resultant
solution prepared by placing in the ice bath, consisting of small unilamellar vesicles, has increased optical transparency at the end of the sonication process, shown in figure 8. After sonication, the solution must be centrifuged for 5 minutes at 2790 relative centrifugal force (rcf) in order to remove metal particulates dislodged from the sonicator probe tip. The supernatant solution was transferred to a new vial, leaving the metal particles from the tip probe at the bottom of the original vial. This phospholipid solution was stored at room temperature and used to coat the inner surface of the capillary; the solutions were found to be viable for at least a week, daily tests of the EOF were conducted to assess viability of the coating.

**Capillary coating and stability**

Prior to use, each new capillary was cleaned by rinsing with methanol (100%), sodium hydroxide (0.1 M), and hydrochloric acid (0.1 M); each solution was rinsed through the capillary for 5 minutes at 5 psi (34.5 kPa), between each solution there was a 2 minutes rinse with water at 5 psi. This was performed to ensure the removal of unwanted particles from the capillary surface and proper deprotonation of the silanol groups on the capillary surface. Following the cleaning of the capillary, the DLPC solution was rinsed through the capillary to coat the internal wall; this was done by rinsing the 50 mM phospholipid solution through the capillary for 20 minutes at 5 psi. Finally, the capillary was rinsed for 2 minutes at 5 psi with 20 mM Tris buffer at pH 7.2 to remove the excess coating solution present inside the capillary. Between the runs, the capillary required only a brief 6 minutes
(5 psi) rinse with DLPC followed by a 2 minutes (5 psi) rinse with the Tris separation buffer; making the total analysis time about 20 minutes once the capillary has been fully coated.

Because phospholipid capillary coating suppresses the EOF, this slower EOF was measured by fast EOF determination method: three neutral marker plugs injection method published by William and Vigh [35] with some modifications suitable with the capillary dimensions, the steps involved are shown in figure 9. First, a plug of neutral marker, mesityl oxide was injected by pressure of 0.5 psi and pushed through the capillary at a pressure of 5 psi for 3 minutes. Then, a second plug of mesityl oxide was injected and a pressure of 5 psi was applied for another 3 minutes. Then 15 kV voltage was applied for 3 minutes so the two mesityl oxide plugs in the capillary migrated along with the EOF. Finally, a third plug of mesityl oxide was then injected and a pressure of peak 5 psi was applied until all three mesityl oxide peaks were detected at UV –Vis 241nm as shown in figure 10. EOF was calculated by the following equation:

$$
\mu_{EOF} = \frac{[(t_3 - t_2) - (t_2 - t_1)]L_dL_t}{t_2t_vV}
$$

(11)

Where $t_1$, $t_2$, $t_3$ are the migration times of mesityl oxide peaks, $t_v$ is the voltage applying time, $L_d$ is the capillary length to the detector, $L_t$ is the total capillary length and $V$ is the Voltage applied. Numerically, the best EOF achieved was $1 \times 10^{-5}$ cm$^2$/V.s with 20 minutes DLPC rinse.
Figure 9. Determination of the magnitude of the suppressed EOF [35].
Figure 10. Electropherogram showing the detection of neutral marker peaks.
Conditions: 50 µm ID X 360 µm OD 42 cm total length; Coating: 20 minutes with 50 mM DLPC in 20 mM Tris-CaCl₂, Run buffer: 20 mM Tris pH 7.2; Voltage: 15 kV.

When 20 mM Tris buffer with 20 mM CaCl₂ pH 7.2 was used as the background electrolyte and a voltage of -15 kV was applied then a faster EOF (1 X 10⁻⁴ cm²/V.s) was achieved. The EOF was measured by injecting a single plug of mesityl oxide at 0.5 psi for 5 seconds followed by a voltage of -15 kV and calculated from the following standard equation in which \( t_m \) is the migration time of the mesityl oxide:

\[
\mu_{EOF} = \frac{L_d L_i}{t_m V}
\]

A linear correlation \( (R^2 = 0.80) \) was observed when the EOF values with three plug Vs single plug method measurements were plotted with the run buffers containing Tris with and without Ca²⁺ keeping the pH same in both. Thereafter,
we used three peak tests to test the freshly prepared DLPC solution to ensure that
the proper EOF was achieved, and for further experiments the standard single plug
method to determine the EOF. As the separation of the HBOCs was found to be
most effective under reversed EOF conditions, the reversed polarity EOF was
measured, using a 20 mM Tris, 20 mM CaCl\(_2\), pH 7.2, separation buffer. The
measurement of the EOF was used as the indicator of the effectiveness of the
phospholipid coating and was tested at the beginning of each day and periodically
if significant changes in sample electropherograms were observed. Well-coated
capillaries were observed to yield EOF values faster than \(-1.4 \times 10^{-4}\) \(\text{cm}^2/\text{V}\cdot\text{s}\). If
the measured EOF was found to be slower than this value, the coating was rinsed
from the capillary with a 5 minutes (5 psi) rinse with methanol, this was followed
by a recoating process consisting of a rinse with Tris buffer followed by DLPC, as
described above. If the recoated capillary still did not yield the appropriate EOF
value, the DLPC solution and capillary were discarded and a fresh phospholipid
solution and capillary were prepared.
Results and Discussion

Sample preparation

Figure 11. Separation of Bovine poly Hb in bovine serum. Electropherogram lower trace belongs to bovine Hb and upper trace belongs to the mixture in 50% diluted bovine serum. Capillary: 50 µm ID X 360 µm OD 42 cm total length; Coating: 7 minutes with 50 mM DLPC in 20mM Tris CaCl2, run buffer: 20mM Tris CaCl2, pH 7.2; Voltage: -15 kV.

In the initial experiments, the polymerization of Hb was done using lyophilized powdered bovine Hb in order to avoid the extra steps involved in purifying the Hb from the intact RBCs and diluted in the bovine serum following the previously reported methods [25, 26]. The polymerization reaction with the glutaraldehyde was successful, however, because the powdered Hb was partially soluble in water the unreacted insoluble Hb was retained in the MWCO filter during
the HBOCs purification process. When CE separation method development was done using these HBOCs, dissolved unreacted bovine Hb was also detected along with its polymerized form, indicates that the reaction is not 100%. Moreover, the undissolved solid Hb particles present in the sample affected the CE separation method development, zero current was seen and resulted in the failed capillaries. The electropherogram in figure 11 was obtained when a mixture of Bovine polymerized hemoglobin and normal bovine hemoglobin solution in 50% Bovine serum diluted with 20 mM Tricine buffer and 30 mM NaCl pH 8.0 was injected and detected at 415 nm UV absorbance (upper trace). When Bovine Hb was diluted in the same solvent and injected (lower trace), the detected peaks were at same migration times as the peaks observed in the mixture sample. From this data it is evident that the sample preparation need to be modified further to remove the Hb solid particles. Therefore, the stock solution of polymerized Bovine Hb was further purified by additional cycles of centrifugal filtration (11,860 rcf for 5 minutes) which reduced the amount of unreacted Bovine Hb present in it but did not remove it completely. Another change was using the human Hb from the freshly lysed RBCs instead of Bovine Hb for the optimization of the CE separation method. Figure 12 shows the separation and detection of the polymerized Bovine Hb and Human Hb in neat bovine serum with 20 mM Tris- 20 mM CaCl\textsubscript{2} buffer (pH 7.2) as the run buffer. This data demonstrated that the CE method conditions are suitable for further optimization toward the separation and detection of HBOCs. To increase the number of injections that could be performed in one capillary column with the DLPC coating, the bovine serum was filtered using the MWCO filter for 10 minutes
at a speed of 11,860 rcf. Filtration of the bovine serum removed the low molecular weight proteins and as a result the number of repeatable injections that could be done in one run sequence improved. These preliminary studies were useful to optimize the sample preparation and revealed the separation difficulties associated with the polymerized powdered Bovine Hb with the Bovine serum as the diluent. Therefore, the sample preparation was improved by incorporating several changes namely, using the Hb from the sheep’s whole blood instead of the powdered Bovine Hb, completely eliminating the use of the Bovine serum and using the human whole blood instead.
Figure 12. Separation of Poly Hb from sheep’s blood in bovine serum. Capillary: 50 µm ID X 360 µm OD 42 cm; Coating: 7 minutes with 50 mM DLPC in 20 mM Tris CaCl<sub>2</sub>; run buffer: 20 mM Tris CaCl<sub>2</sub>, pH 7.2; Voltage: -15 kV. a) To Bovine serum 30 µL of ~3 mg/mL Human hemoglobin stock solution and 30 µL of refiltered polymerized Hb stock solution were added and injected. b) 30 µL of polymerized human Hb diluted in bovine serum was injected individually. c) 30 µL of human Hb diluted in bovine serum was injected individually.

Our modified approach to the detection of HBOCs in the whole blood samples varies significantly from the methods developed by other groups, where the focus was exclusively on detecting HBOCs in the blood serum [21-25]. Because the whole blood sample allows for the detection of HBOCs contained in lipid vesicles and there is an increase in the concentration of any HBOCs in the sample following the filtration, we have chosen to use the whole blood samples. Though the HBOCs contained in the lipid vesicles is only speculative, work has been undertaken to encapsulate Hb in liposomes [36, 37] and a similar approach
could be taken with HBOCs. Should such a liposome be produced, it could easily avoid being detected by the existing methods should it have a density greater than blood serum, as it would be separated from the serum with the red blood cells.

Following the lysis of the cells in the sample, the centrifugal filtration of the sample, with a 100 kDa MWCO filter, is both a necessity and advantage of our process. The filtration is required to decrease the total concentration of protein in the solution; unfiltered samples were found to degrade the separation reproducibility within eight consecutive separations, likely due to the high concentration of proteins disrupting the DLPC coating; when filtered samples were injected, the DLPC coating lasted in excess of 15 consecutive separations without degradation of the separation. The advantage of the filtration process is that the HBOCs are comprised of multiple Hb monomers, as each monomer has a weight of 68 kDa, the filter will retain all the HBOCs while removing the majority of the Hb released from the red blood cells. Additionally, the majority of the cellular and proteins (e.g. albumins 67 kDa) have weights below 100 kDa and will be removed from the sample. This significant reduction in total protein concentration allows the DLPC coating to remain intact over an extended series of separations. The filter is, however, not perfectly efficient, thus along with the higher molecular weight proteins, a portion of the lower molecular weight proteins, including Hb are retained. The majority of these proteins do not interfere with the detection of HBOCs, as the absorbance during separation is monitored at 415 nm, limiting the
detection to those proteins containing heme groups. Consequently, the separation need only be optimized for the differentiation of the HBOCs from the free Hb.

The inefficiencies in the filtration process are also a cause for concern in terms of sample recovery, as loss of HBOCs to the filter could limit the sensitivity of the method or impact the repeatability of the analysis. To assess this, three identical blood samples were prepared, each with 10 μL of whole blood and 521 μg of HBOCs and prepared as described above. The final solutions were all analyzed and the average peak areas for each sample were compared and found to have a percent relative standard deviation of 1.7%, indicating that the repeatability in the filtration process is not a concern to our analysis. In these experiments, the selectivity of the MWCO filtration confirmed the formation of HBOCs polymers combined with the CE migration time of the polymerized Hb, we are convinced that the high molecular weight Hb polymers were produced through our preparation method.

**Separation optimization**

As the isoelectric point (pI) for human Hb is close to a pH of 7 [38], all the separation buffers that were used for this analysis contained calcium chloride in order to yield a reversed and faster EOF that cannot be achieved without the cation [27]. This ensured that even if Hb or the HBOCs were at their pI's they would pass by the detector in a reasonable period of time; without the presence of calcium
ions in the separation buffer, the separation times would be in excess of 40 minutes with the capillary dimensions used in this work.

For an effective CZE separation, there must be a significant difference in the net mobility of the analytes. The difference between monomers and polymers can be very small, as their effective charge to size ratios can remain nearly constant, depending on the form of the polymerization. However, for the separation of Hb from HBOCs, the polymerization process does impact the charge of the resultant protein polymer. The cross-linking reaction with glutaraldehyde takes place at the amine residues of the proteins, such as the basic protein side chains of lysine and arginine [39]. Consequently, this reaction has an impact on the net charge of the polymerized Hb compounds. The removal of the cationic charges from these basic residues will lower the effective pI of the HBOCs, allowing them to have a different charge for the free Hb at select pH values.

With this in mind, a range of pHs were investigated in order to optimize the separation of HBOCs from Hb. Near the pI for free Hb, the HBOCs will maintain a more negative charge than the free Hb; this difference should persist to some extent as the pH is increased further above the pI of free Hb. When the separation was tested in Hepes CaCl$_2$ 20 mM buffer pH 7.0, the separation did not take place. A single peak detected when mixture of poly Hb and normal Hb solution was tested. Furthermore, a range of buffers were prepared using 20 mM of either PIPES, Tris, or CAPS, as appropriate for the buffer pH of interest and 20 mM CaCl$_2$; the pH values tested ranged from 6.7 to 11.2. As it can be seen in figure
13, the net mobility of the HBOCs was greater than that of free Hb in all buffers tested below a pH of 8.8, yielding baseline resolution in each instance, Table 2 shows the resolution obtained.

Table 2. Effect of pH:

<table>
<thead>
<tr>
<th>BGE</th>
<th>EOF (Cm²/V.s)</th>
<th>Sheep's Poly Hb MT (min)</th>
<th>Peak width (min)</th>
<th>Human Hb MT (min)</th>
<th>Peak width (min)</th>
<th>Resolution</th>
</tr>
</thead>
<tbody>
<tr>
<td>PIPES - CaCl₂ (20mM each) pH 6.7</td>
<td>-1.43E-04</td>
<td>8.5</td>
<td>0.703</td>
<td>12.9</td>
<td>1.603</td>
<td>3.81</td>
</tr>
<tr>
<td>TRIS - CaCl₂ (20mM each) pH 7.2</td>
<td>-1.55E-04</td>
<td>7.48</td>
<td>0.63</td>
<td>9.941</td>
<td>0.366</td>
<td>4.94</td>
</tr>
<tr>
<td>TRIS - CaCl₂ (20mM each) pH 7.7</td>
<td>-1.26E-04</td>
<td>7.09</td>
<td>0.425</td>
<td>1) 8.28</td>
<td>2) 9.55</td>
<td>4.33</td>
</tr>
<tr>
<td>TRIS - CaCl₂ (20mM each) pH 8.8</td>
<td>-1.66E-04</td>
<td>6.6</td>
<td>0.367</td>
<td></td>
<td>0.1285</td>
<td></td>
</tr>
<tr>
<td>CAPS - CaCl₂ (20mM each) pH 10.5</td>
<td>-9.55E-05</td>
<td>No peaks detected</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

With a buffer pH equal to or exceeding 8.8, the net mobilities of both the HBOCs and the free Hb were comparable, with no resolution between the different Hb species. For each pH tested, individual tests were conducted with a clean blood sample to identify the native Hb peaks. With the pH of the separation buffer at or below 7.7, the mobility of the HBOCs was significantly greater than the Hb compounds, yielding baseline resolution in all the cases that were tested. It is evident that under these separation conditions, as well as with lower pH buffers, that the Hb yields several species with different mobilities; we hypothesize that these may be dissociated tetramers of the Hb complex. This resulted in two distinct
peaks for the free Hb, however, neither subunit caused interference with HBOC peak. Based upon these results, all further separations were conducted with a 20 mM Tris, 20 mM CaCl$_2$, pH 7.7 buffer, these conditions yielded the best combination of the HBOCs peak intensity, resolution, and separation time.

Figure 13. CE-UV electropherograms of 10 μL whole blood samples doped with 220 μg of HBOCs separated with various pH buffers. The HBOC peak is identified from the Hb peaks when resolved, all other peaks are attributed to Hb. All separation buffers contained 20 mM CaCl$_2$ and 20 mM of the respective buffer: PIPES (pH 6.7), Tris (pH 7.2, 7.7, and 8.8), CAPS (10.8 and 11.2). The capillary was precoated with DLPC solution; total capillary length, 42 cm, 50 μm id; pressure injection for 5 s at 0.5 psi, −15 kV separation voltage; 415 nm absorbance detection.

Hp interference

In all previous published methods for the separation of the HBOCs from Hb and other serum proteins extensive effort was expended in removing interfering
proteins, namely Hp [25]. The Hp protein acts as a scavenger for free Hb in blood serum and will bind to Hb with high affinity [40]. In previous CZE analyses of HBOCs, the Hb-Hp complex was found to interfere with the identification of the HBOC, due to similarities in the migration times [25]. To ascertain if this complex would impact our separations we prepared three samples and ran the separations for each, the electropherograms can be seen in figure 14. All three samples were prepared in the same fashion unless otherwise indicated. Sample A was a typical whole blood sample doped (in vitro) with HBOCs; sample B had the blood serum removed, via centrifugation, prior to the in vitro doping with the HBOCs; sample C was a whole blood sample without any HBOCs doping. As the Hp protein is found in the blood serum, any differences between the electropherograms for samples A and B would be indicative of the impact of the Hp-Hb complex formation on the separation. However, it is clear from the electropherograms that there are no significant differences between the two samples. Sample C was a test to control for the potential for a false-positive result, due to the formation of Hp-Hb complexes. In figure 15 the electropherograms are shown at 214 nm absorbance, trace a shows the serum peak indicated with an asterisk and no change in HBOCs peak when compared to trace b. It is clear from these results that there is no evidence of any signal at the corresponding migration time of the HBOCs, giving us confidence that the potential for a false-positive result is highly unlikely.
Figure 14. CE-UV electropherograms of Hp interference. (A) 10 μL of whole blood doped with 440 μg of HBOCs (B) 10 μL of whole blood doped, serum removed prior to doping with 440 μg of HBOCs (C) 10 μL of whole blood without any dopant. The capillary was precoated with DLPC solution, separation buffer: 20 mM Tris, 20 mM CaCl$_2$, pH 7.7; total capillary length, 42 cm, 50 μm id; pressure injection for 5 s at 0.5 psi, −15 kV separation voltage; 415 nm absorbance detection.
Figure 15. Electropherograms were obtained at wavelength 214 nm from the \textit{in vitro} doped polymerized sheep’s Hb sample.
Trace a) Corresponds to the sample with the human serum, asterisk indicates the serum peak, trace b) Corresponds to the sample without human serum. Capillary: 50 µm ID X 360 µm OD 42 cm total length; Coating: 7 minutes with 50 mM DLPC in 20 mM Tris CaCl$_2$, run buffer: 20 mM Tris CaCl$_2$, pH 7.7; Voltage: -15 KV.

\textbf{Sample volume influence}

As the centrifugal MWCO filtration process is imperfect, allowing substantial amounts of free Hb to be retained along with the HBOCs, the sample volume collected can influence the amount of protein retained by the filter. To ascertain what impact this would have upon the separation that was developed, we tested a range of blood sample volumes mixed with a fixed volume of HBOC solution. For these tests, a 10 µL volume of the HBOCs solution was mixed with increasing volumes of whole blood (10–60 µL) and diluted to the same volume with water. All
the samples were filtered following the same protocol and separated with the 20 mM Tris, 20 mM CaCl$_2$, pH 7.7 separation buffer. It was evident following the centrifugation processes, based on the volume of solution retained in the filter, that increased volumes of whole blood resulted in increased retention of proteins; this was supported by the electropherograms, where the peak areas for the free Hb increased with the whole blood sample volume.

The increase in Hb retention, and associated water, did result in further dilution of the final sample; this could be seen in decrease in the peak area for the HBOCs with increasing whole blood sample volume. There was approximately a 30% decrease in the peak area for HBOCs between the 10 and 40 μL whole blood volume samples. The greatest impact on the analysis from the increase in blood volume sampled was the broadening of the peaks attributed to the free Hb (figure 16). With whole blood sample volumes in excess of 40 μL, baseline resolution was no longer achievable between the HBOCs and the Hb peaks. Consequently, we recommend using sample volumes smaller than, or equal to, 30 μL of whole blood, for analysis.
Figure 16. CE-UV electropherograms of increasing volumes of whole blood sample (10–50 μL) each doped with 220 μg of HBOCs. The capillary was precoat with DLPC solution, separation buffer: 20 mM Tris, 20 mM CaCl₂, pH 7.7; total capillary length, 42 cm, 50 μm id; pressure injection for 5 s at 0.5 psi, −15 kV separation voltage; 415 nm absorbance detection.

**Limit of detection:**

The quantification of the amount of HBOCs in a test sample is unnecessary for antidoping purposes, as any presence of HBOCs constitutes a violation of the WADA Anti-Doping Code. However, it is important that the method be capable of detecting amounts of HBOCs that are equivalent to, or less than, a 5% increase in Hb concentration as this is the lower limit of dopant levels that will yield a performance enhancement. Therefore, an acceptable and effective limit of detection would be one that could identify HBOCs at concentrations of 8 g/L or less in whole blood.
To ascertain the limit of detection for our method, a range of samples were prepared following our established protocol. Each sample was prepared with 10 μL of whole blood to which increasing volumes of the stock HBOC solution (22 mg/mL) are added. The electropherograms in figure 17 represent typical electropherograms from these trials. Analysis of the signal to noise ratio, with the instrument's 32 Karat™ software package, for the HBOCs peak revealed that the threshold ratio of 3:1 was exceeded with 5 μL (110 μg) of HBOCs added to the 10 μL blood sample. This translates into a HBOCs concentration equal to 11 g/L, somewhat above the 5% doping level.

As the HBOCs are concentrated in the sample preparation steps, the sensitivity of this analysis can be improved, within limits, by collecting larger whole blood samples. We tested this by doping 20 μL of whole blood samples each with 5 μL (110 μg) of the stock HBOC solution. This doping level is the equivalent of a 5.5 g/L or a 3.5% increase in Hb mass in an athlete, which yielded a signal to noise ratio in excess of 3 while remaining resolved from the free Hb peaks. Consequently, though sampling can be done with as little as 10 μL of whole blood, it can be beneficial to use larger volumes (≤30 μL) to attain adequately low limits of detection. Lower limits of detection have been reported by others using CE separation methods, with Staub et al. [25] achieving 2 g/L and Donati et al. [26]C reaching 2.5 g/L; though both methods used significantly larger sample volumes at 40 and 100 μL of plasma, respectively.
We also investigated the impact of increasing concentrations of HBOCs in the blood sample, to ensure that higher concentrations would not cause the HBOCs peak to extend into the Hb peak. To this end, increasing volumes of the HBOCs solution was added to several 10 μL samples of whole blood. No detrimental effects were found when the HBOCs volume used was as high as 100 μL (2.2 mg) with a whole blood sample volume of 10 μL. The peak area measure from these tests yielded a linear response for the increasing amount of HBOCs with an $R^2$ value of 0.993.
Animal testing (*In Vivo* testing of HBOCs):

As the potential chemical and physical alterations to the doping agents cannot be predicted or simulated *in vitro* the use of animals in this study is crucial. Mice have been chosen as the test animals due to their adequacy as human models and compatibility of the microliter blood sample volumes required for this analysis. Because the *in vitro* analysis process is refined and functional for the *in vitro* doping tests for the detection of HBOCs below 10 % doping level, *in vivo* experiments were carried out with the mice as the subjects. The *in vivo* tests were performed to ensure that the optimized tests are still valid when the doping occurs in an organism; as the biological processes may alter the analytes in such a way that do not occur *in vitro* that would render the tests ineffective. Individual mice were subjected to HBOCs by intravenous transfusion, 24 hours following the transfusion some of the mouse’s blood was collected and tested to determine if the doping agent can still be detected after it has been present in a living organism for some time.
Anesthetization was accomplished through the use of a precision vaporizer equipped with a non-rebreathing circuit and Isoflurane was used as the anesthetic at a 2-5% dosage level until the mouse is fully anesthetized. Prior to and throughout all the procedures, the mouse was warmed through the use of a heating pad. The mouse was restrained and blood doping was done by injecting with the 320 µL HBOCs solution injection which was done into the tail vein. The injection site was cleaned with an alcohol wipe prior to the injection. The blood doping agent, HBOCs solution was diluted with the phosphate buffered saline solutions; the injections did not exceed 5-10% of the total mouse blood volume which is determined to be 320 µL from the total blood volume of mouse with Hb concentration 26 mg (5% increase) calculated from total Hb content in the mouse blood 0.5312 g. After a minimum of 24 hours, the blood collection was performed on anesthetized doped and undoped mice through the following procedure, as deemed suitable for the volume of blood required. The submandibular bleeding method was performed on anesthetized mice that have been warmed prior to the procedure to facilitate the blood flow. The animal bleeding lancet was used to poke the cheek vein, then the blood droplets (~ 150 µL) were collected into an eppendorph tube containing the anticoagulant.
The mouse RBCs samples were treated following the same procedure as the *in vitro* doping. The main steps involved are the lysis of the cells in the sample, the centrifugal filtration of the sample with a 100 kDa MWCO filter, final dilution with water before the CE separation. The samples were injected in the optimized CE separation conditions, the electropherograms obtained from the samples collected from the doped and normal mouse were similar with no HBOCs detected in the doped sample (figure 18). The reasons for not being able to detect these HBOCs could be: the elimination of significant amount of the HBOCs through the biological processes; the HBOCs retained in the mouse blood could be below the detection limit of the method. This experiment was repeated a few times using new rats and HBOCs were never detected.
Figure 18. CE-UV electropherograms of In vivo doping with the HBOCs. The capillary was coated with DLPC solution, separation buffer: 20 mM Tris, 20 mM CaCl$_2$, pH 7.7; total capillary length, 42 cm, 50 μm id; pressure injection for 5 s at 0.5 psi, −15 kV separation voltage; 415 nm absorbance detection.

Is the CE separation necessary for the detection of perfluorocarbon emulsions in the whole blood sample?

In the initial experiments, we tried to synthesize the PFCEs in our lab by mixing sphingomyelin phospholipid surfactant solution and perfluorodecalin by wand sonication for 20 minutes. Because we did not succeed preparing the stable PFCEs in our lab, commercial perfluorocarbon emulsions ‘Oxycyte,’ was purchased. We aimed at developing a simpler CE method that could separate the PFCEs from the rest of the blood components and detect them in the whole blood samples of doped individuals. The PFCEs were mixed with the human whole blood during the sample pretreatment, because the PFCEs were denser than the whole blood, they settled at the bottom of the eppendorph tube when centrifuged to
remove the unwanted blood components other than the RBCs. Based on these observations it is concluded that the detection of PFCEs does not need a CE separation method and can be detected if the doped human blood sample is centrifuged. It should be noted that the PFCEs are excreted by lungs, therefore, obtaining the doped sample at appropriate time is important, quicker sampling will be beneficial for the detection.
ACKNOWLEDGEMENTS

A version of this chapter has been published as Christopher R. Harrison and Srilatha Vydra Electrophoresis 2012 Volume 33, issue 7, pages 1087-1094.
References


CHAPTER 3

Large volume sample stacking (LVSS) in the zwitterionic phospholipid coated capillary for the separation of peptides and proteins.

Abstract:

Capillary electrophoresis (CE) separation methods, capillary zone electrophoresis (CZE) in particular are being studied and developed in large numbers because of the efficient separations achieved in rapid analysis time at low cost. Because of the low sample volume utilization, CE methods are becoming widely applicable for the analysis of proteins. Most of the CE instruments are equipped with the UV-Vis on-column detection system, which is associated with the difficulty of low concentration sensitivity because of the small path length. To overcome this difficulty, other popular high sensitivity detection systems such as laser-induced fluorescence, mass spectrometry are being used, however, these require high cost, additional sample preparation steps.

The simple approach to improve the detection sensitivity with the CE UV-Vis detection is by utilizing online sample-stacking procedure. Large volume sample stacking (LVSS) is one of the sample stacking methods in which a large volume of sample prepared in a low ionic strength buffer is injected into the capillary containing a high ionic strength run buffer, the analyte ions are stacked into narrow zones, as a result the detection sensitivity improves. In the present studies, a CZE-LVSS method has been developed and optimized to stack the proteins and lower their detection limits using the capillaries coated with a zwitterionic phospholipid.
The zwitterionic phospholipid capillary coating is essential in our CZE-LVSS protein separations to prevent the protein adsorption onto the capillary walls and reduce the electroosmotic flow. A standard anionic protein mixture was tested in this method; the detection sensitivity improved more than 50 fold when compared to the regular CZE method. The detection sensitivity has been improved further by removing the sample matrix by the simultaneous application of the voltage and the back pressure. Double step stacking has improved the signal furthermore, thus the sensitivity of the method improved.

Introduction:

Capillary electrophoresis separation methods, in particular capillary zone electrophoresis are popular because of the efficient separations achieved in rapid analysis time at low cost. Because of the low sample volume utilization, CE methods are becoming widely applicable for the analysis of the biomolecules such as proteins, protein digested peptides and nucleic acids. Most of the CE instruments are equipped with the UV-Vis on-column detection system. Because most of the CE experiments are performed in the capillaries with 25-75 μm inner diameters, the small path length gives rise to difficulty of low concentration sensitivity [1] as explained in Chapter 1. This difficulty becomes worse when the targeted analytes are present in trace amounts in a given biological sample. To overcome this difficulty, other popular high sensitivity detection systems such as laser-induced fluorescence (LIF), mass spectrometry (MS) has been used but these are equipped with highly expensive instrumentation. When compared to the
UV-Vis absorbance/detection, the main limitation of the LIF detection is that it requires sample derivatization because relatively small number of compounds possess natural fluorescence. The main limitation of MS detection includes incompatible capillary wall coatings and surfactants used for the CE protein/peptide separations [2].

The simple and cost effective approach to improve the detection sensitivity with the CE UV-Vis detection is the offline [3-6] or the online sample-stacking methods [2, 7-9]. Because the online sample stacking is done by manipulating the composition of either the run buffer or the sample buffer without the need of modifying the CE instrumentation, it is studied widely and applied to a variety of analytes [10-14]. Use of successful stacking methods in combination with the sensitive detectors such as LIF and MS will be even more beneficial to detect trace amount of biomolecules [2]. The success of online sample stacking methods in CE depends on two factors with which the analyte signal enhancement takes place. Firstly, the success of the stacking method depends on the resultant zone width, the narrower zones increases the peak height and the detection sensitivity of the analytes, and the second is the amount of sample that can be loaded in the given method [8]. The importance of the sample stacking in CE and its applicability is evident from the recent excellent reviews on diverse stacking methods and numerous different names [11-18].

In general, the sample stacking techniques were categorized into four groups based on their operational principles i) application of a pH step ii) MEKC and sweeping [19, 20] developed based on the physico-chemical properties of the
analytes, iii) transient isotachophoresis (t-ITP), iv) Concentration adjustment (Kohlraush adjustment), [10, 12, 14, 21] are developed based on the electrokinetic processes. Several hybrid methods were developed combining the principles of one or more existing approaches, thus the types of online stacking methods increased further [22]. Among these sample-stacking methods, Kohlrausch adjustment [23] in the CZE is a simple approach in which the sample is in a low ionic strength sample buffer is injected hydrodynamically into the capillary containing a high ionic strength run buffer. When a large volume of sample is injected it is known as LVSS [24, 25] and as field amplified sample stacking (FASS) when the injection plug is 5% of capillary volume. Recent review lists the different types of field amplified methods to analyze a variety of analytes including the biomolecules such as proteins [26]. Another related preconcentration technique in which the sample injection is done electrokinetically is the field enhanced sample injection (FESI) [27]. In these LVSS, FASS and FESI electrokinetic processes, the sample ions are stacked into narrow zones at the junction between the sample matrix and the run buffer because of the difference in their electrical conductivities and thereby improve their detection sensitivity in the UV-Vis [28]. Even though the large injection volume in LVSS is an advantage when compared to FASS, the problem with the LVSS method is that the volume of the sample that can be injected is limited. When a large volume of sample is injected, the sample buffer and the run buffers mix and changes the separation conditions such as the generated electrical current, this change in the current decreases the separation efficiency.
The maximum amount (x) of sample that could be injected in the CZE-LVSS method without losing the analytes has been described in the previous studies based on the following equations. The electrophoretic velocity of the analyte i inside the sample buffer as expressed in the following equation (1) [25], it is proportional to the local field strength $E_0 = V/L$ of a uniform system with voltage V across the capillary length L, where $\mu_{epl}$ is the electrophoretic mobility of the ion species i and $x$ is the length of the sample buffer region.

$$v_{epl} = \frac{\mu_{epl} E_0}{x} \quad (1)$$

The electrophoretic velocity of the bulk solution is given in the following equation (2), where $\mu_{eo}$ is the electroosmotic mobility of the sample buffer.

$$v_B = \mu_{eo} E_0 \quad (2)$$

For the ions to be stacked inside the capillary the local electrophoretic velocity of the ions needs to be greater than the velocity of the bulk solution and opposite in direction therefore, it is given with a negative sign.

$$-v_{epl} = v_B \quad (3)$$

The value of x is obtained by substituting equations (1) and (2) in the equation (3), is given as

$$x_{max} = -\frac{\mu_{epl}}{\mu_{eo}} \quad (4)$$

From these equations the amount of sample that could be loaded can be predicted.

The other studies that specifically targeted the protein detection enhancement are FASS method in bubble cell polyvinyl alcohol coated capillary described by Law group [29], an LVSS and reversed pH junction for proteins was
reported by Liu group [30], a simplified approach by Lucy group [31] reported an online sample stacking procedure without polarity switching and pH independent method in the CZE conditions with a zwitterionic surfactant capillary coating, even though this method works in the broad pH range, this approach necessitates that the EOF is suppressed to pump out the sample matrix while the analytes are stacked. In our present studies, this approach may work for the cationic proteins in the cathodic suppressed EOF ($2.0 \times 10^{-5} \text{ cm}^2/\text{V} \cdot \text{s}$) but it will not be applicable for the anionic proteins in the relatively fast anodal EOF [32] generated by DLPC coating. Ken yeung group [33, 34] reported over a 1000-fold protein preconcentration method using pH junction [35] generated with discontinuous buffers in the DLPC coating. Because this procedure traps the protein at its pI and eliminates the other proteins and the sample matrix present in the sample this method will be useful to purify a specific protein in a given sample, the downside of this method is subsequent steps will be necessary to separate if the proteins with close pI are trapped, acidic and basic proteins of interest will be lost.

To improve the separation efficiency, several other online sample stacking methods have been reported before that involves sample matrix removal after the sample has been injected and stacked. One approach in which the sample matrix can be removed from the capillary is the application of the voltage in the opposite direction to the EOF [36]. Recently Kawai group [37] investigated large volume sample stacking with an electroosmotic flow pump method in CZE conditions with a neutral/cationic covalent coating. In this method, application of voltage in the opposite direction to the EOF removed the sample matrix from the capillary, with
this method they were able to achieve a 100-fold sensitivity of the basic proteins. This method is not suitable for our present students because of the combination of buffers that we used in our protein stacking studies. The other approach to remove the sample matrix and focus the analytes is by applying the back-pressure and voltage simultaneously [24, 38], Kowalski group [38] used this sample focusing technique in multi steps before the separation and detected the psychiatric drugs with enhanced sensitivity.

In the present studies, we have developed a simple CZE-LVSS method in the DLPC capillary coating for the protein stacking and separation with and without sample matrix removal. DLPC coating effectively generated reduced reversed EOF that is crucial for the protein stacking method and shielded the capillary surface silanols from protein adsorption. With the CZE-LVSS with the sample matrix, standard myoglobin protein digests and small inorganic ions were separated and detected with enhanced sensitivity. The sensitivity of the method was further improved by removing the sample matrix by applying the voltage and the back-pressure simultaneously. This method was optimized by stacking and separating the acidic pI and neutral pI standard protein mixture and the proteins were detected with improved sensitivity when compared to the normal CZE method.
Experimental:

**Apparatus:**

All the separations were performed on a P/ACE™ MDQ capillary electrophoresis system (Beckman-Coulter, Inc., Fullerton, CA) equipped with a Photo diode array (PDA) detection system using P/ACE™ MDQ 32 Karat™ software (version 8.0). The detection was performed at 214 nm with a 10 nm bandwidth, the data acquisition rate 4 Hz and the separations were temperature controlled at 25°C. Phospholipid solutions were prepared using a Misonix XL-2000 wand sonicator (Qsonica, Newton CT). Solutions were centrifuged using Minispin (Eppendorph, NY) or Accu spin Micro 17 (Fisher Scientific, PA) centrifuges.

**Chemicals:**

All solutions, unless specified otherwise, were prepared using 18.2 MΩ water from a Milli-Q Academic water filtration system (Millipore). Buffer solutions were prepared with Tris (hydroxylmethyl) aminomethane (Tris; Alfa Aesar, MA). 1, 2-dilauroyl-sn-phosphatidylcholine (DLPC) phospholipid was purchased from Avanti Polar Lipids, (Alabaster, AL). All the phospholipid coating solutions contained calcium chloride (Alfa Aesar, MA). The neutral marker, mesityl oxide (Acros Organics, NJ), was used to measure the EOF. Albumin, β- amylase and Myoglobin were purchased from sigma.
**Protein solutions:**

Standard Albumin, β- amylase and Myoglobin were dissolved and further dilutions were done in the 4 mM Tris buffer pH 8.0 for all the LVSS injections. The proteins were dissolved in the run buffers used, such as 20, 40, 60 or 80 mM Tris 20 mM CaCl₂ pH 8.0 buffer for the normal CZE injections.

**Standard myoglobin Peptides:**

The myoglobin digestion was done by using the digestive enzyme trypsin with trypsin to protein 1:100 ratio. The solutions prepared in the 4 mM trypsin pH 8.0 buffer were mixed and incubated at 37°C for 14 hours; the digestion was stopped by adding the trypsin inhibitor in 4 mM Tris buffer solution which is twice the concentration of the trypsin solution. This solution was incubated for 1 hour at 37°C and injected without further dilution.

**Capillary Coating and EOF measurements:**

The DLPC solutions needed for the capillary coating were prepared in the same procedure that has been described in our previous work [32]. New 40 cm total length fused silica capillary (30.2 cm length to the detector), and new 60 cm total length (50 cm to the detector) with 50 µm internal diameter, 360 µm outer diameter were used for the separations. Prior to use each new capillary was rinsed with methanol for 5 minutes at a pressure of 20 psi (34.5 kPa) followed by water for 2 minutes at 20 psi then rinsed with 0.1 M NaOH for 5 minutes at 20 psi followed by water for 2 minutes at 20 psi. The treated new capillary was then coated with the phospholipid by rinsing it with the 50 mM DLPC 20 mM Tris CaCl₂ pH 7.2
solution for 20 minutes at 20 psi. Then the capillary was rinsed at 20 psi with the run buffer for 2 minutes. EOF measurements were performed before the sample injections each day using the mesityl oxide as the neutral marker. The mesityl oxide was injected for 5 seconds at 0.5 psi, and suppressed reversed EOF value of around \(-1.0 \times 10^{-4} \text{cm}^2/\text{V}\cdot\text{s}\) was obtained.

Results and discussion:

On column sample stacking methods are necessary because these are cost effective and will be useful approach in separating and detecting the proteins present in complex biological samples if needed. In the present studies, we have developed online sample stacking LVSS methods with and without sample matrix removal in CZE conditions using DLPC, the zwitterionic phospholipid capillary coating to stack and separate the proteins with the neutral and acidic pI thereby improve their detection sensitivity. DLPC coating is necessary in these studies to reverse the EOF and reduce the run time of the proteins separation with neutral and acidic pI.

**EOF control using DLPC coating:**

Previous studies have shown that DLPC forms a double layered uniform capillary coating, suppresses the EOF, and prevents the protein adsorption effectively in a broad pH range by shielding the negatively charged silanol groups [39]. With this capillary coating the electrophoretic separations were performed in the normal polarity (cathode EOF) for the standard basic proteins and in the reversed polarity (anode EOF) for the standard acidic proteins, reproducible EOF and protein separations were obtained [39]. In our previous work, we have
separated hemoglobin and polymerized hemoglobin which is also called as hemoglobin based oxygen carriers present in the complex whole blood successfully with the DLPC coated capillary [32].

It has been demonstrated before that the reversed EOF is generated in the DLPC coated capillary by adding divalent cations such as Ca$^{2+}$, to the run buffer [39, 40], these divalent ions attach at the phosphate group of the DLPC in the capillary coating and reverses the EOF. The reversal of the EOF gives rapid separation, but the disadvantage to this is the reaction of Ca$^{2+}$ ions with run buffers forming the precipitate. Unlike the large variety of run buffers that can be used in the coatings without these divalent ions, the run buffers that can be used with the DLPC is limited because most of the biological buffers such as caps, borate, phosphate react with the Ca$^{2+}$ ions and form precipitate. Formation of the precipitate even though mild has detrimental effect on the EOF and no separation can be performed. Tris, Pipes and Hepes buffers did not react with the metal ions and the presence of the Ca$^{2+}$ ions did not affect the protein separations as other buffers. In the present studies we have used high ionic strength Tris CaCl$_2$ pH 8.0 as the run buffer and the samples were diluted with low ionic strength Tris buffer pH 8.0 unless specified otherwise.

**LVSS of standard proteins with sample matrix:**

As observed in the previous studies [37] and our studies, LVSS works better if the mobilities of the analyte ions are greater than the electroosmotic flow. In our studies, the DLPC capillary coating has a unique property and that is the change in the direction and magnitude of the EOF which is dependent on the presence of
the Ca\textsuperscript{2+} ions in the sample/run buffer. The Ca\textsuperscript{2+} ions were absent in the sample buffer, as a result the EOF in the injected large sample zone is weak and opposite in direction when compared to the run buffer which has the Ca\textsuperscript{2+} ions in it. Because of the combined effect of this setup and the overall charge of the analyte ions it is observed that the electrophoretic mobilities of the analyte ions in the sample zone is faster than the EOF of the sample zone. As the analyte ions approach the run buffer they moved slowly, therefore stacked at the boundary between the sample buffer and the run buffer. The stacking did not take place when the sample and the run buffer contained equal amount of the Ca\textsuperscript{2+} ions. Moreover, if the Ca\textsuperscript{2+} ions were absent in the run buffer a normal slow cathodic EOF (1 X 10\textsuperscript{-6} cm\textsuperscript{2}/V.s) was seen and the migration times of the analytes is predicted to be slower than the migration times in the reversed EOF.
Figure 19. LVSS in DLPC coating with reversed EOF.
1. Shows the large volume sample injection. 2. Upon application of separation voltage, the analyte ions (proteins, peptides or inorganic ions) are stacked at the junction between run buffer and the sample buffer. 3. The separation of proteins in the new EOF as a result of buffer mixing.

Figure 19 describes the LVSS method that was used to stack the anionic proteins, the DLPC coated capillary was filled with the high ionic strength Tris CaCl$_2$ pH 8.0 run buffer and a range of different run buffer concentrations were tested. With the DLPC coating and the calcium ions present in the run buffer suppressed reversed EOF (-1 X 10$^{-4}$ cm$^2$/V.s) was obtained. A large volume (150 nL) of anionic protein solution prepared in the dilute Tris buffer pH 8.0 was injected and upon application of the voltage, the ions present in the low conductivity high electric field strength sample buffer, moved faster toward the detector. As the analyte ions reached the run buffer with high conductivity and low electric field strength, their mobilities reduced significantly therefore they stacked as shown in
step 2. The separation was observed in the final step as the ions migrated toward the detector with different velocities.

For the LVSS injections the capillary was rinsed with DLPC for 12 minutes at 20 psi then the excess DLPC was pumped out with run buffer rinse for 2 minutes at 20 psi then the protein solution was injected for 2 minutes, 2.5 minutes or 3 minutes, the stacking and separation was done with -20 kV for 25 minutes. Figure 20 shows the separation of the following three standard proteins albumin (pI 4.7), β-amylase (pI 4.8) and myoglobin (pI 7.0) in the described LVSS conditions, in these conditions all the three proteins migrated as anions. It can be seen that in this method, large volume of sample proteins are stacked and separated successfully and the signal enhancement factor of ~30 is obtained for the three proteins when compared to the normal CZE 0.5 psi, 5 seconds injection (5 nl), which is 30 times less than the LVSS injection. For the normal CZE injections, the capillary was rinsed with the DLPC for 12 minutes at 20 psi then the excess DLPC was pumped out with run buffer rinse for 2 minutes at 20 psi then the sample was injected for 5 seconds at 0.5 psi and the separation was done by applying -20 kV. The LVSS injections are repeatable with the migration time RSD values less than 0.5% (n=4) for the three peaks as shown in figure 21. The signal enhancement factor is calculated by using the following equation (5), where $h_{LVSS}$ and $C_{LVSS}$ are the analyte peak amplitude at maximum height and the concentration respectively, tested in the LVSS conditions. The $h_{conv}$ and $C_{conv}$ is the analyte peak amplitude at maximum height and the concentration respectively tested in the normal conditions.
\[ \text{SEF} = \frac{h_{\text{LVSS}}}{h_{\text{conv}}} \times \frac{C_{\text{conv}}}{C_{\text{LVSS}}} \]  \hspace{1cm} (5)

The signal enhancement factor was calculated by using the peak height instead of peak areas and the comparison of this calculation is shown in table 3. Although the peak areas of the proteins increased with sample stacking method, the increase is not proportional to the amount injected, therefore these values were not useful to estimate the stacking effect.

![Graph showing protein separation and detection](image)

**Figure 20.** Comparison of separation and detection of proteins in regular versus LVSS.

CZE conditions: 50 µm ID 360 µm OD, 60 cm total length, 50 cm length to the detector, 60 mM Tris 20 mM CaCl₂ pH 8.0 used as the run buffer Separation voltage: - 20 kV a) Shows the stacking and the separation of the protein mix injected for 2.5 minutes 0.5 psi in the CZE-LVSS conditions with sample matrix 4 mM Tris pH 8.0 sample buffer present.
Table 3. LVSS signal enhancement comparison:

<table>
<thead>
<tr>
<th></th>
<th>Albumin</th>
<th>B-amylase</th>
<th>Myoglobin</th>
</tr>
</thead>
<tbody>
<tr>
<td>150 seconds</td>
<td>26</td>
<td>25</td>
<td>15</td>
</tr>
<tr>
<td>180 seconds</td>
<td>29</td>
<td>33</td>
<td>20</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th></th>
<th>Albumin</th>
<th>B-amylase</th>
<th>Myoglobin</th>
</tr>
</thead>
<tbody>
<tr>
<td>150 seconds</td>
<td>30</td>
<td>34</td>
<td>100</td>
</tr>
<tr>
<td>180 seconds</td>
<td>35</td>
<td>36</td>
<td>114</td>
</tr>
</tbody>
</table>

Figure 21. Repeatability of separation and detection of proteins in LVSS. CZE conditions: 50 µm ID 360 µm OD, 60 cm total length, 50 cm length to the detector, 60 mM Tris 20 mM CaCl₂ pH 8.0 used as the run buffer. Separation voltage: -20 kV. 150 seconds injections shows the stacking and the separation of the protein mix injected for 2.5 minutes 0.5 psi in the CZE-LVSS conditions with sample matrix 4 mM Tris pH 8.0 sample buffer present. 5 seconds injections are named as regular injection.
Optimization of LVSS method with sample matrix:

The electropherograms obtained with the -15 kV, -20 kV, -25 kV potential shown in figure 22 were compared based on the migration time of the proteins, when -15 kV potential was applied the separations were slow and slow separation is unfavorable to the low mobility ions, which could be lost. In -25 kV faster separation is achieved but the high current generated is not desirable for the protein separations, high current is responsible for the nonuniform heating of the bulk solution which affects the local EOF. Because with the -20 kV the separation was faster than -15 kV and generated less output current than -25 kV, thereafter -20 kV potential was used for further optimization.

Figure 22. Comparison of protein mixture separation with change in the applied voltage.
CZE-LVSS conditions: 50 μm ID 360 μm OD, 60 cm total length, 50 cm length to the detector, 60 mM Tris 20 mM CaCl₂ pH 8.0 used as the run buffer; Separation voltages: -15 kV, -20 kV or -25 kV; Injection volume 149 nl (150 seconds, 0.5 psi).
In these studies, the following injection plug volumes were tested: 119 nL (120 seconds, 0.5 psi), 148 nL (150 seconds, 0.5 psi), 178 nL (180 seconds, 0.5 psi), it is observed that as the injection plug length increased the resolution between the peaks decreased. For further optimization studies 148 nL injection plug length was used.

**Effect of run buffer and sample buffer concentration:**

To determine the effect of the run buffer concentration on the stacking and separation of the stacked protein mix, we tested the separation with various concentrations of Tris such as 20, 40, 60, 80 mM and kept the amount of CaCl$_2$ 20 mM same in all. It is observed that the analyte stacking is taking place in all the various buffer concentrations tested as shown in figure 23, as the amount of Tris in the run buffer increased the run time increased and the resolution improved. Comparing the separations obtained in the run buffer containing 60 mM and 80 mM Tris, the intensity of the myoglobin peak in the run buffer with 80 mM is less than 60 mM. It could be because of the slower EOF in the 80 mM, some of the slow moving myoglobin present in the 4 mM Tris buffer could be lost by migrating toward the inlet vial.
Figure 23. Comparison of protein mixture separation in various run buffer concentrations.
CZE-LVSS conditions: 50 µm ID 360 µm OD, 60 cm total length, 50 cm length to the detector, 60 mM Tris 20 mM CaCl$_2$ pH 8.0 used as the run buffer; Separation voltage: -20 kV; Injection volume 149 nl (150 seconds, 0.5 psi).

With 60 mM Tris 20 mM CaCl$_2$ pH 8.0 run buffer, the protein mix solutions prepared in the DI water, 4, 8, 12 mM Tris buffer pH 8.0 were injected. There is no significant differences observed in the 4, 8 and 12 mM sample buffer stacking, however, some differences were observed between the DI water and the 4 mM Tris buffer (figure 24). With the DI water as the solvent, the run time is longer than the 4 mM Tris sample buffer and slow moving myoglobin peak was less intense because of the sample loss as observed with 80 mM Tris 20 mM CaCl$_2$ pH 8.0 run buffer.
Figure 24. Comparison of protein mixture separation in various sample buffer concentrations.
CZE-LVSS conditions: 50 µm ID 360 µm OD, 60 cm total length, 50 cm length to the detector, 60 mM Tris 20 mM CaCl$_2$ pH 8.0 used as the run buffer; Separation voltage: -20 kV; Injection volume 149 nl (150 seconds, 0.5 psi).

Effect of separation length:

There is no effect of the column length on the resolution between the peaks with the normal injection (5 nL) plug length, because of the minimal effect of band broadening. However, with the increase in the injection volume in LVSS there are changes occurring inside the capillary, such as, the change in the separation distance after the stacking has been taken place, change in the EOF because of the presence of high amount of the sample buffer. Figure 25 shows the comparison of sample stacking and separation in the 40 cm and 60 cm DLPC coated capillaries, with the 60 mM Tris 20 mM CaCl$_2$ pH 8.0 run buffer. The applied voltage was -15 kV in the 40 cm capillary and – 20 kV in the 60 cm capillary to obtain close EOFs in both, the EOF in the 40 cm is $-1.25 \times 10^{-4}$ cm$^2$/V•s and in the 60 cm capillary it is $-1.3 \times 10^{-4}$ cm$^2$/V•s. The protein stacking took place in both the
capillaries when the same amount of large plug of protein mix was injected. In the 40 cm capillary the resolution between the peaks is smaller than the 60 cm capillary because of the dominance of the long sample matrix over the EOF of the run buffer, therefore the analyte ions would not have separated in the short separation distance while migrating from the stacking zone toward the detector. With the increase in the separation length, there is increase in the separation distance therefore, the resolution between the peaks improved significantly. This experiment indicates that the separation of the proteins is taking place as they migrated toward the detector, therefore, the longer the distance of migration after stacking better resolution is obtained between the proteins in our setup.
Figure 25. Effect of capillary length on the stacking.
The separation of the protein mix injected for 2.5 minutes 0.5 psi in the CZE-LVSS conditions with sample matrix 4 mM Tris pH 8.0 and 60 mM Tris 20 mM CaCl₂ pH 8.0 used as the run buffer. Separation and detection of proteins in the CZE conditions: 50 µm ID 360 µm OD, 40 cm total length, 30 cm length to the detector, separation voltage: -15 kV shown in the electropherogram named 40 cm capillary. The separation of the protein mix in the CZE conditions: 50 µm ID 360 µm OD, 60 cm total length, 50 cm length to the detector, separation voltage: -20 kV shown in the electropherogram named 60 cm capillary.

LVSS of standard peptides with sample matrix:

The myoglobin peptides were used to investigate the applicability of the present CZE-LVSS method for the enhanced detection of the peptides. The peptides were obtained from the proteolytic digestion of the standard horse heart myoglobin protein by trypsin digestion. Previous studies have demonstrated that the myoglobin undergoes partial trypsin digestion under normal conditions without the thermal denaturation [41]. In our studies, we used the normal trypsin digestion, as a result some of the myoglobin was retained intact but the number of peptide
peaks detected were sufficient for the CZE-LVSS method optimization purpose. When compared to the 5 seconds normal injection in the 40 cm capillary, the peaks in the 40 seconds and 60 seconds injections are not resolved which could be due to the band broadening with the increase in the sample plug length. As shown in the figure 26, it can be seen that the resolution between the peaks decreased as the injection plug length increased. Moreover, as the sample plug length increased, the sample and the run buffers mixed and as shown in the figure 27 the output current is changed which in turn slowed down the EOF significantly. These changes in the EOF increased the migration time which can be seen in the 40 seconds and 60 seconds injection in figure 26. The detection enhancement took place with poorly resolved peaks.
Figure 26. Comparison of separation and detection of standard myoglobin peptides.
CZE conditions: 50 µm ID 360 µm OD, 40 cm total length, 30 cm length to the detector, 60 mM Tris 20 mM CaCl$_2$ pH 8.0 used as the run buffer Separation voltage: -20 kV a) Shows the stacking and the separation of the peptides injected for 5 seconds 0.5 psi in the CZE-LVSS conditions with sample matrix 4 mM Tris pH 8.0 sample buffer present. b) Shows the separation of the peptides injected for 40 seconds 0.5 psi in the CZE conditions with sample matrix 60 mM Tris 20 mM CaCl$_2$ pH 8.0. c) Shows the separation of the peptides injected for 60 seconds 0.5 psi in the CZE conditions with sample matrix 60 mM Tris 20 mM CaCl$_2$ pH 8.0.
Figure 27. Comparison of output current generated during the separation and detection of myoglobin peptides. CZE conditions: 50 µm ID 360 µm OD, 40 cm total length, 30 cm length to the detector, 60 mM Tris 20 mM CaCl$_2$ pH 8.0 used as the run buffer. Separation voltage: -20 kV a) Shows the current for the 5 seconds 0.5 psi injection in the CZE conditions with sample matrix 4 mM Tris pH 8.0. b) Shows the current for 40 seconds 0.5 psi injection in the CZE-LVSS conditions with sample buffer 4 mM Tris pH 8.0. c) Shows the current for the 60 seconds 0.5 psi injection in the CZE-LVSS conditions with sample matrix 4 mM Tris pH 8.0.

The resolution between the peptides improved when the separation length was increased from 40 cm capillary to 60 cm capillary as observed in the protein separation. However, because of relatively more number of peaks detected with the peptide sample than with the protein mixture, the total peptide sample plug length that could be injected is less than protein mixture because of the loss in the resolution between the peaks.
LVSS of standard proteins without sample matrix:

As described in the LVSS methods done before [25, 36] and based on the results obtained so far in our present studies, the presence of large amount of sample matrix decreased the separation efficiency and limits the amount of sample that could be injected. To improve the detection sensitivity of the present method, we have examined the removal of the sample matrix by switching the polarity by applying 10 kV voltage for 1 minute, before applying -20 kV for the separation. This method did not work because of the following reasons, when the 60% capillary volume was filled with 4 mM Tris buffer pH 8.0 and 10 kV voltage was applied, the fast moving anionic analytes albumin and β-amylase were not detected, because of the fast migration toward the inlet vial before the slow moving 4 mM Tris buffer pH 8.0 sample matrix with the EOF $7.8 \times 10^{-5} \text{cm}^2/\text{V}\cdot\text{s}$ removed and only myoglobin was detected. If a plug of run buffer would have been injected between the sample plug and the inlet vial, upon application of 10 kV voltage, the sample loss can be remedied but the sample and run buffer mixing takes place and overall EOF changes, the sample injection plug length cannot be increased and therefore the sensitivity enhancement will be poor.

a) Single step LVSS stacking

To remove the sample matrix and improve the amount of sample that could be injected, we examined the simultaneous application of the voltage and back pressure as shown in figure 28. To optimize the voltage and the required back pressure application time, the current generated needed to be monitored. In the
normal CZE, with the run buffer 60 mM Tris 20 mM CaCl$_2$ pH 8.0 and -20 kV applied voltage, ~ 45 µA current is generated. With the simultaneous application of the back pressure and voltage, if the current reached 40 µA (90%) or above then sample loss is observed, therefore while optimizing the time required to remove the sample matrix by the simultaneous application of back pressure and the voltage, it is necessary to stop this step at appropriate time to avoid the sample loss. In this step, the current is close to ~20 µA initially, then slowly increases as the sample matrix is removed, then drastically increases which indicates that most of the sample matrix has been removed, that is the time when the simultaneous application of back pressure and the voltage needs to be stopped. For the LVSS single step process without sample matrix injections the capillary was rinsed with DLPC for 12 minutes at 20 psi then the excess DLPC was pumped out with run buffer rinse for 2 minutes at 20 psi then the protein solution was injected for 1 minute at 5.2 psi. The sample matrix removal and stacking was achieved by applying -20 kV and -1.5 psi simultaneously for 3.6 minutes. The separation was done with -20 kV for 25 minutes. With – 20 kV and 1.5 psi reverse pressure for 3.65 minutes, the signal enhancement is as follows: ~100 fold for Albumin, ~90 fold for β amylase and ~ 60 fold for the myoglobin proteins when compared to the normal CZE injection as shown in figure 29. From the data it can be seen that in the single step stacking some amount of myoglobin is also removed along with the sample buffer. In the single step LVSS with the sample buffer removed the calculated SEF for myoglobin is ~60 whereas it is ~120 with sample buffer present. Moreover, the peak intensity of myoglobin with sample buffer present is more than
the myoglobin peak intensity with sample buffer removed. This data complies with the prediction of maximum amount of sample that could be injected as shown in equation 4. From the migration time of myoglobin from figure 30, the electrophoretic velocity of myoglobin was calculated to be \(-1.56 \times 10^{-4} \text{ cm}^2/\text{V.s.}\); the EOF of sample buffer is close to 50\% of the electrophoretic velocity of the myoglobin, therefore only 50\% of the capillary can filled to avoid the sample loss. Because the capillary is filled upto 60\% in these experiments some of the myoglobin is lost. The single step LVSS injections are repeatable as shown in figure 30 with the migration time RSD values less than 1\% (n=4) for the three peaks.

Figure 28. Single step LVSS without sample matrix in DLPC coating with reversed EOF.
1. Shows the large volume sample injection about 60\% capillary volume filled. 2. Simultaneous application of voltage and back-pressure to remove the sample matrix, the proteins are stacked at the junction between run buffer and the sample buffer while the sample matrix is removed and the sample plug also pushed toward the inlet vial. 3. The application of the separation voltage and the proteins separation.
Figure 29. Comparison of separation and detection of proteins in single step stacking.

CZE conditions: 50 µm ID 360 µm OD, 60 cm total length, 50 cm length to the detector, 60 mM Tris 20 mM CaCl$_2$ pH 8.0 used as the run buffer, separation voltage: -20 kV a) Shows the stacking and the separation of the protein mix injected for 60 seconds at 5.2 psi in the single step CZE-LVSS conditions with sample matrix 4 mM Tris pH 8.0 removed by the simultaneous application of -20 kV and -1.5 psi for 3.65 minutes. b) Shows the separation of the protein mix injected for 2.5 minutes 0.5 psi in the CZE conditions with sample matrix present. c) Shows the separation of the protein mix injected for 5 seconds 0.5 psi in the CZE conditions with the sample matrix 60 mM Tris 20 mM CaCl$_2$ pH 8.0.
Figure 30. Repeatability of single step stacking: separation and detection of proteins.
CZE conditions: 50 µm ID 360 µm OD, 60 cm total length, 50 cm length to the detector, 60 mM Tris 20 mM CaCl₂ pH 8.0 used as the run buffer, separation voltage: -20 kV. The stacking injection was done for 60 seconds at 5.2 psi, sample matrix (4 mM Tris pH 8.0) was removed by the simultaneous application of –20 kV and -1.5 psi for 3.65 minutes. Regular injections of the protein mix injected for 5 seconds 0.5 psi in the CZE conditions with the sample matrix 60 mM Tris 20 mM CaCl₂ pH 8.0.

b) Double step LVSS stacking

To improve the detection sensitivity further, we repeated the sample injection and sample buffer removal steps twice successively before the separation voltage application as shown in figure 31. For the LVSS double step process without sample matrix injections the capillary was rinsed with DLPC for 12 minutes at 20 psi then the excess DLPC was pumped out with run buffer rinse for 2 minutes at 20 psi then the protein solution was injected for 1 minute at 5.2 psi. The sample
matrix removal and stacking was achieved by applying -26 kV and -1.7 psi simultaneously for 3.27 minutes. The sample injection and the sample matrix step was repeated, then the separation was done with -20 kV for 25 minutes. With -26 kV and 1.7 psi for 3.27 minutes, in this double stacking process the sensitivity enhancement obtained is as follows: ~200 fold for Albumin, ~220 fold for β amylase and ~ 270 fold for the myoglobin proteins when compared to the normal CZE injection (figure 32). When compared to single step stacking with sample matrix removal, in the double step stacking more amount of sample can be injected in a single run, therefore the detection sensitivity improvement is significant. From the current generated it is observed that in the double step stacking the sample matrix was not completely removed therefore the myoglobin was not removed and the sensitivity increased, however the migration time increased and resolution between the peaks decreased when compared to the single step stacking. The double step LVSS injections are repeatable as shown in figure 33 with the migration time RSD values less than 1% (n=4) for the three peaks.
Figure 31. Double step LVSS without sample matrix in DLPC coating with reversed EOF.

a) Shows the large volume sample injection about 60% capillary volume filled. b) Simultaneous application of reverse voltage and back-pressure (p,v) -26 kV, -1.7 psi 3.27 minutes to remove the sample matrix, the proteins are stacked at the junction between run buffer and the sample buffer while the sample matrix is removed and the sample plug also pushed toward the inlet vial. c) The steps 1 and 2 repeated. d) Application of the separation voltage and the proteins separation.
Figure 32. Comparison of separation and detection of proteins in double step stacking versus the normal CZE injection.
CZE conditions: 50 µm ID 360 µm OD, 60 cm total length, 50 cm length to the detector, 60 mM Tris 20 mM CaCl₂ pH 8.0 used as the run buffer, separation voltage: -20 kV. Two step stacking shows the overlay of the stacked and separated protein mix injected for 60 seconds at 5.2 psi followed by removal of the sample matrix by applying – 26 kV, -1.7 psi for 3.27 minutes. This injection and sample matrix removal steps were repeated in the same conditions. The stacked proteins were separated by applying -20 kV voltage. 5 second injection was done at 0.5 psi.
Figure 33. Repeatability of double step stacking.
CZE conditions: 50 µm ID 360 µm OD, 60 cm total length, 50 cm length to the detector, 60 mM Tris 20 mM CaCl$_2$ pH 8.0 used as the run buffer, separation voltage: -20 kV. 2 step stacking injections shows the overlay of the stacked and separated protein mix injected for 60 seconds at 5.2 psi followed by removal of the sample matrix by applying – 26 kV, -1.7 psi for 3.27 minutes done twice. The stacked proteins were separated by applying -20 kV voltage. Regular injection 5 seconds at 0.5 psi.

Conclusions:

In these studies we have been able to develop an online protein stacking process with UV-Vis in the DLPC coating frequently used for the protein separations in CZE conditions. In the LVSS with sample matrix 4 mM Tris pH 8.0 present and with 60 mM Tris 20 mM CaCl$_2$ pH 8.0 as the run buffer, upto 30 fold signal enhancement is observed for the proteins. The standard myoglobin peptides were stacked and separated with a resolution comparable to the normal injection in the 60 cm capillary and detected with enhanced sensitivity. When the sample
matrix is removed by simultaneous application of the voltage and back-pressure and the proteins were stacked in a single step, a significant increase in signal is observed for albumin and β-amylase but the slow mobility myoglobin is removed along with the sample matrix. The detection sensitivity of all the three proteins increased in double step stacking process, more than 200 fold signal enhancement is observed when compared to the normal CZE separation.

Because this method has been developed in the commercial CE instrument and because of its simplicity it can be easily adaptable. Being able to stack and separate the proteins and detect them with enhanced sensitivity in one method is the main advantage of these studies. Not limiting to the standard proteins and peptides as described in these studies, this method can be applicable to separate and detect a large variety of analytes such as proteins present in the biological samples, their peptides, inorganic anions with enhanced sensitivity in the UV-Vis detection if necessary. Other potential applications of this method could be combining it with the high sensitive MS detection for proteomics analysis and make them even more sensitive.
ACKNOWLEDGEMENTS

A version of this chapter is in preparation for publication as Srilatha Vydhia, Christopher R Harrison; “Large volume sample stacking in the zwitterionic phospholipid coated capillary for the separation of proteins”. 
References:


[34] Li, T., Booker, C. J., Yeung, K. K. C., *Analyst* 2012, 137, 4766-4773.


CHAPTER 4

Autologous blood doping test method development for the analysis of the erythrocyte surface proteins by capillary electrophoresis:

Abstract:

Autologous blood transfusion is a potential blood doping method carried out by transfusing the athlete’s own stored blood. Because there is no direct method to detect this type of transfusion, we investigated the applicability of CZE for the detection of the transfused blood marker glycated proteins present in the doped blood as described in this chapter. Through these studies, the standard glycated proteins were successfully separated and detected using the DDAB and DLPC coated capillaries. This chapter also describes the successful isolation of RBC membrane ghosts and trypsin digestion of membrane ghosts proteins. A significant signal enhancement has been observed with the trypsin digests of RBCs membrane ghosts in the normal CZE-LVSS when compared to the CZE. When the tryptic digests of new ghost proteins and stored ghost proteins were tested, a small difference in the peak intensities were observed, however, these differences are very small and did not confirm any storage induced changes. From the results it was impossible to conclude whether the blood sample contained any stored blood or not. Moreover, because of the presence of intrinsic glycated proteins in normal individuals the glycated protein test is not appropriate test for detecting doping directly.
Introduction:

Blood transfusions used for doping is WADA’s forbidden process by which the athletes increase the number of red blood cells (RBCs) in their bloodstream in order to improve the athletic performance [1]. Because the RBCs carry oxygen from the lungs to the muscles, a higher concentration in the blood can improve an athlete’s aerobic capacity and endurance. Blood transfusion for doping purposes can be carried out in two different ways; they are homologous blood transfusion in which the blood of a donor of same blood type is transfused to a doping athlete. This type of transfusion can be detected from the methods such as flow cytometry [2, 3]. Another way is the autologous blood transfusion [4], that is, using the athlete’s own stored blood. To implement this form of doping, athletes collect their own blood, store it for weeks and infuse themselves prior to a sport event [5]. Unlike the homologous transfusion, there is no direct method capable of detecting and confirming the autologous transfusion if the doping of this type happens [6]. Trials are underway to detect these through the indirect methods such as determining the total hemoglobin mass [7], urine analysis for the detection of plasticizer related molecules which could serve as potential biomarkers for transfusion [8], [9], athlete’s biological passport [10-15] and several other approaches listed in the recent review [16]. The other neat WADA funded approach under development is testing for the RBCs age dependent size distribution and the impact of storage after autologous transfusion by capillary electrophoresis [17]. Other potential methods which are under development and
WADA funded are the proteomics methods which evaluates the changes occurring in the RBCs membrane proteome because of the storage [18].

Chang group [19] recently investigated the detection of neocytolysis, which is the lysis of youngest circulating RBCs to maintain the hemoglobin mass levels, which enables the rapid adaptation to a new environment. They are the first group to demonstrate this process in the astronauts returning from space. Their goal was to investigate the differential expression of cell adhesion molecules or cell surface receptors (RBC surface proteins) in the young and old RBCs which could be the targets for the neocytolysis by using flow cytometry. Their thought is that these RBC surface proteins could be the discriminating markers for the young and old RBCs. From their findings, they suggested that neocytolysis could be occurring in the athletes after blood doping which could be responsible for the troublesome health effects. Therefore, they proposed that reproducible tests for the neocytolysis by screening the RBCs surface proteins could be powerful tools to detect the autologous blood transfusion [16] used for doping. With this proposed route in mind for the detection of blood doping in combination with the storage effects on the RBCs in particular the glycation of the stored RBC proteins [20], we explored the detection of these possible changes in the RBCs proteome by using CE in our present studies.

The stored RBCs undergoes numerous biochemical and morphological storage induced changes, one such effect is the in-vitro non-enzymatic glycation of the proteins present on the surface of the stored RBCs [20]. When the blood is stored in dextrose/glucose (reducing sugar) rich storage solution, the high amount
of glucose present in the solution glycates the RBC proteins including the surface proteins during the storage. In this reaction, the sugars condense with the amino groups of the proteins to form Amadori products [21] (figure 34), which are also called as advanced glycation end products [22]. Because of the occurrence of the in vitro chemical modifications in the stored blood, the transfused blood for doping will contain these glycated proteins whereas these will be absent in the healthy normal circulating RBCs. The presence of these glycated proteins in the blood sample confirms the presence of the stored (aged) RBCs in the circulation along with the normal human RBCs, and indicates the autologous blood transfusion used for doping. Because similar glycation reaction of proteins occurs in the patients with diabetes mellitus [23], these glycated proteins could be used as the markers of doping in the non-diabetic individuals only. The human blood sample is complex, therefore the identification and detection of the trace amounts of the glycated proteins is impossible without a method to separate these from the nonglycated proteins and other interfering components present in the sample prior to the detection. Therefore, it is necessary to develop a separation method capable of separating and detecting these non-enzymatically glycated proteins cleaved from the surface of stored RBCs present in the doped athletes’ blood sample.

Several analytical techniques have been reported before that detected the glycation end products of non-enzymatic glycation [24-26]. Prebyl group [27] and Wollenberger [28] developed electrochemical methods for the detection of the glycated hemoglobin. Ahmed group [29] developed HPLC assays with fluorimetric detection of advanced glycation end products with intrinsic fluorescence, another
assay which used derivatization [30], other HPLC methods can be found in the review by Misciagna group [31]. It has been noted before that the HPLC methods for the glycated Hb lacks specificity, meaning, different values of glycated Hb was reported for the same sample in different HPLC conditions because of coelution with the impurities [26].

**Advanced glycation end products: Maillard reaction**

![Diagram of Maillard reaction](image.png)

Figure 34. Non- enzymatic protein glycation reaction.

CE has proven to be very efficient method for the protein analysis because of lower sample consumption, shorter analysis time when compared to other separation methods such as HPLC and slab gel electrophoresis. In the previous CE methods Marie group developed a CE-MS method for the analysis of glycation
products of human serum albumin [32, 33], Creamer group summarized the CE and microchip CE methods for the therapeutic proteins and peptides which includes glycated proteins [33]. Shen group used a hydrophilic cellulose coated capillary to separate the glycated Hb [34], Koval group developed a CZE method in which the cis diol interactions were explored and a CIEF method to detect glycated Hb [35], Hempe group developed another dynamic CIEF method for the detection of glycated Hb [32, 36]. From these efforts toward the detection/quantitation of the nonezymatic glycated proteins, it is evident that these molecules are clinically important and inhibition of these molecules is necessary to avoid disease complications [21]. Whereas the main focus of most of these studies is to quantitatively determine the amount of glycated Hb in diabetics and normal human blood samples, the main focus of our studies is to develop a CE method which will detect the glycated surface proteins and determine the blood doping.

In our present studies, three standard glycated proteins which were obtained by the in-vitro non-enzymatic glycation were successfully separated from their native proteins left unreacted in the sample by using the CZE method with the DDAB coated capillaries. With the same CE separation method, the stored RBCs were tested to detect the storage induced RBCs surface glycated proteins, however, this approach was not successful because of the cell lysis of stored RBCs. Therefore, RBCs membrane ghosts were trypsin digested and tested with the conventional CZE and CZE-LVSS methods with the DLPC coating for the storage induced protein glycation. The results showed slightly different peak intensities when stored sample and normal fresh RBCs samples were compared.
Significant improvement in the signal intensities were observed in the CZE-LVSS method when compared to the CZE method in the same run buffer. The amount of the sample that could be injected in the CZE-LVSS method became limited because of the increase in the baseline signal and severe loss in resolution. In another attempt to further ensure the *in vitro* glycation process and CE separation method is applicable the human Hb was glycated *in vitro* and separated using CZE method, results showed the separation of glycated protein but further information such as extent of glycation became obscure.

**Experimental:**

**Apparatus:**

The separations were performed on a P/ACE™ MDQ capillary electrophoresis system (Beckman-Coulter, Inc., Fullerton, CA) equipped with a Photo diode array (PDA) detection system using P/ACE™ MDQ 32 Karat™ software (version 8.0). The detection was performed at 415 nm with a 10 nm bandwidth and the data acquisition rate 4 Hz. Separations were temperature controlled at 25°C and the. Solutions were centrifuged using Minispin (Eppendorph, NY) or Accu spin Micro 17 (Fisher Scientific, PA) centrifuges; centrifugal filtration was performed using Amicon Ultra 0.5mL MWCO 100 kDa (Millipore, location) centrifugal filters. Human blood was withdrawn with Unistik 3 Lancets and red blood cells were lysed using sonicator water bath FS20 (Fisher Scientific). Phospholipid solutions were prepared using a Misonix XL-2000 wand sonicator (Qsonica, Newton CT).
Chemicals:

All solutions, unless specified otherwise, were prepared using 18.2 MΩ water from a Milli-Q Academic water filtration system (Millipore). Buffer solutions were prepared with either Tris (hydroxymethyl) aminomethane (Tris; Alfa Aesar, MA) Boric acid, Ammonium bicarbonate, Sodium Phosphate didodecyldimethylammonium bromide. All the phospholipid coating solutions contained calcium chloride (Alfa Aesar, MA). Trypsin (Fisher scientific) was used for the protein digestion and Trypsin inhibitor was used to stop the it. Ribonuclease A, Cytochrome C, Lysozyme The neutral marker, mesityl oxide (Acros Organics, NJ), was used to measure the EOF.

Methods:

Standard protein glycation reaction and sample preparation:

The in-vitro nonenzymatic glycation reaction of the standard proteins was carried out following a previously reported procedure with some changes [36]. The Ribonuclease A (3 mg/ml), cytochrome c (3 mg/ml), and lysozyme (3 mg/ml) were dissolved in the citrate phosphate dextrose (cpd) solution prepared in the lab with the composition similar to the commercial cpd solution: citric acid, anhydrous (3.3 mg/ml), sodium citrate dihydrate (26.3 mg/ml), sodium dihydrogen phosphate dihydrate (2.5 mg/ml), D-glucose (25 mg/ml). A blank salt solution (named as cpd blank) was prepared with all the reagents except the D-glucose and used to prepare each protein solution with the same concentration as in the cpd solution. To expedite the glycation reaction of these proteins the solutions prepared in the
cpd and the cpd blank were incubated at 37°C for at least 72 hours before the CE separation.

Sample preparation:

An aliquot of protein solution in cpd and cpd blank solutions (glycated and nonglycated respectively) were filtered with the ultra-centrifugal 3 kDa molecular weight cutoff filters at a speed of 14,000 g for 30 minutes to get rid of the excess salts and unreacted glucose present in the samples. To ensure that all the unreacted glucose has been filtered, the retained solution was replenished with water and filtered again at the same speed. The concentrated centrifugal filtered protein solutions were diluted with water and used for the CE separation.

Whole blood sample preparation for the autologous blood doping:

Human blood was drawn, aliquoted with 100 µL each in the 1.5 mL sterile eppendorph tubes containing 14 µL of cpd (citrate phosphate dextrose) solution and stored in the refrigerator at 4 °C until further use.

Extraction of the surface proteins from the intact RBCs:

The stored and freshly drawn RBCs (100 µL each) taken in 1.5 mL eppendorph tubes were washed in 1 mL of PBS by centrifuging the solution for 5 minutes at 2790 rcf to remove the plasma proteins, white blood cells and the platelets. The supernatant was discarded and washing was repeated two more times with the new PBS solution. To the RBCs settled at the bottom of the tube, 7 µL of the trypsin 4 mg/ml in the 50 mM ammonium bicarbonate pH 8 was added
and incubated for 3 hours, then the reaction was stopped by adding the trypsin inhibitor 8 mg/ml. Stored RBCs lysed upon the addition of the trypsin, therefore RBC ghosts were prepared.

**Preparation of RBC membrane ghosts:**

The red blood cells (stored and freshly drawn) were washed in 1 mL of PBS by centrifuging the solution for 5 minutes at 2790 rcf to remove the plasma proteins, white blood cells and the platelets. The supernatant was discarded and the RBCs were lysed in 1 mL 10 mM phosphate buffer pH 7.5. The cells were sonicated in the water bath for about 30 seconds to ensure complete cell lysis and the solution was centrifuged at 11,860 rcf for 30 minutes. Supernatant is carefully removed and the retained ghosts were collected, the ghosts were washed three times with the phosphate buffer followed by three times with the 20 mM Tris buffer pH 7.5 to remove the hemoglobin adhered to the RBC membranes by centrifuging for 5 minutes at 11,860 rcf each time, discarding the supernatant and replenishing with the new buffer. To the washed RBCs membrane ghosts settled at the bottom of the tube, 7 µL of the trypsin 4 mg/ml in the 50 mM Ammonium bicarbonate pH 8 was added and incubated for 15 hours (overnight), then the reaction was stopped by adding the trypsin inhibitor 8 mg/ml. centrifuged for 20 minutes at 11,860 rcf and the supernatant was tested by CE for the presence of membrane surface proteins.
Results and discussion:

**CZE separation of standard glycated proteins from the unreacted native protein:**

As a starting point to develop a method which could detect the storage induced glycation, we undertook the *in-vitro* glycation reaction of the standard proteins in our lab. In the initial experiments, a sample preparation method for the non-enzymatic glycation reaction of three basic standard proteins Ribonuclease A, Lysozyme and Cytochrome c was developed. To ensure that the glycation reaction takes place, the proteins were dissolved in a salt solution containing excess of glucose (0.14 M) which is 10 times higher concentration than the blood banking conditions. A CZE separation method was developed to evaluate the glycation reaction and separate the glycated protein from the left over unreacted/ unglycated native protein.

For this CE separation method to be successful it is necessary to use a capillary coating to prevent the basic protein adsorption on to the capillary wall. Previous studies have demonstrated that DDAB, a double chained cationic surfactant with critical vesicle concentration of 0.035 mM at 25°C in water forms a flat bilayer on the surface of the capillary wall. This coating provides the reversed EOF because of the cationic head group and effectively prevents the protein adsorption [37, 38]. The other main advantage of using the DDAB is that it forms a semi-permanent, stable coating and the coating need not be present as a buffer additive, which eliminates any possible coating- analyte interactions.
Figure 35. Electropherogram shows the separation of the unglycated and the glycated Lysozyme. The peak named glycated protein belongs to the glycated lysozyme and the most intense peak belongs to the unglycated lysozyme. Capillary: 50 µm ID X 360 µm OD 42 cm total length; Coating: 5 minutes with 0.1 mM DDAB; Run buffer: 120 mM Borate buffer pH 9.5; Voltage: -15 kV; UV absorbance: 214 nm.

Figure 35 shows the overlaid electropherograms of the glycated lysozyme sample and the blank (unglycated) lysozyme. The glycated protein peak is very well separated from the unglycated lysozyme; the difference in the peak intensities of the unreacted lysozyme in the sample and the blank indicates the glycation reaction. The repeatable injections are shown in the figure 36. The DDAB capillary coating (0.1 mM in water) generated a fast reversed EOF with the 120 mM boric acid pH 9.5 run buffer, the lysozyme with isoelectric point of 11 is positively charged in these conditions. On the other hand, because of the glycation reaction
(figure 35 upper trace) the charge on the glycated lysozyme is relatively negatively charged therefore detected faster. Figure 37 b shows the reaction of the borate ions from the run buffer and the glycated protein molecules through the cis-diol interactions which results in the formation of the anionic complexes. Therefore, the separation is achieved based on the collective effect of the charge and the size of the borate- glycated protein complex, predominantly the negative charge.

Figure 36. Electropherogram shows the repeatability of the separation of unglycated (blank) and the glycated Lysozyme (sample). Capillary: 50 µm ID X 360 µm OD 42 cm total length; Coating: 5 minutes with 0.1 mM DDAB; Run buffer: 120 mM Borate buffer pH 9.5; Voltage: -15 kV; UV absorbance: 214 nm.
Because there is a possibility that the free glucose from the cpd solution may retain in the sample solution, it is necessary to ensure that the free unreacted glucose molecules are completely eliminated and does not interfere with the separation. To attain this, the centrifugal filtration with the 3 kDa MWCO filter was repeated and the samples were tested in the same CE method (data not shown). The results showed the separation but some sample loss was observed because of the repeated filtration. Because there are no free glucose molecules in the solution, there is no competitive cis-diols formation that could affect the separation. From the results it has been observed that the non-enzymatic glycation reaction of standard proteins worked when the protein solutions were incubated at 37°C atleast 72 hours before the CZE analysis and the glycation reaction did not take place when the sample was placed at the room temperature.
Figure 37. Protein glycation reaction and the cis-diol interactions.
   a) Shows the *in vitro* protein glycation reaction. b) Shows the cis-diol interactions between the glycated protein and borate ions.

As it can be seen in the figure 38 similar separation results were observed with the Ribonuclease A (pl) 9.6 glycation. The repeatability of the separations are shown in the figure 39. The resolution between the glycated Ribonuclease A and its native form is less than the Lysozyme, the reason could be the number of glycation sites present in the protein molecule. It is reported before that the glycation reaction (figure 37a) takes place at the ε- amino groups of the lysine belonging to the protein chain [39], therefore more the number of sites glycated more difference in their charge to size ratio and better resolution observed. It has also been noted that as the glycation reaction incubation time at 37 °C was
increased there was increase in the glycated protein peak signal (data not shown) but on long standing (more than a week at 37 °C) the glycated peak disappeared which indicates that the glycated protein is either converted to its native form or the other compounds collectively named as advanced glycation end products (figure 34).

Figure 38. Electropherogram shows the separation of the unglycated and the glycated Ribonuclease A. The peak named glycated protein corresponds to the glycated Ribonuclease A and the most intense peak belongs to the unglycated Ribonuclease A. Capillary: 50 µm ID X 360 µm OD 42 cm total length; Coating: 5 minutes with 0.1 mM DDAB Run buffer: 120 mM Borate pH 9.5; Voltage: -15 kV; UV absorbance: 214 nm.
Figure 39. Electropherogram shows reproducible separation of the unglycated (blank) and the glycated Ribonuclease A.
Capillary: 50 µm ID X 360 µm OD 42 cm total length; Coating: 5 minutes with 0.1 mM DDAB Run buffer: 120 mM Borate pH 9.5; Voltage: -15 kV; UV absorbance: 214 nm.
Figure 40. Electropherogram shows the separation of the unglycated and the glycated Cytochrome c. Arrow points the detected glycated cytochrome c peak and the unglycated protein is the most intense peak. Capillary: 50 µm ID X 360 µm OD 42 cm total length; Coating: 5 minutes with 0.1 mM DDAB; Run buffer: 120 mM borate pH 9.5; Voltage: -15 kV; UV absorbance: 214 nm.

In figure 40 the electropherograms shows the separation of the glycated and unglycated Cytochrome C (pI 9.63), the migration times of the proteins, and the detection of glycated protein as observed with the other proteins. The repeatable injections are shown in the figure 36. Because all the three proteins are cationic in the pH 9.5 boric acid run buffer, and because of the faster EOF, the separations were successful. Moreover, it has been observed that the migration times of the peaks were repeatable in the successive runs done on the same day and intraday with slight variation. When Hb with pI 7.0 was tested in the same conditions, it was not detected because the Hb is negatively charged in the pH 9.5
run buffer therefore it could have bound to the cationic DDAB capillary coating. Because the successful separation was observed with the standard proteins biological sample testing was undertaken to verify the applicability of the method.

Figure 41. Electropherogram shows the reproducible separation of the unglycated (blank) and the glycated Cytochrome c (sample). Arrow points the detected glycated cytochrome c peak and the unglycated protein is the most intense peak. Capillary: 50 µm ID X 360 µm OD 42 cm total length; Coating: 5 minutes with 0.1 mM DDAB; Run buffer: 120 mM borate pH 9.5; Voltage: -15 kV; UV absorbance: 214 nm.
CZE analysis of intact whole blood samples after trypsin digestion:

Figure 42. Electropherograms shows the separation of the 3-hour trypsin digested solution of intact fresh RBCs. Only trypsin and trypsin inhibitor detected. Capillary: 50 µm ID X 360 µm OD 42 cm total length; Coating: 5 minutes with 0.1 mM DDAB; Run buffer: 120 mM borate pH 9.5; Voltage: -15 kV.

Figure 42 shows the separation of the trypsin-digested solution of the freshly drawn intact RBCs and a blank containing the same amount of the mixture of trypsin and trypsin inhibitor as in the RBCs. As the data shows the peaks present in the sample electropherogram are the same as the blank and there were no other proteins detected. The detected peaks could be the Trypsin pI (10.8), Trypsin inhibitor pI (10.5) which will be cationic under these conditions and would not adsorb to the capillary surface. The trace amount of Hb present in the sample is not detected because of the possible interactions of the negatively charged Hb with the positively charged DDAB coating. In this experiment, when the trypsin
solution was added to the stored RBCs, the cells lysed completely because of the storage effects on the RBCs cell membrane [40]. It is impossible to separate and detect the trace amounts of surface proteins present in the cell lysate containing the proteins from the hemolysate. To get around the difficulties with the intact RBCs alternate approach was tested, in the further experiments the stored and new RBCs were lysed completely, the cell components were removed, then the membrane ghosts were trypsin digested and separated by the CZE.

**CZE analysis of RBC membrane ghosts samples after trypsin digestion:**

The RBCs were lysed completely in the further experiments, the stored and new cells were washed and lysed completely after the removal of the plasma, platelets and extracellular proteins present in the whole blood sample. The hemolysate was centrifuged for 30 minutes to separate the membrane ghosts, then these membrane ghosts were subjected to the trypsin digestion for 14 hours (overnight), centrifuged to obtain the digested proteins in the supernatant. The supernatant was then injected in the same CZE conditions with the 120 mM boric pH 9.5 as the run buffer, the proteins/ protein digests were not detected in these conditions, as with the standard Hb samples the data was not repeatable because of the interaction of the negatively charged analytes (proteins /protein digests which are also known as peptides) with positively charged DDAB capillary coating.
Figure 43. Electropherograms shows the separation of the Ribonuclease A glycated and native protein.
Capillary: 50 µm ID X 360 µm OD 42 cm total length; Coating: 7 minutes with 50 mM DLPC; Run buffer: 20 mM Tris-CaCl$_2$ buffer pH 7.7; Voltage: -15KV.

Because there was no detection of acidic and neutral proteins with the DDAB capillary coating and boric acid run buffer pH 9.5, the separation method was modified. Previous studies have demonstrated that in the DLPC capillary coating acidic, basic and neutral proteins were separated efficiently [41]. In our previous studies, the DLPC worked for the neutral and acidic protein separation [42] therefore standard glycated Ribonuclease A and a blank without glycation were tested for the separation. As it can be seen in the figure 43 the glycated protein separated from the native form with DLPC capillary coating with pH 7.7 Tris buffer. Because the standard protein separation was successful in these
conditions, further experiments with RBCs membrane ghosts’ protein digest solutions were done with the 50 mM DLPC capillary coating in the 20 mM Tris-CaCl$_2$ pH 7.7 run buffer.

![Graph showing electropherograms](image)

Figure 44. Electropherograms shows the separation of the overnight trypsin digested solution of RBCs membrane ghosts. New and stored (old) ghosts have the same peaks. Capillary: 50 µm ID X 360 µm OD 42 cm total length; Coating: 7 minutes with 50 mM DLPC; Run buffer: 20 mM Tris-CaCl$_2$ buffer pH 7.7; Voltage: -15KV. UV absorbance: 214 nm.

The data in the figure 44 shows the separation of the overnight trypsin digested solution of RBCs membrane ghosts. It can be seen in the electropherograms that the peptide peaks are detected but they are not significantly different from each other in the stored and the new RBCs ghost solutions. There were no additional target glycated peaks observed in the stored sample. The hemoglobin peaks are smaller in the stored RBCs sample because
the hemoglobin detached easily from the old membranes. The experiment was repeated several times with different age stored RBCs, however, there were no additional peaks detected other than hemoglobin from the cell lysate. To further improve the detection of the peptides and obtain conclusive results it became necessary to improve the detection sensitivity. Thus, the CZE-LVSS method was developed by using the standard proteins as described in the chapter 3 using the DLPC capillary coating with 60-80 mM Tris- 20 mM CaCl₂ as the run buffer and 4 mM Tris as the sample buffer.

The RBCs membrane ghosts stored and new were prepared separately in the 4 mM Tris and trypsin digested overnight. The reaction was stopped with the trypsin inhibitor. The supernatant containing the RBCs protein digest was separated by centrifugation; injected and separated in the CZE-LVSS conditions for 15 seconds in the 60 cm capillary using the 60 mM Tris- 20 mM CaCl₂ pH 8.0 run buffer. The detection signal has improved when compared to the conventional injections with the 5 seconds injection time in 40 (figure 44) and 60 cm capillaries (figure 45). In the 15 seconds injection, the resolution between the peaks is same as the resolution in the 5 seconds injection. The data from the successive multiple runs showed interday and intraday migration time repeatability. When the stored RBCs ghost sample electropherogram is compared with that of the new sample, there were slight variation in the intensities of the adjacent peaks as it can be seen in the figure 45 shown in the arrows. These peak intensity differences were repeatedly seen in the multiple samples tested in the same conditions in the same method.
Figure 45. Electropherograms shows the separation of the overnight trypsin digested solution of RBCs membrane ghosts. New and stored (old) ghosts have the slightly different peaks. Capillary: 50 µm ID X 360 µm OD 60 cm total length; Coating: 12 minutes at 20 psi with 50 mM DLPC; Run buffer: 60 mM Tris-CaCl$_2$ buffer pH 8.0; Voltage: -20 KV. Injection time: 15 sec at 0.5 psi; UV absorbance: 214 nm.

In another experiment the sample injection volume was increased to 20 seconds keeping all the other experimental conditions same as in 15 seconds injections. The results obtained were same as 15 seconds injections with slight variation in the peak intensities of the peptides as shown in the figure 46. Moreover, as the sample injection time was increased from 15 seconds to a higher injection volume, the peak intensities improved with the severe loss in the resolution because of the increase in the baseline signal. Keeping the injection volume 15 sec when an *in-vitro* 30% doped sample (70 new: 30 stored RBCs mixed) was injected no significant difference was noted when the doped sample injections were compared to the fresh normal RBCs sample injection done in the
same conditions (data not shown).

In the previous studies De Palma group [43] extracted surface proteins from the intact RBCs by trypsin digestion for 30 minutes. In our present studies, in order to examine the effect of the partial trypsin digestion on the separation and detection of the peptides obtained from the digested RBCs membrane ghosts, and hoping to obtain larger polypeptides, the trypsin digestion reaction was allowed to take place only for 30 minutes by incubating the samples at 37°C. The partial digestion electropherograms had fewer peaks when compared to the fully digested samples, but the signal intensity of the detected peaks remained same as the previous experiments.

Figure 46. Electropherograms shows the separation of the overnight trypsin digested solution of RBCs membrane ghosts. New and stored (old) ghosts have the slightly different peaks. Capillary: 50 µm ID X 360 µm OD 60 cm total length; Coating: 12 minutes at 20 psi with 50 mM DLPC; Run buffer: 60 mM Tris-CaCl₂ buffer pH 8.0; Voltage: -20 KV; injection time: 20 sec at 0.5 psi; UV absorbance: 214 nm.
Figure 47. Electropherograms shows the separation of the overnight trypsin digested solution of RBCs membrane ghosts with different injection times. From the top to bottom the order is: New and stored (old) ghosts have the slightly different peaks. Capillary: 50 µm ID X 360 µm OD 60 cm total length; Coating: 12 minutes at 20 psi with 50 mM DLPC; Run buffer: 60 mM Tris-CaCl$_2$ buffer pH 8.0; Voltage: -20 KV; injection time: 20 sec at 0.5 psi; UV absorbance: 214 nm.

From the comparison of figure 45 and figure 46 it has been observed that as the sample injection time increased, the baseline absorbance increased and loss in the resolution of the peptides. This could be due to the presence of the lipid molecules from the RBCs membranes. To improve absorbance of the baseline the sample matrix was removed as it was done with the standard proteins in the chapter 3 by applying the simultaneous back pressure for 30 seconds. The data is shown in the figure 47, the resolution did not improve and sample loss was observed.
CZE analysis of human Hb for glycation:

Figure 48. Electropherograms shows the separation of the *in-vitro* glycated human hemoglobin. Capillary: 50 µm ID X 360 µm OD 60 cm total length; Coating: 12 minutes at 20 psi with 50 mM DLPC; Run buffer: 60 mM Tris-CaCl$_2$ buffer pH 8.0; Voltage: -20 kV; injection time: 20 sec at 0.5 psi; UV absorbance: 214 nm.

There are several capillary isoelectric focusing methods developed for the detection of glycation in the Hb [35] for clinical uses such as assessing the diabetes. Figure 48 shows the suspected glycated Hb peak, figure 49 shows the repeatable injections detected with the DLPC coating and 60 mM Tris-CaCl$_2$ buffer pH 8.0 as the run buffer. The human RBCs were lysed and the Hb was incubated in excess cpd solution at 37°C, and tested in the CZE conditions. By testing the highly abundant RBC protein it was concluded that the glycated Hb will be an indirect marker for surreptitious transfusion used for doping and not as a direct test
because glycated Hb is intrinsically present in the normal individuals and considered normal if it is below 7%.

Figure 49. Electropherograms shows the reproducibility of separation of the in-vitro glycated human hemoglobin.  
Capillary: 50 µm ID X 360 µm OD 60 cm total length; Coating: 12 minutes at 20 psi with 50 mM DLPC; Run buffer: 60 mM Tris-CaCl₂ buffer pH 8.0; Voltage: -20 kV; injection time: 20 sec at 0.5 psi; UV absorbance: 214 nm.

Conclusions:

From the successful separation of standard glycated proteins in these studies it is demonstrated that the CZE methods could be used for the detection of clinically important glycated proteins in the biological samples. Significant signal enhancement has been observed with the trypsin digests of RbCs membrane
ghosts in the normal CZE-LVSS with slight variation in the signals when new and stored RBCs membrane ghosts digests are compared. However, this separation sensitivity cannot be improved further because of the interference absorbance of the sample matrix. Even though this data does not conclude autologous doping with the CZE alone but this separation could be a powerful approach when combined with a highly sensitive detector such as a mass spectrometer. From the separation of in-vitro glycated Hb it has been noted that because of the presence of intrinsic glycated Hb in the normal individual it will not be a direct marker for doping, repeated tests will indicate the variation in the levels. Furthermore, just like Hb all the other proteins in the RBCs could be intrinsically glycated and it is far from reach to detect and differentiate the intrinsically glycated low abundant proteins with the present CZE method.
ACKNOWLEDGEMENTS

A version of this chapter is in preparation for publication as Srilatha Vydha, Christopher R Harrison; “Autologous blood doping test method development for the analysis of the erythrocyte surface proteins by capillary electrophoresis”. In Preparation.
References


CONCLUSIONS AND THE FUTURE WORK:

The capillary zone electrophoresis is simple and easy to use with a wide range of separation possibilities, as evidenced by its applications in diverse areas of research and the increase in the number of research publications over the past years. The work presented in this thesis regarding the blood doping analysis using the CZE hopefully convinced that the CZE methods could be used for the routine analysis of the doping samples. The CZE applications presented here will hopefully motivate for the further exploration of the CE methods, thereby meet the ever increasing demand for the new analytical methods to detect the surfacing doping methods and compounds. It has been demonstrated in our studies that the CZE in-vitro blood doping analysis and the detection can be accomplished with the microliter volume of whole blood eliminating the necessity for the vein puncture when multiple sampling is required for the confirmation of the blood doping. The separations presented in this studies are rapid when compared to the previous reported lengthy and multi-step analytical methods. The CZE method with the DLPC coating was not successful in detecting the in-vivo doping using the mouse blood when it was HBOCs doped. However, because the CZE-LVSS has been developed successfully in the later studies for the analyte preconcentration and signal enhancement, the in-vivo analysis may be repeated in the CZE-LVSS should the necessity arise.

The CZE-LVSS method developed in the present studies demonstrates that it is the most cost effective approach to preconcentrate the analytes and thereby
increase the detection sensitivity. A number of sample stacking methods have already been developed over the period since it was introduced. Even though the diverse sample stacking methods are developed which works on a few basic principles, development of the new methods is always a necessity and a challenge. This necessity arises because most of the methods are different in one or other CE method variables, such as the composition of the run buffer, pH, buffer additives, capillary coatings or others, therefore, needs to be optimized. In the present studies, the CZE-LVSS method has been optimized successfully and removal of the sample matrix improved the signal enhancement. The results obtained from these studies demonstrated a tradeoff between the resolution between the detected proteins and their signal enhancement. These CZE-LVSS signal enhancement approaches could be very useful for the evaluation of the clinically important proteins without the necessity of additional sample extraction methods.

The autologous blood doping test with the focus on the glycated proteins has led to the development of successful CZE method capable of separating the glycated proteins from their native forms by using the Cis-diol interactions. The DDAB coating worked for the cationic proteins and the separations showed repeatable migration times, however, the protein with overall negative charge might have adsorbed to the capillary wall, therefore no separations were obtained. Because of the cell lysis of the stored RBCs, intact RBCs trysin digestion was not useful, the already challenging autologous blood doping became even more complicated. As an alternative, the RBCs membrane ghosts’ protein digests were
tested in the capillaries coated with the DLPC because of the limited applicability of the DDAB coating. In the conventional CZE experimental conditions the peptides were detected with poor sensitivity. The detection sensitivity was improved significantly by using the CZE-LVSS method. Because of the absorbance interference of the sample matrix the amount of sample that could be injected became limited and the difference between stored and fresh cells could not be proven. The *in-vitro* glycation of human Hb reminded that glycation of proteins occurs intrinsically in the normal healthy humans, this test will be an indirect approach and requires repetitive testing of suspicious doping.

**Future work:**

**Further improvements of CZE HBOCs separation:**

In this thesis, the CZE protein separation method development for the blood doping analysis is presented. The CE separations are rapid when compared to the HPLC and gel electrophoresis, however, to be able to apply the methods in the anti-doping labs, high throughput methods will be beneficial with even shorter analysis time. Therefore, further optimization of the method to reduce the analysis will be beneficial. For the *in-vivo* HBOCs doping, the cross-linked protein need to be purified with a solid phase extraction method to remove the unreacted Hb and lyophilization is necessary to obtain a sterilized sample. If the HBOCs are prepared in the lab these steps will be necessary, on the other hand if it is purchased from the commercial vendor these steps are not needed. To achieve significant
improvement in the detection sensitivity, the doped samples can be tested in the CZE-LVSS method, successful stacking method may detect the HBOCs with nanoliter volume of sample.

**Further improvements of CZE-LVSS separation:**

In the CZE-LVSS method presented here only the anionic proteins were stacked and separated. Because DLPC works in a broad pH range of run buffers, the cationic protein stacking need to investigated. To eliminate additional separation steps, it will be much more beneficial to see the separation of cationic and anionic proteins separated in a single step with enhanced signal. Because the DLPC coating requires Calcium in the coating buffer, the compatible buffers that does not form chelating complexes with calcium need to be explored. The increase in the number of non-chelating buffers over a broad pH range will further increase the applications of already established DLPC coating for protein separations.

**Further improvements of autologous blood doping test:**

The main challenge with the autologous blood doping test with RBCs membrane ghosts’ protein digests separation is the interference of the sample matrix UV absorbance leading to poor resolution. A solid phase extraction step to remove the sample matrix might be beneficial to improve the detection signal of the peptides several fold without the loss in the resolution. The major challenge with this process could be the loss of the analyte along with the sample matrix. Moreover, every additional step in the analysis adds time and cost to the
procedure. The other important variable that needs to be tested with the autologous blood doping test is the blood type. It has been demonstrated before that most of the human blood types possess surface proteins (antigens) but some types of blood don't have these surface antigens to begin with which is why these blood types are universal donors. If a person is doped with this type of blood, the doping will not be detected by this method because the main focus of the present studies is to be able to detect the glycated surface antigens.

Even though the data presented in this thesis does not conclude autologous doping with the CZE alone but this separation could be a powerful approach when combined with a highly sensitive detector such as a mass spectrometer which is popular for the bottom up proteomics. Furthermore, from the separation of in-vitro glycated Hb it has been noted that because of the presence of intrinsic glycated Hb in the normal individuals it will not be a direct marker for doping, repeated tests will indicate the variation in the levels. Moreover, just like Hb all the other proteins in the RBCs could be intrinsically glycated and it is far from reach to detect and differentiate the intrinsically glycated low abundant proteins with the present CZE method alone. However, these will be easy to detect with the combination of a mass spectrometer.