

Analysis of MicroRNAs in Biological Samples

by

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Abstract

MicroRNAs (miRNAs) are a class of small, single-stranded, non-protein coding RNA molecules that regulate cellular messenger RNA (mRNA) and protein levels by binding to specific mRNAs. Aberrant miRNA expression has been shown to be implicated in several diseases, including cancer. Extracellular miRNAs have been found to circulate in the bloodstream and some of their levels have been associated with different diseases. Furthermore, they hold promise as tissue- and blood-based biomarkers for cancer classification and prognostication. Blood-based biomarkers are attractive for cancer screening due to their minimal invasiveness, relatively low cost and ease of reproducibility.

New miRNA analysis techniques will add toward the understanding of their biological functions. In this thesis, I investigate the utility of capillary electrophoresis (CE) and mass spectrometry (MS) for analysis of miRNAs through proof-of-concept experiments. In the first part of this work, we developed a Protein-Facilitated Affinity Capillary Electrophoresis (ProFACE) assay for rapid quantification of miRNA levels in blood serum (see List of publications (6)). We also implemented a capillary electrophoresis with laser induced fluorescence detection (CE-LIF) method with online sample pre-concentration for detection of endogenous microRNAs in human serum and cancer cells.

3' modification of miRNA is a physiological and common post-transcriptional event that shows selectivity for specific miRNAs and is observed across species. Recent studies have shown that post-transcriptional addition of nucleotides to the 3' end of miRNAs is a mechanism for miRNA activity regulation. For example, such modifications in plants and *C. elegans* influence miRNA stability. In humans, effects on miRNA stability and on mRNA target repression have both been observed. Thus, there is a need for miRNA detection techniques which are direct and multiplexed, require minimal sample preparation and provide qualitative information regarding these modifications. We developed a multiplexed miRNA detection technique based on capillary electrophoresis coupled on-

line with electrospray ionization mass spectrometry (CE-ESI-MS). This method allowed a label-free, direct detection of multiple miRNAs extracted from cancer serum as well as their post-transcriptional modifications with a high mass accuracy.

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Dedication

For Samuel and Monika, each equally my pride and joy.

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List of Abbreviations

Symbol	Description
36-FAM	3' 6-FAM (Fluorescein)
3AlexF488N	3' Alexa Fluor 488 (NHS Ester)
ACS	American Chemical Society
AD	Alzheimers disease
AGO	Argonaute protein
AMI	Acute myeloid leukaemia
APCI	Atmospheric pressure chemical ionization
API	Atmospheric pressure ionization
APPI	Atmospheric pressure photo ionization
BACE1	Beta-site amyloid protein precursor-cleaving enzyme 1
BCL2	B cell lymphoma 2 protein
B-CLL	B-cell chronic lymphocytic leukemia
BGE	Background electrolyte
BODIPY	Boron-dipyrromethene
<i>C. elegans</i>	<i>Caenorhabditis elegans</i>
CBD	Chitin binding domain
CGE	Capillary gel electrophoresis
CIEF	Capillary isoelectric focusing
CIRV	Carnation Italian Ring spot virus
CITP	Capillary isotachopheresis
cDNA	Complementary DNA
CE	Capillary electrophoresis
CLL	Chronic lymphocytic leukemia
CZE	Capillary zone electrophoresis
Da	Dalton

DGCR8	DiGeorge syndrome chromosomal region 8
DIG	Digoxigenin
DMEM	Dulbeccos Modified Eagle Medium
DNA	Deoxyribonucleic acid
ds	Double stranded
DTT	Dithiothreitol
E-coli	Escherichia coli
EDTA	Ethylene diamine tetra acetic acid
EOF	Electroosmotic flow
ESI	Electrospray
GLD-2	Germ-line development regulatory protein-2
H1N1	Hemagglutinin Type 1 and Neuraminidase Type 1
H5N1	Hemagglutinin Type 5 and Neuraminidase Type 1
HCC	Hepatocellular carcinoma
HCV	Hepatitis C virus
HDL	High-density lipoprotein
HepG2	Human hepatocellular liver carcinoma cell line
Huh7	Human hepatocellular liver carcinoma cell line
i.d.	Inner diameter
IPTG	Isopropyl -D-1-thiogalactopyranoside
LC-MS	Liquid chromatography - mass spectrometry
LDL	Low-density lipoprotein
LEKC	Liposome electrokinetic chromatography
LIF	Laser Induced Fluorescence
LNA	Locked nucleic acid
LOD	Limit of Detection

m/z	Mass over charge ratio
MALDI	Matrix assisted laser desorption ionization
MBP	Maltose binding protein
MEEKC	Micro emulsion electrokinetic chromatography
MEKC	Micellar electrokinetic chromatography
miRISC	MicroRNA-induced silencing complex
miRNA	MicroRNA
mRNA	Messenger RNA
MS	Multiple sclerosis
MS	Mass Spectrometry
NAFLD	Nonalcoholic fatty liver disease
ND	Neurodegenerative diseases
NGS	Next-generation sequencing
NPM1	Nucleophosmin 1
NSERC	Natural Sciences and Engineering Research Council of Canada
o.d.	Outer diameter
ORF	Open reading frame
PACT	Protein ACTivator of the interferon-induced protein kinase
PCR	Polymerase chain reaction
PD	Parkinsons disease
PDCD4	programmed cell death 4 gene
piRNA	PIWI-interacting RNAs
Pitx3	Pituitary homeobox 3
PIWI	P-element induced wimpy

Pre-miRNA	Precursor microRNA
Pri-miRNA	Primary microRNA
ProFACE	Protein facilitated affinity capillary electrophoresis
PTEN	Phosphatase and tensin homologue gene
qRT-PCR	Real-time reverse transcription-PCR
RCF	Relative centrifugal force
RISC	RNA-induced silencing complex
RNA	Ribonucleic acid
RNAi	RNA interference
rRNA	Ribosomal RNA
RT	Reverse transcription
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
siRNA	Small interfering RNA
SLE	Systemic lupus erythematosus
ss	Single stranded
SSB	Single stranded DNA binding protein
SYBR Green	N', N'-dimethyl-N-[4-[(E)-(3-methyl-1,3-benzothiazol-2-ylidene) methyl]-1-phenylquinolin-1-ium-2-yl]-N-propylpropane-1,3-diamine
TAR	trans-activation response
TCEP	Tris-2-carboxyethyl phosphine
TOF-MS	Time-of-flight mass spectrometry
TRBP	TAR RNA binding protein
tRNA	Transfer RNA
UTR	Untranslated regions
UV	Ultra-violet
ZCCHC11	Zinc finger, CCHC domain containing protein11

Thesis outline

The following is a brief outline of research work presented in this thesis: In Chapter 2, I discuss miRNA detection methods developed in our lab, using capillary electrophoresis with laser induced fluorescence detection for rapid quantification of miRNA levels in serum and cells. Since the quality and quantity of extracted RNA is important for reproducibility and accuracy in miRNA-profiling studies, in Chapter 3, I evaluated different miRNA extraction methods to select a suitable method for miRNA sample preparation from serum and cells for mass spectrometric analysis. Chapter 4 discusses a CE-MS method for detecting multiple endogenous microRNAs and their modifications in cancer serum.

In our laboratory, three different electrochemical miRNA detection methods were developed. I performed all miRNA purifications from human serum, and I conducted all qRT-PCR experiments to validate the data obtained from the biosensor experiments. The data was published in peer reviewed journals, two of them in *Analytical Chemistry*, and the other in the *Journal of the American Chemical Society*. (see List of publications (2, 4, and 5)). The biosensor data are not included in this thesis.

Chapter 1

Introduction

1.1 Introduction to Small RNAs

Several types of small RNAs have evolved in eukaryotes to silence undesirable genetic materials and transcripts [1,2]. Based on their origin, small RNAs are classified into three classes: microRNAs (miRNAs), small interfering RNAs (siRNAs) and PIWI-interacting RNAs (piRNAs) [1,3]. MicroRNAs are derived from a double-stranded RNA region of short hairpin RNAs through sequential processing by Drosha and Dicer (RNase III-type polymerase), whereas siRNAs (~ 21 nucleotides long) are generated from long double-stranded RNAs or long stemloop structures processed by Dicer. siRNAs play an important role in antiviral defence and facilitate the post-transcriptional suppression of transcripts and transposons [1]. Oligomers of 24-30 nucleotides-long piRNAs are independent of RNase III-type polymerase and are generated by an endonuclease called Zucchini (in humans, this is known as mitochondrial cardiolipin hydrolase) from single-stranded precursors [2,4–6]. Although the roles of some piRNAs are still unknown, the main function of piRNAs is to silence transposable elements in germline cells [6]. piRNAs bind to PIWI proteins, whereas miRNAs and siRNAs are associated with the Argonaute (AGO) proteins. In most somatic tissues, a significant amount of small RNAs are comprised of miRNAs [7].

MicroRNA molecules are generally characterized by their short sequences (19-23 nt). They are evolutionarily conserved single-stranded, non-protein coding RNA molecules, found in both plants and animals [8]. They regulate messenger RNA and protein levels by binding to specific mRNAs. It is estimated that miRNAs regulate approximately 30% of the human protein-coding gene involved in a range of cellular processes including proliferation, differentiation, apoptosis and development [8–10].

1.1.1 MicroRNA Discovery

MicroRNAs were the first endogenous small RNAs to be discovered [11–15]. While doing genetic analysis of developmental mutants of *Caenorhabditis elegans*, Ambros and his group observed that the translation of lin-14 mRNA into LIN-14 protein was inhibited during its developmental stage transition by a small transcript of 22 nt in length [11]. Around the same time Ruvkun and his group observed a similar phenomenon [16], although Ruvkin’s research focus was lin-14 gene, while Ambros’ research was on the lin-4 gene. In 1993, by joint efforts of Ambros and Ruvkun groups, the first miRNA lin-4 was discovered [11, 16]. Seven years after the discovery of the first miRNA, the second miRNA, let-7 was found in *C. elegans* by Reinhart *et al.* [17] in 2000. It has been shown that in contrast with lin-4, let-7 sequence is conserved across species from flies to humans [18]. This finding effected the study of miRNAs in other organisms [12, 19] and prompted an insurgency in the research of this new class of small non coding RNAs called miRNAs. Since then, thousands of miRNAs have been identified in humans, as well as in other species. An online sources, for sequences, such as the miRbase database, was also made available since then [20–22]. In Figure 1.1, selected hallmarks of microRNAs history are illustrated.

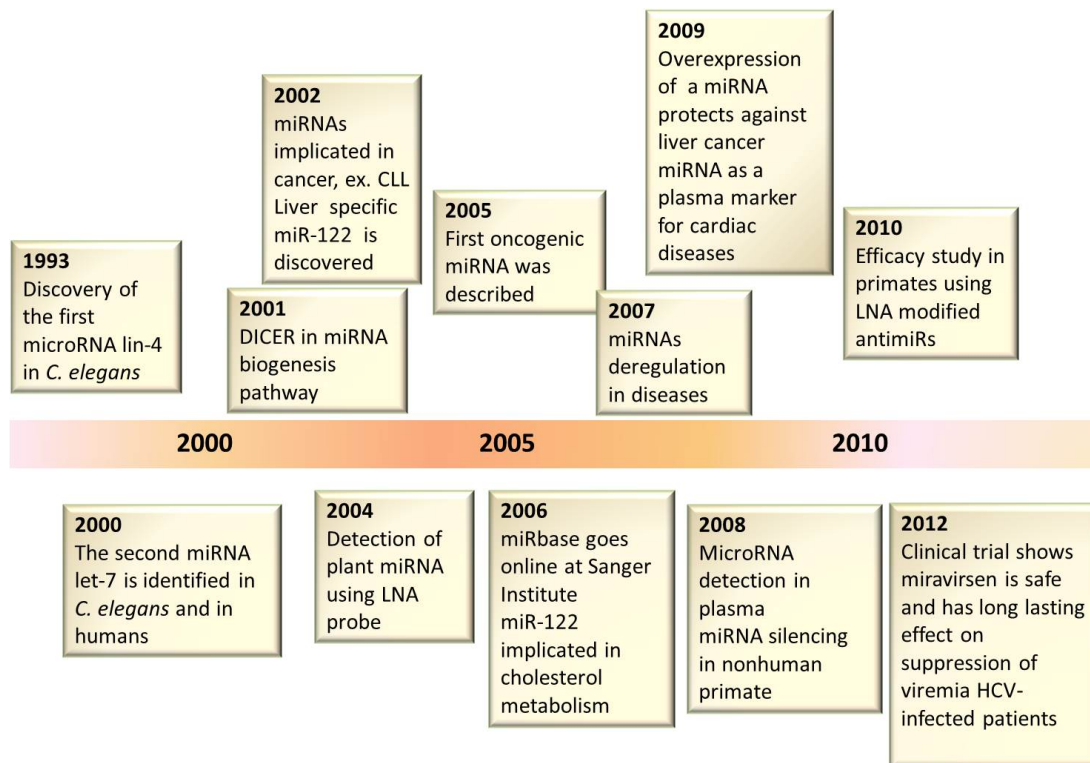


Figure 1.1: Historical point of view on the evolution of our understanding of miRNAs and breakthrough discoveries in miRNA biology.

1.1.2 MicroRNA Biogenesis and Function

It has been established that a multi-step process is necessary to generate a mature form of miRNA [23]. As shown in Figure 1.2, miRNAs are initially transcribed in the cell nucleus by RNA Polymerase II forming long primary transcripts (pri-miRNA) that have a cap structure at the 5'-end and are poly-adenylated at the 3'-end. Pri-miRNA is then processed by RNase III enzyme Drosha and its RNA binding partner, Pasha/DGCR8, into a 60 to 110-nucleotide long pre-miRNA precursor product. It is then transported from the nucleus to the cytoplasm by Exportin-5 for final processing. The pre-miRNA is then processed by another RNase III enzyme, called Dicer, along with its double stranded RNA binding cofactor TRBP/PACT proteins, to generate a short transient ~ 22 -nucleotide miRNA:miRNA* duplex [24, 25]. This duplex is then loaded into the miRNA-associated RNA-induced silencing complex (miRISC). This pre-RISC, containing Argonaute (AGO) proteins associated with miRNA duplexes, rapidly removes the passenger strand (miRNA*) to generate a mature RISC (Figure 1.2, light blue), comprising of Argonaute proteins and single stranded miRNA. Generally, unwinding of miRNA duplexes promoted by mismatches that are present in the guide strand at nucleotide positions 2-8 and 12-15 [26, 27]. The guide strand is determined based on two criteria: relative thermodynamic stability of the two ends of the small RNA duplex and the sequence of the first nucleotide. Guide strands are selected by Argonaute proteins with a uracil at a nucleotide position one. The passenger strand is degraded rapidly upon being released [13, 28, 29]. The mature miRNA (Figure 1.2, red) then binds to the target mRNA genes, either with perfect or imperfect complementarity, consequently negatively regulating gene expression. The binding sites are found depending on the degree of complementarity between the miRNA and its target. When miRNA binds to its target mRNA with perfect (or nearly perfect) complementarity, the binding sites are typically found in the open reading frame (ORF) of the target mRNA, resulting in a degradation of target mRNAs (Figure 1.2, lower right). This mechanism of gene silencing is observed in plants [30–32]. Animal miRNAs use a different mechanism of gene regulation,

where miRNA binds to 3' untranslated regions (3' UTRs) of target mRNA genes, resulting in a negatively regulated gene expression at the level of protein translation (Figure 1.2, lower left), similar to the one that is used for the RNAi pathway [11,16,33–36]. The major function of miRNAs is to inhibit protein production, either by repression of translation, or mRNA degradation. Guo *et al.* conducted a study using ribosome profiling, where they simultaneously measured overall effects on protein production and mRNA levels. They found that destabilization of target mRNAs reduces the protein level [37]. Moreover, miRNAs have shown to activate target mRNA translation [38,39], and to act as molecular decoys for RNA-binding proteins [40]. It has been shown that on average about 500 genes are targeted by one miRNA [41,42]. On the contrary, it is estimated that about 60% of the mRNAs have one or more evolutionarily conserved sequences that are predicted to interact with miRNAs. [41–44].

1.1.3 Post-Transcriptional Modification of MicroRNAs

Though a lot of recent studies have focused on miRNA biogenesis [7,45], our understanding of the mechanism of post-transcriptional regulation of mature miRNAs are still very poor. Generally, mature miRNAs exist within a complex with AGO proteins. Except for being in protein complexes, mature miRNAs also undergo protective modifications. In plants, such as *Arabidopsis thaliana*, at the 3' ends of miRNAs, addition of methyl groups by HEN1 methyltransferase is observed, which is shown to prevent uridylation and destabilization of miRNAs [46,47]. However, unlike plant miRNA, animal miRNAs undergo various nucleotide substitutions, additions and deletions, but no 3' methylations [48–51]. Recent studies have shown that post-transcriptional addition of nucleotides to the 3' end of miRNAs is a mechanism for regulation of miRNA activity. For example, such modification in plants and *C. elegans* influences miRNA stability [52–55]. In humans, effects on miRNA stability and on mRNA target repression have both been observed. For instance, miR-122 was shown to be adenylated by the RNA nucleotidyl transferase GLD-2

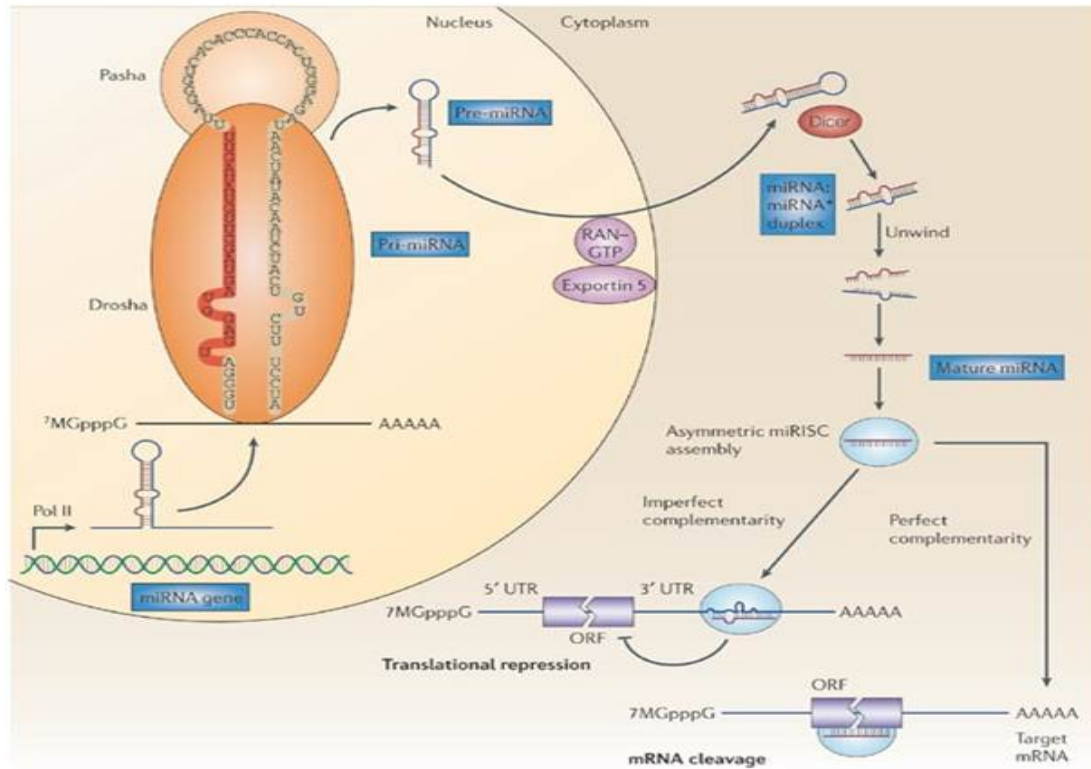


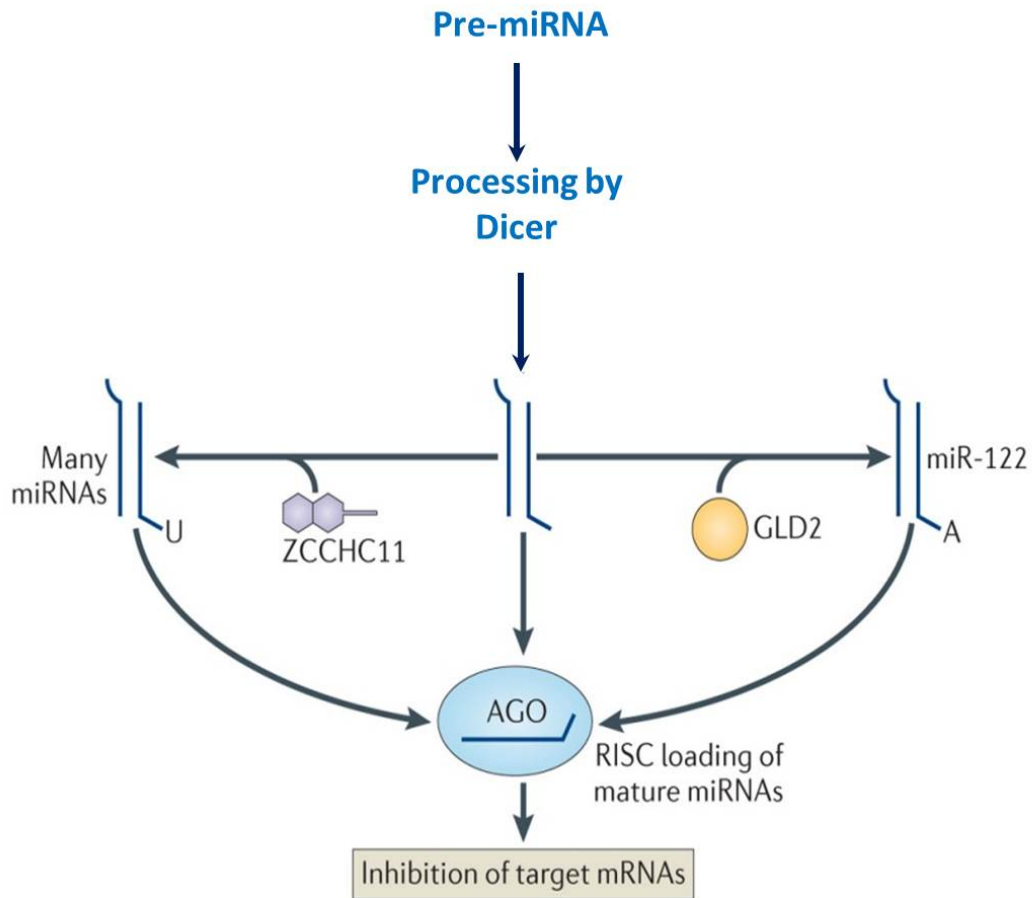
Figure 1.2: The biogenesis of microRNAs. MicroRNA genes are transcribed by RNA Polymerase II in the nucleus to form pri-miRNA transcripts. Pri-miRNA is then processed by the RNase III enzyme Drosha and its co-factor, Pasha, into a 60 to 110-nucleotide pre-miRNA precursor product. It is then transported to cytoplasm by exportin-5. Then, the pre-miRNA is processed by another RNase III enzyme, Dicer, to generate a transient 22-nucleotide miRNA:miRNA* duplex. This duplex is then loaded into the miRNA-associated RNA-induced silencing complex (miRISC) (light blue), comprising of Argonaute proteins and single stranded miRNA (red). Mature miRNA (red) then binds to the 3' untranslated regions (3' UTRs) of target mRNA genes with imperfect complementarity, consequently, negatively regulating gene expression at the level of protein translation (lower left). MicroRNA can also bind with perfect (or nearly perfect) complementarity to the open reading frame (ORF) of the mRNA target. Reprinted by permission from Macmillan Publishers Ltd: [Nature Reviews Cancer] [35], copyright (2006).

in humans and mice (see Figure 1.3), which resulted in an increase in the stability of the miRNA [56]. In contrast, uridylation of miR-26a had no effect on miRNA stability, but had an effect on the ability of miR-26a to inhibit its mRNA target [57]. The 3' modification of miRNA is a physiological and common posttranscriptional event that shows selectivity for specific miRNAs and is observed across species, ranging from *C. elegans* to humans [45]. Thus detecting these modifications of miRNA is necessary for understanding the post-transcriptional regulation of miRNA function.

1.2 MicroRNA Involvement in Human Diseases

MicroRNAs play a crucial role in almost every aspect of cell biology, including developmental timing, proliferation, and apoptosis [11, 59–61]. Moreover, miRNAs are involved in various cellular activities, such as insulin secretion [62], immune response [63–65], neurotransmitter synthesis [66], and viral replication [67]. Thus, aberrant miRNA expression has an impact on those critical processes, and consequently, leads to a number of pathological and malignant conditions [68, 69]. Here, I summarize miRNA related studies on diseases which have a very few treatment options, with a special emphasis on cancer:

Viral diseases: Viruses are pathogens causing chronic diseases, as well as some of the most deadly pandemics in our history. Viral-encoded miRNAs were discovered by bioinformatics, cloning, or combined approaches in multiple virus species including adenovirus [70], polyoma virus [71], and a number of subtypes of the herpes viruses [72–74]. Viral infection has been shown to change the miRNA expression profile. For instance, in animal experiments, Li *et al.* demonstrated that miR-21 and miR-223 were highly up-regulated in lethal infections of H1N1 influenza virus in mice and H5N1 avian influenza virus in macaques [75, 76], compared to animals infected with less pathogenic viruses. In more recent work, Zhang *et al.* showed that the expression of miR-155 was increased in HCV-infected patients which in turn may activate the Wnt signaling pathway and con-



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Figure 1.3: Possible mechanism of nucleotide addition to miRNAs. After mature miRNA is loaded onto an Argonaute protein (AGO) to form the RNA-induced silencing complex (MiRISC), ZCCHC11 and GLD2 act in a miRNA-specific manner adding single uridylyl or adenylyl residues, respectively, to miRNA 3' ends. Modified and reprinted by permission from Macmillan Publishers Ltd: [Nature Reviews Cancer] [58], copyright (2013).

tribute in part to HCV-induced hepatocarcinogenesis [77]. MicroRNA expression patterns could be useful to make treatment plans for patients infected with higher or lower virulent pathogens.

Immune-related diseases: MicroRNA signatures were identified in many common immune-related diseases, such as type I/II diabetes, multiple sclerosis (MS), systemic lupus erythematosus (SLE), and nonalcoholic fatty liver disease (NAFLD). [78–81]. miR-145 expression alone was found to discriminate affected MS patients compared to the healthy control, with high specificity and sensitivity [79]. Higher expression of miR-34a, miR-155 and miR-326 was detected in MS lesions, and an increased severity of MS was shown to be correlated with an increased expression of miR-326. [82, 83]. Luo and Lofgren’s group independently found a correlation between decreased levels of miR-146a and an increased risk for SLE among east Asian and European populations, using hundreds of SLE patients and healthy controls [80, 84]. Differential expression profiling of miRNA has also been observed in type 2 diabetes, which includes miR-144, miR-146a, miR-150 and miR-182 [78]. In an experiment with diet induced NAFLD in rats, Alisi *et al.* observed increased levels of miR-200a, miR-200b and miR-429, and decreased levels of miR-122, miR-451 and miR-27 [81].

Neurodegenerative diseases: As the pathogenesis of neurodegenerative diseases (ND) such as Parkinson’s disease (PD) and Alzheimer’s disease (AD) on a molecular level is still not well understood, effective treatments are not yet available. Recent developments in studies revealing miRNA involvement with neurodegenerative diseases have increased our knowledge on disease pathogenesis and might lead to innovative treatment approaches. By using a systemic miRNA profiling method in peripheral blood mononuclear cells from Parkinson’s disease patients, Martins *et al.* showed the association of miR-30b, miR-30c, and miR-26a with the susceptibility of the disease [85]. It has been shown that the miR-133b-Pitx3 feedback loop is necessary for retaining dopaminergic neurons in the brain, thus deregulation of miR-133b expression might be associated with the pathogenesis of PD [86].

Hebert *et al.* showed that decreased levels of miR-29a, miR-29b-1 and miR-9 in AD patients, ultimately resulted in aberrantly highly expressed BACE1, their target protein [87], which has been shown to play an important role in pathogenesis of the Alzheimer's disease [88]. Taking together these finding we can appreciate the importance of research and understanding miRNAs and their involvement in the pathogenesis of neurodegenerative diseases which would facilitate and enable future medical interventions.

1.2.1 Abbarent Expression of MicroRNAs in Cancer

Cancer is believed to develop and progress due to genetic alterations that disrupt normal cellular processes such as cell cycle, DNA damage response, survival and migration [89]. In cancer, each of these cellular processes is affected by dysregulated miRNAs [90]. First indication of miRNA's involvement in cancer was reported by Calin *et al.* in which they showed that a frequent deletion of miRNA genes, miR-15 and miR-16, occurs in 60% of patients affected with B-cell chronic lymphocytic leukemia (B-CLL) [91]. Following that report, distinctive miRNA expression signatures were found in other malignant tissues from cancer patients [92,93]. By performing genome-wide profiling, authors of these studies demonstrated that miRNA expression signatures can be used to discriminate different types of cancer with high accuracy, whereas mRNA profiles showed very inaccurate indication of tissue or cancer type [92–94]. It was observed that selective groups of miRNAs are commonly down-regulated or up-regulated in different types of human cancers and were frequently associated with cytogenetic abnormalities [93]. For example consistent up-regulation of miR-17 and miR-21 was observed in prostate, colon, stomach, lung and pancreatic tumours and miR-155 was identified to be up-regulated in lung, breast and colon cancer [93]. On the contrary, it was reported that miR-29 was down-regulated in CLL, acute myeloid leukaemia (AML), mantle cell lymphoma, breast, lung and liver cancer [95–100]. Additionally miR-15a and miR16-1-3p was identified to be down-regulated in CLL, prostate and pituitary adenomas [91, 101, 102] and let-7 family members were found

to be down-regulated in breast, lung, colon, ovarian and stomach cancer [98, 99, 103–105].

1.2.2 MicroRNAs as Oncogene and Tumor Suppressor Genes

Because of a common characteristic that was observed in miRNA expression patterns, it has been suggested that deregulation of these miRNAs were not caused by random events in cancer. This led to the assumption that up-regulated miRNAs may act as oncogenes and down-regulated miRNAs may act as tumour suppressors [90]. Figure 1.4 represents a basic model of miRNAs acting as tumour suppressors and oncogenes proposed by Garzon *et al.* [90]. They proposed that, when a miRNA down-regulates the expression of an oncogene, it can be categorized as a tumor suppressor and is frequently observed to be lost in tumor cells. The loss of function of these miRNAs may occur due to any abnormality in the miRNA biogenesis, such as mutation, deletion or promoter methylation. These might produce an abnormal expression of the target oncogene, which then contributes to tumor formation, which will ultimately induce cell proliferation, invasion, angiogenesis and decreased cell death (Figure 1.4, A). For example, it has been shown by Calin *et al.* that miR-15a-miR-16-1 cluster located in the chromosome 13q14 region is a negative regulator of the B cell lymphoma 2 protein (BCL2), in patients with chronic lymphocytic leukemia (CLL) due to a genomic deletion of this region. [91, 95, 106]. On the contrary, if a miRNA that down-regulates a tumor suppressor gene is overexpressed, it might contribute to a tumor formation by angiogenesis and invasion by preventing apoptosis and increasing genetic instability (Figure 1.4, B). It has been reported that overexpression of the oncogenic miRNAs, mir-21, mir-155 and mir-17-92 cluster are linked with tumor initiation and progression by repressing the expression of tumor suppressor genes, such as phosphatase and tensin homologue (PTEN), and programmed cell death 4 (PDCD4) [107–110]. miR-17-92 cluster is one of the first oncogenic miRNAs that was identified by Di Leva *et al.*, which consists of seven miRNAs: miR-17-5p, miR-17-3p, miR-18a, miR-19a, miR-20a, miR-19b-1, and miR-92-1 [69]. The role of miR-17-92 cluster as an oncogene was confirmed by L He

et al. in the E μ -Myc transgenic mouse model for B cell lymphoma [111]. Another oncogenic miRNA, miR-21, is up-regulated in almost all types of cancer. It's oncogenic activity was shown by J Liu *et al.* in transgenic mouse models with alternated miRNA-21 function combined with a mouse model for lung cancer [112]. It should be noted though that miRNA function depends on the expression of its target. Depending on the cell type, some miRNAs could function as oncogenes, as well as tumor suppressors. Presumably, miRNAs are not responsible for a specific phenotype by acting on a single target; instead they are involved in complex interactions with the transcriptome machinery and can simultaneously target multiple mRNAs. [90].

1.3 Challenges of MicroRNA Expression Analysis

According to the latest miRBase release (v20, June 2013, <http://www.mirbase.org/>), more than 30,000 mature miRNA sequences are listed, with ~ 2500 human miRNAs identified up to date, which can target more than 30% of the human genome [113]. Additionally Wu *et al.* experimentally showed that multiple miRNA clusters (from the same family) can target the same mRNA, which adds to the complexity of the miRNA gene regulation [114]. Given the role miRNA plays in human diseases, recent studies have shown that miRNA expression profiles can serve for diagnostic tests on a molecular level for diseases, as well as bases for novel therapeutics [115, 116]. Most importantly, recent identification of circulating miRNAs, have shown great potential of their use as biomarkers since they are readily available in blood samples [117, 118]. MicroRNA analysis, however, presents many challenges due to their low abundance, small size and sequence similarity between miRNA family members. Their small size makes their analysis more difficult compared to mRNAs, particularly with conventional molecular biology methods, such as polymerase chain reaction (PCR) and hybridization-based assays. The small size of probes that are used greatly affects the efficiency of these methods because of a very low melting temperature [119, 120].

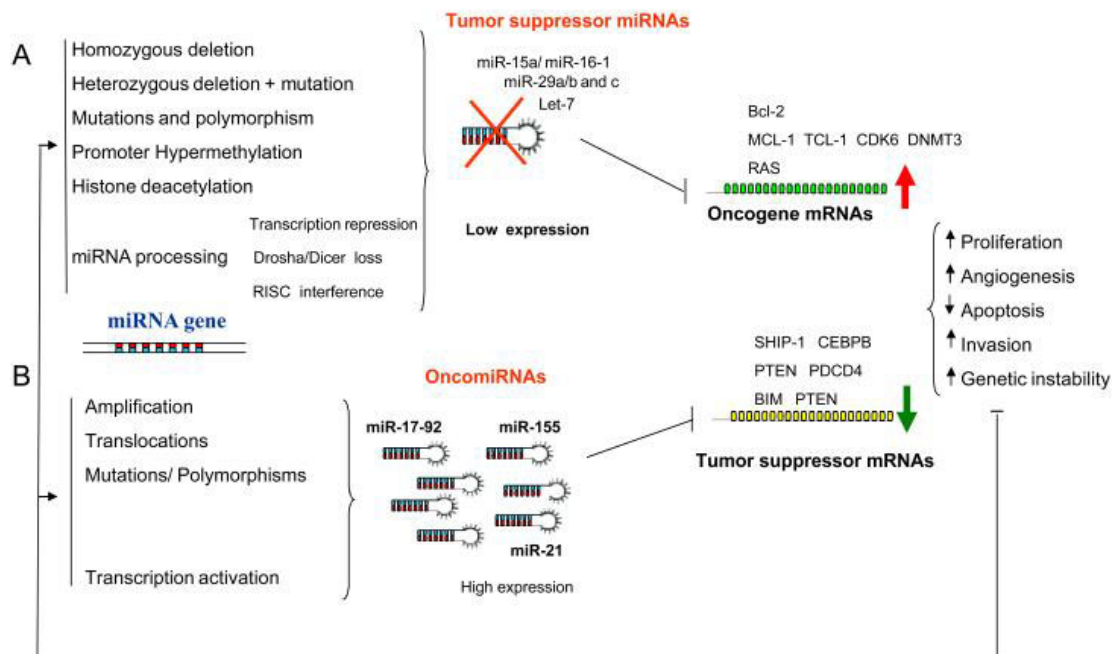


Figure 1.4: A basic model of miRNAs acting as tumour suppressors and oncogenes proposed by Garzon *et al.* [90]. a) When a miRNA down-regulates the expression of an oncogene, it can be categorized as tumor suppressor and is frequently seen to be lost in tumor cells. Loss of function of these miRNAs may occur due to any abnormalities in the miRNA biogenesis such as mutation, deletion or promoter methylation. These might produce an abnormal expression of the target oncogene, which then contributes to tumor formation, which will ultimately induce cell proliferation, invasion, angiogenesis and decreased cell death. b) MicroRNA that down-regulates tumor suppressor genes can be defined as an oncogene (oncomiR). If it is overexpressed, it might contribute to tumor formation by angiogenesis and invasion or by preventing apoptosis and increasing genetic instability. Modified and reprinted by permission from Macmillan Publishers Ltd: [Nature Reviews, Drug Discovery] [90], copyright (2010).

In this section the most widely used miRNA detection methods will be discussed.

Northern blotting is the standard method for microRNA detection and used for detection of both mature and precursor forms of miRNA, since it involves a size-based separation step. The Northern blotting protocol involves miRNA extraction, followed by polyacrylamide gel electrophoresis. Afterwards, separated sample is transferred to the blotting membrane, followed by visualization via hybridization with a complementary radioactively labeled probe. Though this method is relatively time consuming and requires a high amount of sample, it is widely used to validate data obtained with newer techniques [12,91,121,122]. Traditional northern blotting protocols have gone through a number of improvements in recent years, including the incorporation of locked nucleic acid (LNA) and digoxigenin (DIG)-labeled oligonucleotide hybridization probes, decreasing the detection limit to 0.05 fmol and reducing the exposure time by almost 1000-fold [123–127].

Microarray-based detection is one of the most common technique used for miRNA profiling [128]. A widely used strategy for microarray analysis is fluorescent labeling of miRNA followed by hybridization to capture probes on the array. Like northern blotting, by incorporating LNA capture probes to target miRNAs, the specificity of a microarray increases significantly. More importantly, through selective integration of LNAs, the melting temperature normalizes across all of the captured probe-target duplexes [129]. miRNA microarrays have been effectively used to study the biogenesis of miRNAs, differential miRNA expression profiles, disease characterizations, and stem cell development to name a few applications [120]. Despite having the benefit of usually being less expensive and allowing a large number of parallel measurements, microarray lacks specificity, sensitivity and the ability to perform absolute quantification of miRNA abundance. Thus, it is rather used as a miRNA screening method and not as a quantitative assay platform.

Real-time reverse transcription-PCR (qRT-PCR) is considered as the gold standard for gene expression analysis [130]. qRT-PCR-based miRNA profiling involves reverse transcription of miRNA to cDNA, followed by real time PCR of product. Reverse transcription

of miRNA to cDNA can be achieved either by using miRNA-specific reverse transcription primers, or by polyadenylation of all miRNAs by *Escherichia coli* poly(A) polymerase, followed by reverse transcription using universal primers consisting of an oligo (dT) sequence on its 5' end [131–134]. However, both of these approaches have reduced ability to detect miRNA modifications, such as 3' methylation and/or nucleotide substitutions [135, 136]. Over the years, qRT-PCR methods have improved the sensitivity of miRNA detection [137]. qRT-PCR is frequently used for miRNA expression profiling and validation of results found using other methods such as northern blotting, microarray [138].

Since the development of massively parallel/next-generation sequencing (NGS), there has been an increase of miRNA identification and discovery [139, 140]. NGS involves preparation of a cDNA library from the RNA sample, followed by the sequencing of millions of individual molecules. Obtained sequence reads undergo a bioinformatical analysis to identify and quantify (relative abundance) both known and novel miRNAs using application tools such as miRDeep [140, 141]. Although NGS is a high-throughput assay for miRNA expression profiling, disadvantages of this technique includes the high cost, the high amount of RNA used, and the sequence-specific biases due to enzymatic steps in cDNA library preparation [141]. Nonetheless, NGS has been recently used for differential expression analysis of miRNAs in different diseases, including ovarian cancer [142] and Huntington's disease [143], suggesting the usefulness of this technique for diagnostics and early detection.

1.4 Detection of Circulating MicroRNA

It has been reported that miRNAs are found in body fluids, such as blood, saliva and urine, of both diseased and healthy people. These extracellular circulating miRNAs exist in a stable form and are resistant to endogenous RNase activity, as well as extreme pHs and temperatures [117, 118]. The stability of circulating miRNAs are proven to be due to the fact that they are found to be packaged in the microvesicles, such as microparticles

and exosomes [144, 145], or associated with RNA binding proteins, including Argonaute 2 (AGO2) and nucleophosmin (NPM1) (see Figure 1.5) [146, 147]. Aberrant expression of circulating miRNAs in different diseases, such as stroke, cardiovascular diseases, breast cancer, ovarian cancer, gastric cancer, lung cancer, colorectal cancer, diabetes, hepatocellular carcinoma (HCC) and drug induced liver injury has been reported [118, 148–152]. Given that miRNAs are deregulated in different human diseases, they have great potential to be used as a non-invasive biomarker for disease diagnostics, particularly when they are easily detectable in biofluids, such as serum or urine [153]. miR-21 was the first serum-miRNA biomarker and was discovered by Lawrie and his group, in diffuse large B cell lymphoma. They showed that the levels of miR-155, miR-210 and miR-21 in diffuse large B cell lymphoma patient’s blood were higher than in healthy controls; and increased levels of miR-21 are thought to be associated with a relapse-free survival [154].

For circulating miRNA identification and quantification in human body fluids, high throughput profiling techniques, such as miRNA microarray and sequencing combined with qRT-PCR, have been applied [118, 155–160]. Due to a very low number of these molecules, miRNA measurements are challenging and affected by a number of issues, including RNA extraction efficiency, successful execution of methods and data analysis. In recent years, new technology-based methods have been reported [161, 162]. For example, Wang *et al.* proposed a label-free, nanopore sensor, based on the α -haemolysin protein, as a non-invasive method for early detection of cancer [162]. The method uses a programmable oligonucleotide probe to generate a target-specific signature signal that quantifies miRNAs associated with cancer and can distinguish single-nucleotide differences between miRNA family members. Even though the above-mentioned method is label-free, the use of a probe is necessary for such a method, making the miRNA detection indirect.

In Chapter 4 of this thesis, I describe a direct and multiplexed miRNA detection technique based on capillary electrophoresis-electrospray ionization-mass spectrometry (CE-ESI-MS).

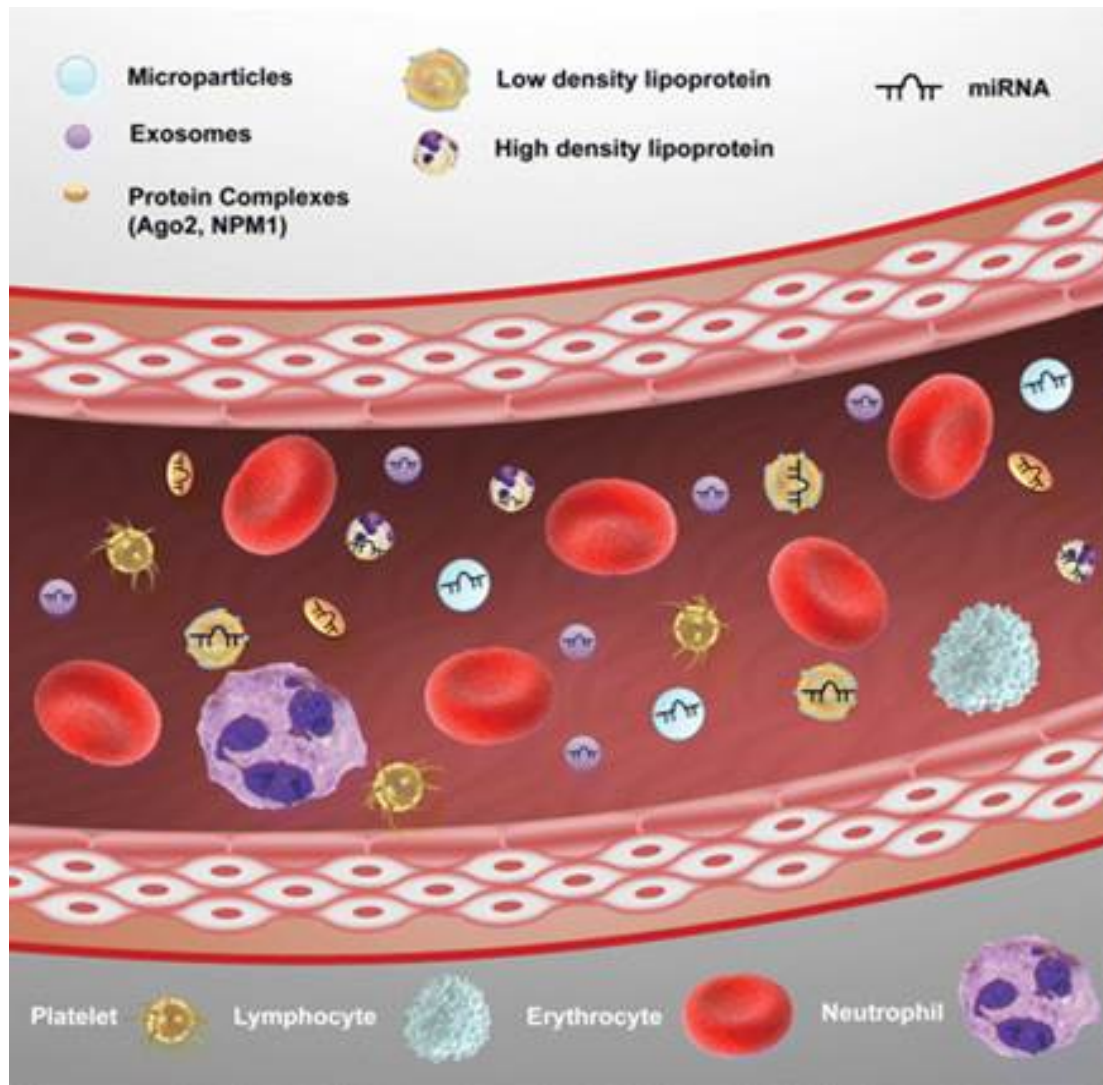


Figure 1.5: Compartmentalization of circulating miRNAs. Circulating miRNAs are contained within vesicles (exosomes, microparticles, apoptotic bodies), in protein complexes (AGO2, NPM1), and in lipoprotein complexes (HDL and LDL complexes). Although various tissues contribute to the circulating miRNA pool, most miRNAs are probably derived from blood cells. In response to injury, cardiac-specific miRNAs, which are otherwise undetectable, are released into the circulation. Reprinted by permission from Oxford University Press: [Cardiovascular Research] [163], copyright (2012).

1.5 Capillary Electrophoresis for MicroRNA Analysis

1.5.1 Background

Instrumentation in CE system is very simple compared to complexity of high performance liquid chromatography (HPLC) system. The separation in CE involves using an open or gel-filled capillary, whereas in HPLC prepacked columns are required that are expensive and has limited lifetime. The separation mechanism of HPLC involves partitioning of analytes between a liquid mobile phase and stationary phase. The diffusion characteristics of the analytes play an important role in HPLC separation. The separation efficiency is decreased with the increased size of the analyte, since larger molecules show relatively slow diffusion characteristic, which in turns works in CEs favor [164]. Moreover unlike traditional chromatographic methods, capillary electrophoresis (CE) has the advantage of high throughput, good separation efficiency, low sample consumptions ability, robustness and fast analysis in nucleic acid research [165,166]. Additionally, the popularity of CE in regular analytical application is due to the simplicity of the instrumentation and easy automation of analytical procedures. Furthermore, in recent years there has been an increase of application of CE with laser induced fluorescence (CE-LIF) for detection detection of trace amounts of miRNA in biological samples without amplification [167–173]. CE-LIF alone is well suited for low level miRNA detection using appropriate probes. Although multiplexing is difficult in CE detection, it has a number of features that makes it a method of choice such as the possibility of simultaneous pre-concentration and separation of analytes [174].

1.5.2 Capillary Electrophoresis: Instrumentation and Principles

The terminology "electrophoresis" is defined as the migration of charged molecules in an electrolyte induced by an applied electric field [175]. In an electric field, only charged species begin to move, while neutral species stay unaffected. Positively charged ions move toward

the cathode and negatively charged ions move toward the anode. Capillary electrophoresis is essentially an electrophoretic separation performed in an electrolyte (ie: buffer)- filled narrow (25-150 μm , id) capillary tube made of fused silica. Since the first introduction of CE in 1981, by Jorgenson and Lukacs [176], the technique has evolved into a versatile and powerful separation tool in analytical chemistry. Currently, CE is routinely applied in diverse analytical and bio analytical fields, such as forensic science [177], food analysis [178, 179], pharmaceutical sciences [180–182] and clinical diagnosis [183, 184].

Capillary electrophoresis is applied in various modes, each with a different separation mechanism related to electromigration. The most common technique is free solution capillary zone electrophoresis (CZE). Other modes include capillary isotachopheresis (CITP) [185, 186], often applied for sample pre-concentration, and capillary isoelectric focusing (CIEF) [187], utilized mostly for the separation of proteins in a pH gradient according to their isoelectric points. Moreover, by adding either liposomes [188, 189], micelles [190] or micro emulsions [191] to the electrolyte, nonionic compounds can be separated. In these cases, a pseudostationary phase is created that enables the separation through its electromigration; the modes are called liposome electrokinetic chromatography (LEKC), micellar electrokinetic chromatography (MEKC), and micro emulsion electrokinetic chromatography (MEEKC), respectively. In capillary gel electrophoresis (CGE) [192, 193], separation of analytes based on their size and shape occurs in a capillary filled with a gel or a polymer solution. In this thesis, CZE (referred to as CE from this point onward) was used for microRNA analysis in biological samples.

Capillary electrophoresis instrumentation is relatively simple, as shown in Figure 1.6, and consists of the following: two buffer (electrolyte) vials connected to a high-voltage power supply, two electrodes (a cathode and an anode), a fused silica capillary, and an on-column detector connected to a computer for data collection. After rinsing the capillary, sample is injected into the capillary pre-filled with buffer solution, from the inlet (opposite side of the detector). The separation is performed by applying a voltage of 10-30 kV across

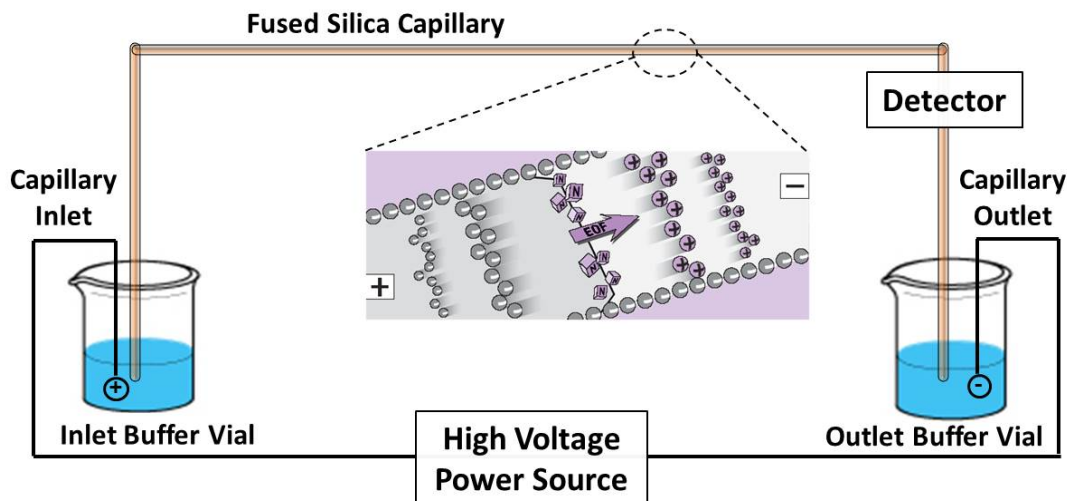


Figure 1.6: Schematic diagram of CE instrumentation. A zoomed view of the section of the capillary shows the direction of the electrophoretic mobility and the electroosmotic flow.

the length of the capillary as shown in Figure 1.6.

During the CE separation, in the presence of an electric field, a bulk flow of buffer occurs, which is known as the electroosmotic flow (EOF). Both the small diameter of the capillary and surface charges on the interior of the capillary wall contribute to the generation of EOF. Silanol groups (SiOH) present on the capillary wall deprotonate under aqueous conditions above pH 2.0 to 3.0. As a result, the inner surface becomes negatively charged (SiO^-). Cations from the buffer are electrostatically attracted to the negatively charged wall and bind tightly to the silanate groups, whereas a loose diffuse-layer (Figure 1.7, A) is formed by the rest of the buffer cations forming an electrical double-layer at the interface of silica-buffer. When voltage is applied to the buffer-filled capillary, the hydrated cations in the diffuse layer move towards the cathode placed at the outlet of the capillary, dragging the bulk solution along. During this process, no pressure drops are encountered along the capillary, resulting in a flat profile of EOF (Figure 1.7, B). This characteristic of EOF can help minimize zone broadening, as such resulting in high separation efficiencies.

In CE, separation of analyte ions occurs based on differences in their velocity due to

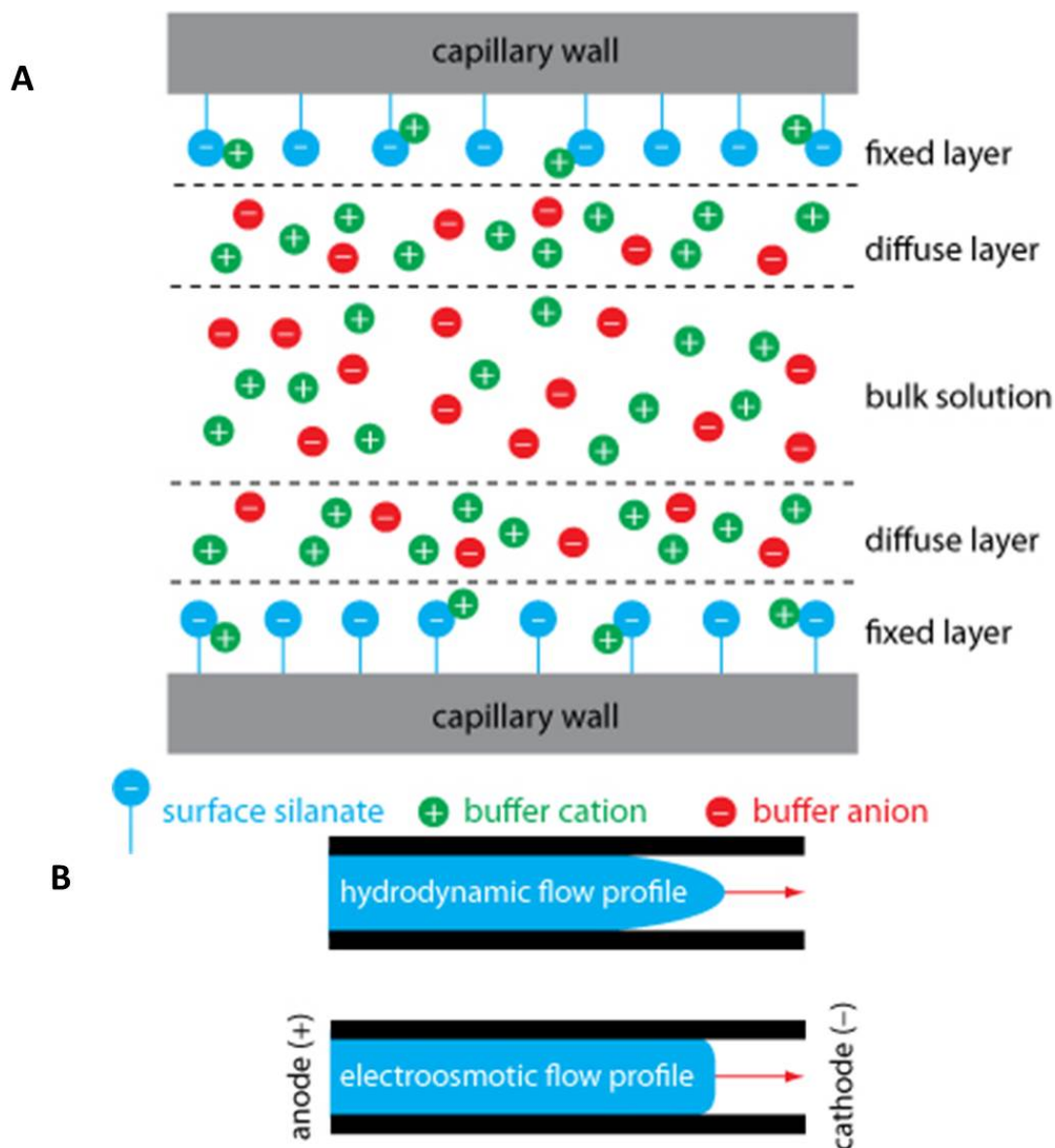


Figure 1.7: (A) Schematic diagram of EOF generation. (B) Schematic diagram of the EOF profile. EOF flat flow profile (bottom) and laminar flow profile (upper). Courtesy of David Harvey (2013), <http://community.asdlib.org>.

differences in their charge-to-size ratios. The higher the ratio, the quicker the ion migrates in an electric field. The net velocity of an ion (v_i) is proportional to its net mobility and the applied potential [194] as in the following equation 1.1:

$$\nu = \mu_{\text{app}} \times E \quad (1.1)$$

where E is the applied electric field (the ratio of applied voltage over capillary length) and μ_{app} is the apparent mobility of the ion. The latter affected by the electroosmotic mobility μ_{eo} of the buffer and the intrinsic electrophoretic mobility of the ion μ_{ep} , as in equation 1.2:

$$\mu_{\text{app}} = \mu_{\text{ep}} + \mu_{\text{eo}} \quad (1.2)$$

The magnitudes of μ_{app} and μ_{eo} can be directly determined from the migration time of charged analytes and the neutral EOF marker, respectively. It should be noted that μ_{eo} is not dependent on the electric field. For a buffer with a specific pH, the electrophoretic mobility (equation 1.3) of a charged ion can be described by the Debye-Huckel-Henry theory,

$$\mu_{\text{ep}} = \frac{q}{6\pi\eta r} \quad (1.3)$$

where q represents the net charge of the ion at a given pH, η is the buffer viscosity, and r is the hydrated radius of the charged analyte. From this relationship, it can be concluded that analytes that are small and highly charged have high mobilities, and analytes that are large and less charged have low mobilities.

The charge q can be determined by the buffer pH and the acid dissociation constants of the analyte, whereas the radius r is affected by many factors including buffer ionic strength, counter-ion effects, non-spherical shape or other non-ideal behaviors of molecules.

In free solution CE (FSCE), neutral analytes migrate with the same velocity and in the same direction as the EOF, in the time window between anions and cations and cannot be separated. On the other hand, anions and cations are separated based on differences in their apparent mobilities (μ_{app}). Cations move in the same direction as the EOF, have the same sign for electrophoretic (μ_{ep}) and electroosmotic mobility (μ_{eo}), thus apparent mobility of a cation is greater than electrophoretic mobility. The anions, on the contrary, move in the opposite direction than EOF and have the opposite signs for μ_{ep} and μ_{eo} . At moderate pH, EOF is higher than electrophoretic flow hence the apparent mobility of an anion is net cathodic and causes anions to elute towards the cathode (outlet). Thus, with buffer solution at pH greater than 3, large anions of -1 charge will migrate first, followed by small anions of -1 charge, whereas, small anions of -2 or more charge will migrate later. In our work, this basic principle of CE was applied to separate two nucleic acids using affinity CE.

1.5.3 Capillary Electrophoresis of Nucleic Acids

Instrumentation in the CE system is very simple compared to the complexity of high performance liquid chromatography (HPLC) system. The separation in CE involves the use of an open or gel-filled capillary, whereas in HPLC pre-packed columns are required, which can be costly and of a limited lifetime. The separation mechanism with HPLC involves partitioning of analytes between the liquid mobile and stationary phases. Diffusion characteristics of the analyte play an important role in HPLC separation. The separation efficiency of HPLC is decreased when the analytes size increases since larger molecules have a relatively slow diffusion characteristic, whereas this works in CE's favor [164]. Moreover CE is faster to run and more cost effective than HPLC. Capillary electrophoresis has been used for analysis of nucleic acids for over 30 years. [165, 166].

Capillary gel electrophoresis (CGE) is a powerful technique for the separation of large

nucleic acid with identical charge-to-mass ratios mainly due to its high efficiency and resolving power. In this technique cross-linked polyacrylamide gel or entangled polymer solutions such as cellulose derivatives is used as molecular sieving materials. Despite the advantage of CGE in large DNA separation it suffers some intrinsic systematic disadvantages such as the stability of gel-filled capillaries. Free solution CE separation is based on EOF and electrophoretic mobility of analytes, thus the difference between the size-to-charge ratios of ionic solutes results in good resolution [195].

The separation of nucleic acid (single or double stranded) in FSCE would be impossible due to the same size-to-charge ratio, as the nucleic acid chain grows, the charge increases proportionally since the ratio of phosphate group to base is always one to one. However, shorter nucleic acids (approx. 25bp), with different sequences can be partially separated by FSCE, because of the small differences in mass (or hydrodynamic ratio) and because for anions the net velocities as well as μ_{app} are low. Beyond a certain length, this relative difference is too small to completely resolve the peaks in FSCE. Therefore, large ssDNA has the same electrophoretic mobility as dsDNA of the same length, because their size-to-charge ratio is almost identical. Since miRNAs are relatively short oligonucleotides, FSCE is an appropriate technique for their analysis which is usually performed.

In CE, analytes, that form complexes through molecular interactions, can be simultaneously separated as long as the mobilities of the complexes differ. This technique is known as affinity CE (ACE), in which molecules participate in specific or non-specific affinity interactions during CE separation [196, 197]. Affinity CE is classified in three modes: (a) non-equilibrium capillary electrophoresis of equilibrium mixtures (NECEEM), in which target and ligand are first mixed off-column and the sample is then introduced into the capillary pre-filled with separation buffer; (b) dynamic equilibrium affinity electrophoresis or equilibrium capillary electrophoresis of equilibrium mixtures (ECEEM), in which a sample containing the target molecule is injected into the capillary and the ligand is added to the separation buffer; (c) affinity-based capillary electrochromatography (CEC)

separations using immobilized species, in which a ligand molecule is immobilized on the walls of a capillary, leading to affinity-capture of the target, its release from adsorbed state and separation by CE [196]. These techniques are used to analyze a variety of analytes in biological samples. In our work, ECEEM for protein-DNA and protein-RNA interactions was used.

Ultraviolet (UV) absorbance is the most common detection method used in CE; however, it lacks sensitivity because the effective absorbance path length across the capillary is small. On the other hand, laser induced fluorescence (LIF) is a sensitive method with low detection limits [198]. However, unlike with UV detection, fluorescent labeling of the analyte of interest is usually required. Detection of nucleic acids (DNA and RNA) by CE-LIF involves the use of fluorescent intercalating dyes or direct labeling. In the first approach, the dye added to the sample or CE buffer interacts with dsDNA or dsRNA by inserting itself between the base pairs of DNA or RNA. When excited by an appropriate wavelength, the DNA-dye or RNA-dye complex fluoresces strongly compared to the dye alone. In the latter approach direct labeling of the 3' end or 5' end of oligonucleotide probes complementary to the target DNA or RNA with suitable fluorophore permits detection upon hybridization by exciting with the appropriate laser excitation wavelength. Probes are either synthesized with a fluorescent dye, or labeled post synthesis with labeling kits. For nucleic acid detection, the most common fluorophores are fluorescein, modified fluoresceins such as fluorescein isothiocyanate (FITC), Alexa Fluor, Texas Red or tetramethylrhodamine [199]. In order to excite fluorescein dyes, 488 nm is used whereas for rhodamine dyes, 543.5 nm or 514.5 nm laser sources are used.

Capillary gel electrophoresis is considered to be a useful technique for the separation of DNA and RNA, ever since its use for DNA sequencing for the Human Genome Project in 1990s [200]. Several groups tried to replace the gel matrix with sieving polymers and achieve reproducible separation of DNA. Yin et al. performed a single-nucleotide polymorphism analysis using the CE-LIF system with poly-N,N-dimethylacrylamide (PDMA) instead of

cross-linked polyacrylamide gels. This polymer was used to overcome the problem observed with cross-linked polyacrylamide gels such as high viscosity and long separation time [201]. In the field of RNA research, various CE-LIF applications were also reported. Al-Mahrouki and co-workers [202] utilized a fluorescein labeled DNA probe to hybridize mRNA which was then directly analyzed and quantified by free solution CE-LIF. Affinity separation was mediated by single stranded DNA binding protein (SSB). In miRNA analysis, CE-LIF has been emerging as a method of choice due to its simplicity, reproducibility and sensitivity [167–173].

In this work, we used the CE-LIF system to detect miRNAs; however detection of endogenous miRNAs that have a low abundance in serum and cells is very difficult, even with the use of this sensitive technique. One practical way to make this possible is to concentrate samples using an on-line chemical enrichment approach. This can be accomplished by manipulating the background electrolyte (buffer) and sample composition along with sample injection procedures, without altering the instrumentation. One such example is sample stacking [203]. Briefly, sample stacking occurs as a result of the movement of sample ions across a boundary that separates the sample ion region from the background buffer region [203, 204]. Sample stacking can be conducted in both the electrokinetic and hydrodynamic injection mode. Here I will discuss hydrodynamic injection mode of sample stacking. The simplest sample stacking mode is the normal stacking mode (NSM) which is performed by preparing the sample in a low conductivity matrix and injecting hydrodynamically. Sample focusing occurs at the interface of the low-conductivity sample matrix and the high conductivity buffer. When the separation voltage is applied across the capillary, the sample region experiences a higher resistivity compared to the rest of the capillary. Consequently, a high electric field is set up in the sample region. As a result, the ions migrate rapidly under this high field toward the steady-state boundary between the lower concentration plug and the higher concentration run buffer. Once the ions pass the concentration boundary between the sample and the run buffer, they immediately ex-

perience a lower electric field and slow down, thus causing a narrow zone of analyte to be formed. Sample stacking of extremely large sample volumes is done by injecting the sample that has been dissolved in a low-conductivity matrix or water. Since in this mode, a larger volume of sample is introduced, the sample solvent (matrix) is moved out of the capillary to preserve the efficiency of the separation. The polarity of voltage during sample stacking is chosen based on the charge of the sample ion. A detailed theoretical and experimental sample stacking procedure is described elsewhere [203].

The utility of CE-LIF in different biological fluids, including serum, was reported in many studies [205,206]. For example, Hu and co-workers used CE-LIF to analyze fluorescently labeled noradrenaline (NA) and dopamine (DA) from human serum. For LIF detection, both compounds were labeled with a fluorescent reagent, 5-(4,6-dichloro-s-triazin-2-ylamino) fluorescein, which readily reacts with an amine group under mild conditions [205]. In another study, amines and amino acids in human serum were also analyzed, where indoleamines were detected by using a gold nanoparticle-based extraction with CE/laser-induced native fluorescence [206]. To our knowledge, no other work was reported on miRNA analysis in serum using CE-LIF.

There is a number of other CE detection techniques used as well. Among them, mass spectrometry (MS), owing to its high specificity and sensitivity. MS has for many years been an important technique for the analysis of different biomolecules, specifically in combination with HPLC and more recently with CE. In this thesis, CE with LIF detection as well as CE coupled on-line to MS through electrospray ionization (ESI) as the ion source were used for detecting very low amounts of miRNAs in biological samples.

1.6 Mass Spectrometry for Analysis of MicroRNA

1.6.1 Background

For the detection and characterization of various molecules, mass spectrometry (MS) has become an essential analytical technique due to its selectivity, sensitivity and accuracy [207,208]. MS has had a huge impact in almost all aspects of protein research. On the contrary, not so much impact in the field of nucleic acid research has been made, mostly because the traditional methods for the analysis of nucleic acids are quite powerful in many aspects. DNA or RNA can easily be amplified by PCR for detection and further analysis. Moreover, the next generation sequencing technique is able to sequence an enormous number of nucleotides in a short period of time with quantitative information. However, these techniques use polymerization-based copying of DNA or RNA. As a result, information on original modifications can be lost in newly produced nucleic acids [209]. Therefore, by detecting the characteristic mass change as a result of modification, MS becomes a valuable tool, offering a direct way of identifying multiple miRNA modifications. CE-MS has been applied in the field of proteomics, but rarely for analysis of small RNAs from biological matrices [210–212]. Recently MS has been used to screen siRNA in plasma samples for doping control purposes [213,214], although no endogenous small RNA has been detected with this method.

Mass spectrometry is a separation technique that separates ions based on their mass-to-charge ratio (m/z) in the gas phase. MS provides information on the analyte's molecular weight, and often allows the identification of molecules that have co-migrated in the upstream chromatography or electrophoresis separation. Even though different MS instruments are available with different capabilities and mass analysers (ie: quadrupole, ion trap, time-of-flight, etc), in general they are all composed of same major components, shown in Figure 1.8: an ion source, a mass analyzer, a vacuum pump, a detector and a data processing system. In this thesis, CE coupled online with an MS system having an electrospray

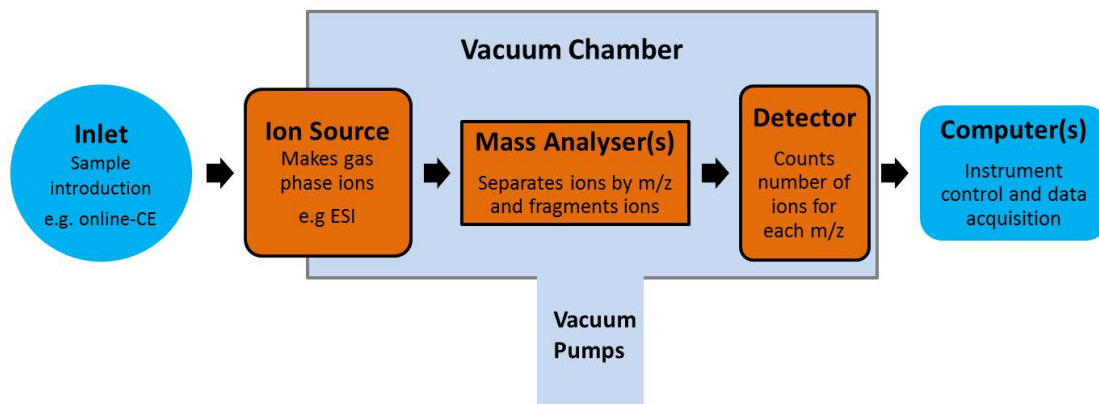


Figure 1.8: Simplified schematic for typical components of a mass spectrometer.

ion source (ESI) and a coupled quadrupole-time-of-flight analyser (Synapt G2 HDMS) was used to detect miRNA modifications.

1.6.2 Mass Spectrometry of Nucleic Acids

Most MS studies on nucleic acids use negative ion ESI mode, generating a series of ions of the $[M-nH]^{n-}$ -type, although use of the positive ion ESI mode generating $[M+nH]^{n+}$ ions of nucleic acids has also been reported [215]. However, negative-ion ESI-MS yields a better signal for nucleic acids. The maximum charge state that can be observed is equal to the number of phosphodiester bonds, or one less than the number of bases in an oligonucleotide. Since the phosphate group of the sugar unit has metal ion-binding sites, clusters corresponding to $[M-(n+m)H+mNa/K]^{n-}$ are also seen in the ESI spectrum. These metal ion adducts are disadvantageous in ESI-MS analysis since they decrease the analyte ion signal affecting mass measurement accuracy. Therefore, sample purity is essential for a successful ESI-MS analysis of oligonucleotides. An effective method used to suppress the signal from alkali-adducted ions is the addition of organic bases, such as trimethylamine, triethylamine, piperidine, imidazole or ammonium acetate in ESI sheath liquid [216].

1.6.3 Coupling of Capillary Electrophoresis to Mass Spectrometry

Though MS is capable of analyzing a variety of liquid phase samples, with the increased complexity of biological matrices, the quality of MS data can be significantly degraded. Thus to analyze biological samples, and to achieve the best sensitivity and selectivity, a separation step prior to MS is required. In CE-MS coupling, CE serves as a separation tool and resolves components from a complex analyte mixture. Although in direct infusion composition of the analytes entering the instrument is constant, there is a risk of ion suppression that potentially decreases sensitivity, particularly in the analysis of complex biological species that are low in abundance. Therefore, coupling of CE to MS is advantageous to analyze these types of samples.

Coupling of a fast and efficient CE separation technique with highly selective and sensitive MS detection makes CE-MS a powerful analytical tool, enabling determination of low abundance species. In 1987, Olivares *et al.*, described this hyphenation for the first time [217]. Since then, CE-MS has progressively been used for a variety of analytical studies. It is appropriate for analytes that are highly polar and charged, and which might be difficult for LC-MS methods to analyze [218]. The use of CE-MS for specific applications including metabolomics [219], proteomics [220–222], environmental [223], forensic [224], and clinical sciences [225] has been reported. CE-MS primarily uses electrospray ionization (ESI), a soft ionization technique, which under low-flow conditions (nL/min), allows charged or polar molecules to be efficiently transferred into the gas phase for MS analysis [226]. Except for ESI, a few other ionization techniques at atmospheric pressure (API) can also be coupled to CE, for example, atmospheric pressure photo ionization (APPI) and atmospheric pressure chemical ionization (APCI) [227,228]. Matrix assisted laser desorption ionization (MALDI) is another soft ionization technique that offers sensitive analysis for a variety of analytes, including nucleic acids. Since MALDI is a solid-state, pulsed technique, it cannot easily

be coupled online with liquid-based upstream separation methods such as CE and LC. Moreover, due to the need of maintaining high vacuum in the MALDI source, it greatly limits the online interfacing of CE with MALDI-MS [229]. Nevertheless, with independent optimization of the CE and MS conditions, off-line coupling can be used.

1.6.4 Electrospray Ionization

Electrospray ionization (ESI) was first coupled to MS by Malcolm Dole in 1968 [230] and later in 1980s, John B. Fenn and co-workers developed it for analysis of macromolecules [231, 232]. Earlier work on ESI by Dole [230] contributed to the work of Fenn for which shared the Nobel Prize with Koichi Tanaka in 2002 [231, 233]. Being a soft ionization technique, along with versatility and effectiveness, ESI is one of the most frequently used ionization techniques for charging large biomolecules in the gas phase [234]. The ESI process involves converting solution-phase analytes into gas-phase ions under the influence of an electric field [235, 236]. One major feature of ESI is that it produces multiply-charged ions of large molecules, without fragmentation (soft ionization). As a result, m/z values are lowered to a range that can be easily measured by different types of mass analyzers [237]. Nevertheless, multiply-charged ions produce more complex mass spectra, making their interpretation harder, and also reducing sensitivity. A strong electric field (3-6kV) under atmospheric pressure is applied between the capillary, which carries the analyte solution, and the skimmer cone at the entrance to the mass analyser. Depending on the polarity of analytes, aerosolized droplets formed by the electrospray may contain either an excess of positively or negatively charged ions. The source of these excess charges may be due to either oxidation or reduction reactions, for positive or negative modes respectively [238]. Once the coaxial sheath gas (dry N_2) is added the solvent from the charged droplets will evaporate. This process decreases the droplet size, which leads to coulombic repulsion between droplet charges. Further splitting of these droplets forms individual gas phase analyte ions. Under the influence of a very high electric field, ions are then directed

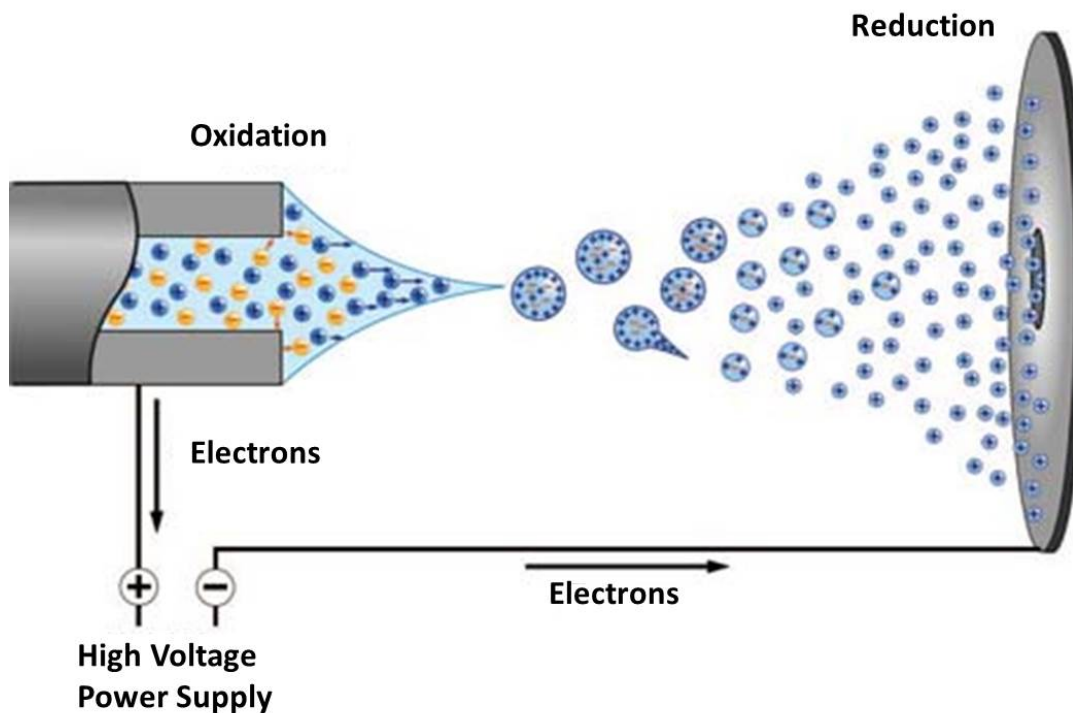


Figure 1.9: Schematic representation of an electrospray ionization source in positive ion mode. Generated ions are transferred into a gas phase. Courtesy of Andreas Dahlin (2008), <http://www.adorgraphics.com>.

towards the entrance of the mass spectrometer [239] (see Figure 1.9).

When interfacing CE to MS using ESI, a few important parameters need to be considered. For sample analysis, it is essential to maintain an electrical potential across the capillary in order to have a steady electrophoresis occurrence. To achieve this, most commonly, a sheath-flow CEMS interface (Figure 1.10) [240] is used. In this arrangement, a coaxial sheath liquid containing an organic solvent with an electrolyte is used to establish the electrical contact. This delivers a higher flow rate, maintaining stable potential for both electrophoresis and electrospray ionization. Buffer for CE separation also has to be optimized, independently of this interface. The main drawback of co-axial sheath flow interface is the possibility of analyte dilution and reduced sensitivity. However, since during the spray process sheath liquid is also evaporated, this effect may not be significant [241]. Stability and limit of detection of ESI-MS can be greatly improved by performing careful

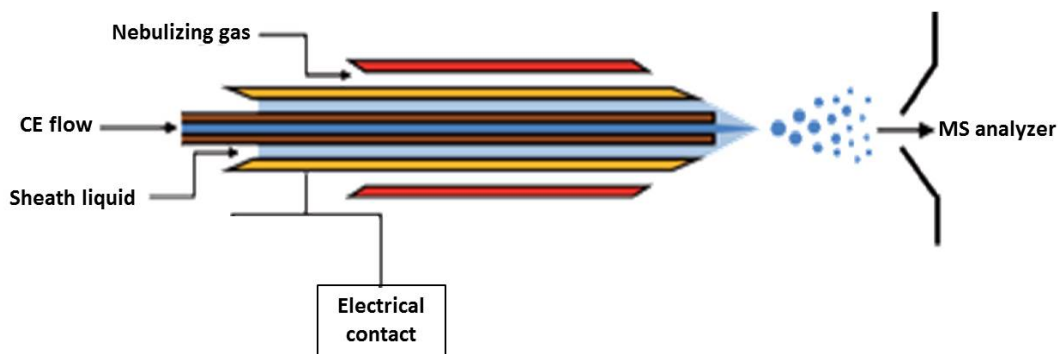


Figure 1.10: Schematic representation of a co-axial sheath-flow CE-MS interface. Courtesy of Rob Haselberg (2012).

optimization of the sheath-liquid composition, the flow rate and the position of the CE capillary tip with respect to the ESI capillary tubing. In this work (Chapter 3 and 4), for the on-line coupling of CE to ESI-MS, the co-axial sheath flow interface was used.

1.6.5 Mass Analyzers

Quadrupole Mass Analyzer

In this work, we used a quadrupole mass analyzer in conjunction with time of flight mass analyser (Q-TOF) (chapter 3 and 4). In this configuration, ions generated by ESI pass through the quadrupole mass filter into the high-resolution TOF analyzer for mass analysis. A quadrupole mass analyzer comprises of four parallel metal rods as depicted in Figure 1.11. Two opposite rods are paired and have equal voltages with opposite signs applied to them. A combination of variable direct current (dc) and radio frequency (rf) voltages that is applied will allow filtering of ions according to their mass-to-charge ratio [242]. Since all other ions will not have a stable trajectory through the quadrupole mass analyzer, they will collide with quadrupole rods, and thus will never reach the detector. After ion generation by electrospray, parent ions can be selected by using a quadrupole mass filter and then subject to fragmentation, or not, followed by separation by TOF.

Time of Flight Mass Analyzer

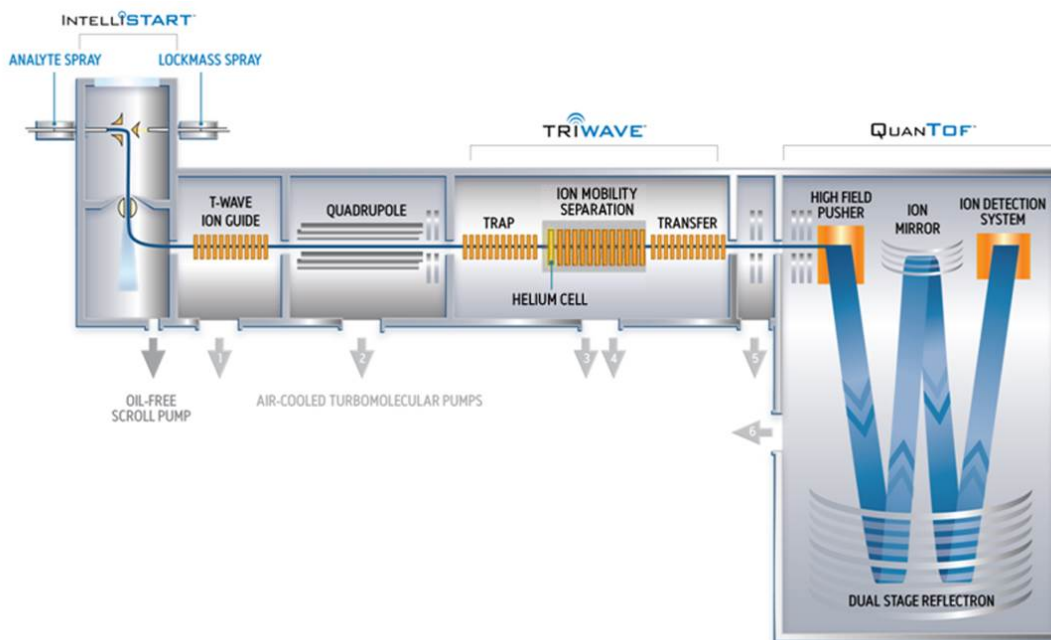


Figure 1.11: Schematic representation of the Waters Synapt-G2 HDMS. Courtesy of Kate Yu and Jeff Goshawk, Waters Corporation.

Time of flight mass analyzers (TOF) separates ions that move in a same direction with a constant kinetic energy but have different masses. By measuring the time ions require to travel a specific distance within a flight tube, we can determine the mass-to-charge ratio. The voltage (V) that is applied, the length of the flight path (L), and the time (t) that an ion takes to reach the detector, can directly be related to the ion's m/z according to the following equation 1.4: [241]:

$$t = \frac{L}{\sqrt{2V}} \times \sqrt{\frac{m}{z}} \quad (1.4)$$

In this work, we used a Q-TOF mass spectrometer, containing a high field pusher and a dual stage reflectron (see Figure 1.11). In this arrangement, ions are first selected by using the quadrupole mass filter, then travel down the flight tube to the dual stage reflectron, then reflect them towards the ion mirror, which, in turn, reflects the ions back to the dual stage reflectron, finally reflecting the ion to the detector (Figure 1.11). As a result, the

resolution of TOF-MS increases by increasing the distance that ions travel to reach the detector (i.e. the flight path), and by correcting the variation in initial velocity of ions of the same mass [243]. Ions with a lower mass-to-charge ratio fly faster than ions with a higher mass-to-charge ratio, thus separating them from each other.

Chapter 2

Analysis of MicroRNAs by Capillary Electrophoresis with Laser Induced Fluorescence Detection (CE-LIF)

2.1 Objectives

The objectives of this project was to develop a quantitative detection method for endogenous miRNAs using capillary electrophoresis with laser induced fluorescence detection (CE-LIF).

2.2 Introduction

A substantial part of data presented in this chapter (section 2.4.1 - 2.4.3) has been published in a peer-reviewed journal, *Analytical Chemistry* [172].

Many bioanalytical methods that have been established to identify and quantify miRNAs include northern blotting [244], in-situ hybridization [245], small-RNA library sequenc-

ing [12], bead arrays [246], microarray hybridization [247], and reverse transcription polymerase chain reaction (RT-PCR) [248]. A few methods were capable of detecting miRNA directly from cell lysate without RT-PCR [249–251]. Some miRNA species have low copy numbers per cell making it difficult to detect small changes in their expression [244]. With such minute differences in miRNA expression between normal and cancerous cells, high sensitivity is essential.

In this part of the project, we introduce two methods for protein-facilitated affinity capillary electrophoresis (ProFACE) assay of hybridization products for sensitive and quantitative detection of small RNAs. CE-LIF [198] is a promising technique for miRNA analysis as miRNAs are easily separated from most of the other biomolecules, except for other nucleic acids, due to their strong negative charge. Some advantages of ProFACE-LIF over other methods include high sensitivity and selectivity toward the targeted molecules, rapidity of CE separation, and the ability to screen complex samples.

In our two ProFACE-LIF methods, two different affinity ligands (proteins) were used in conjunction with two hybridization probes complementary to the target miRNA were used for annealing (referred to as hybridization henceforth) miRNA to form a miRNA-probe duplex through hydrogen bonding. One of the probes was fluorescently-labeled single-stranded DNA and the other one was fluorescently-labeled single-stranded RNA. The probes were added in excess to ensure complete hybridization of target miRNA. The hybridization products (i.e. miRNA - DNA or miRNA - RNA duplex) and the excess (i.e. non-annealed) probes are separated by ACE. We used two proteins, single-stranded DNA binding protein (SSB) for the first method and an RNA binding protein p19 for the second method, as affinity ligands to enhance the separation between excess fluorescently labeled probe and the miRNA-probe duplex (Figure 2.1).

Previously, SSB has been used to distinguish single stranded DNA from double stranded (ds) DNA using CE [252]. Protein SSB binds to ssDNA ($K_d < 50$ nM) as well as to ssRNA of eight bases or more but does not bind to dsDNA, dsRNA, or dsDNA-RNA

hybrid [253, 254]. Furthermore, the affinity of ssDNA binding to SSB is about 10 times higher than that of ssRNA to SSB [255]. When ssDNA probe binds to the bulky and neutral SSB, it forms a complex which migrates at a faster rate than the free ssDNA which in turn significantly increases the separation time between the excess probe and miRNA-probe duplex (Figure 2.1A).

The protein used for the second assay, p19 (19 kDa), is expressed by plant viruses to function as a suppressor of the RNA silencing pathway by binding and sequestering small dsRNAs [256]. The p19 protein binds only to dsRNA in a size-specific manner [257, 258]. The length of the double stranded (miRNA-RNA probe) is critical for high affinity binding. The p19 protein binds tightly to 21-23 base pair dsRNA but progressively weaker to 24-26 base pair dsRNA and poorly to 19 base pairs and less. The basis of the binding is the electrostatic and hydrogen bonding interactions between the β -sheet formed by the p19 homodimer and the sugar phosphate backbone of dsRNA, thereby making its binding independent of the RNA sequence but dependent on RNA total length. The p19 protein acts as a molecular caliper for selected miRNAs based on the length of the duplex region of the RNA. Binding is enhanced with miRNAs containing 5'-phosphate groups since they interact with tryptophan residues on the end capping helices of the p19 dimer for stabilization. The p19 protein does not bind to ssRNA, rRNA, mRNA, ssDNA, or dsDNA. Unlike SSB, p19 binds to the miRNA-RNA probe duplex directly, allowing its separation from the excess probe in CE (Figure 2.1B) since p19 has minimal affinity toward single stranded species and DNA.

In this work two different p19 proteins were used: one (p19 protein) for the ProFACE assay, the other (p19 magnetic beads) for the extraction of specific miRNA from serum. We developed a novel ProFACE miRNA detection method without PCR amplification in serum that applies the unique binding properties of p19 to a liver-specific miRNA, miR-122 whose expression has been shown to be repressed in liver cancer [259]. The expression of miR-122 is 70% of all miRNAs expressed in the human liver [260]. We also explored two

other CE-LIF methods involving sample preconcentration but not affinity CE, as a means to detect lower amount of endogenous miRNAs.

2.3 Materials and Methods

2.3.1 Materials

Chemicals and Materials: Single-stranded DNA binding protein (SSB) from *E. Coli* was purchased from Sigma-Aldrich (Oakville, Canada). Carnation Italian Ring spot virus (CIRV) p19 protein was expressed and purified as described below in section 2.3.3. The masking DNA (20 nt scrambled synthetic oligonucleotide) was purchased from IDT DNA technologies (Coralville, USA) and yeast tRNA was purchased from Sigma-Aldrich (Oakville, Canada). Buffer components were obtained from VWR (Mississauga, Canada). The bare silica capillary was purchased from Polymicro Technologies (Phoenix, USA). All solutions were made using Milli-Q-quality deionized water and filtered through a 0.22 μm filter.

MicroRNAs and Hybridization Probes: For method optimization and serum experiments the miRNA called miR-122 and its complementary fluorescently (referred to as FAM: 3',6'-dihydroxyspiro[2-benzofuran-3,9'-xanthene]-1-one) labeled hybridization probes (DNA and RNA) were used. For endogenous miRNA detection, Alexa Fluor 488 (referred to as Alexa 488; chemical name: (2Z)-3-[6-[5-[[2-[(E)-[(2R,3S,5S,8S,9S,10R,13S,14S,17R)-17-[(2S,3R,4R,5S)-3,4-dihydroxy-5,6-dimethylheptan-2-yl]-2,3-dihydroxy-10,13-dimethyl-1,2,3,4,5,7,8,9,11,12,14,15,16,17-tetradecahydrocyclopenta[a]phenanthren-6-ylidene]amino]oxyacetyl]amino]pentylamino]-6-oxohexyl]-2-[(2E,4E)-5-[3,3-dimethyl-5-sulfo-1-(3-sulfopropyl)indol-1-ium-2-yl]penta-2,4-dienylidene]-3-methyl-1-(3-sulfopropyl)indole-5-sulfonic acid) labeled hybridization probes (only DNA) complementary to the miRNAs miR-122, miR-21 and miR-16 were used. All miRNA analyte standards and hybridization probes were custom synthesized by IDT DNA Technologies (Coralville, USA).

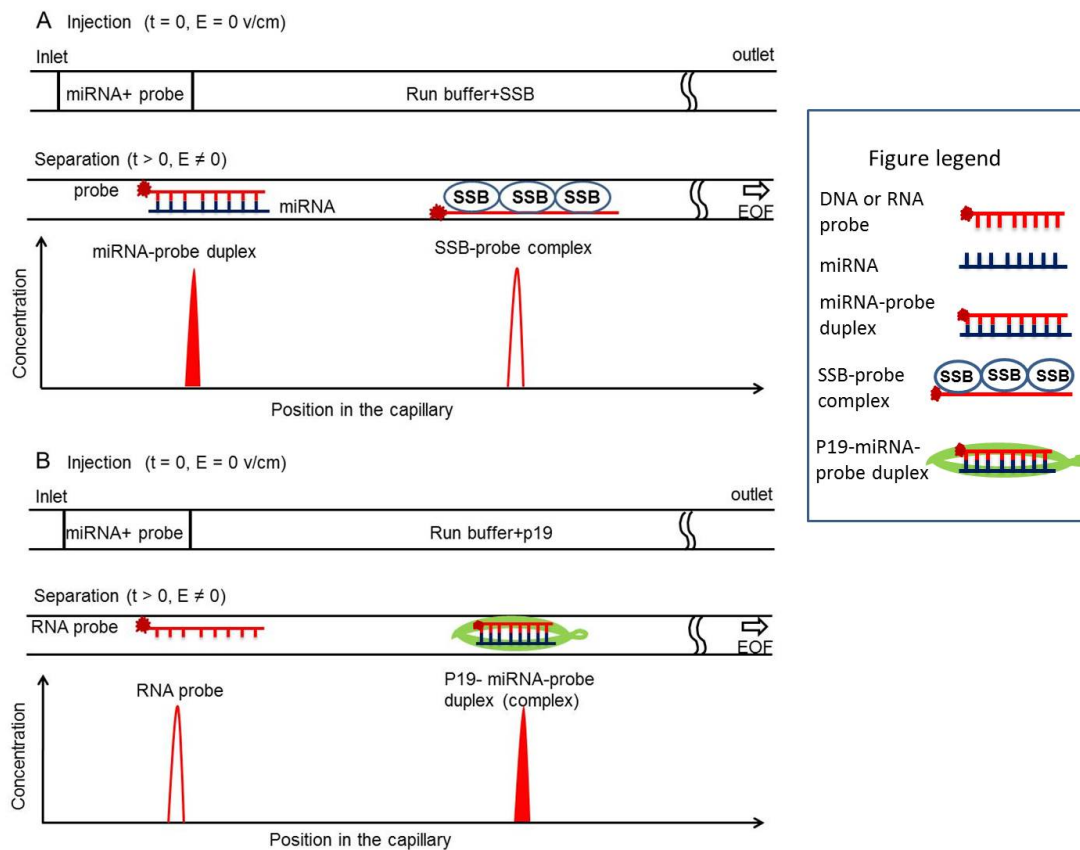


Figure 2.1: Schematic diagram of the protein-facilitated affinity capillary electrophoresis (ProFACE) assay for miRNA detection. A mixture of a miRNA and excwss of fluorescently labeled ssDNA or ssRNA probe complementary to miRNA is injected as a small plug into a capillary and subjected to electrophoresis. (A) When SSB is added in the run buffer, it binds only to the unhybridized DNA or RNA probe and enhances the separation of the miRNA-probe duplex from the excess probe. (B) When p19 is present in the run buffer, it binds only to the miRNA-RNA probe duplex and shifts the latter's mobility far away from the excess free probe, increasing resolution and sensitivity of the assay. (Reproduced and modified from [172] with permission from ACS publications).

The standards miR-122 and miR-21 and miR-16 had the following sequences respectively:

5’-/Phos/UGGAGUGUGACAAUGGUGUUUG-3’

5’-/Phos/UAGCUUAUCAGACUGAUGUUGA-3’ and

5’-UAGCAGCACGUAAAUAUUGGCG-3’.

The DNA and RNA probes complementary to miR-122 (used in ProFACE experiments) had the following sequences respectively:

5’-/Phos/CAAACACCATTTGTCACACTCCA/36-FAM/-3’ and

5’-/Phos/CAAACACCAUUGUCACACUCCA/36-FAM/-3’.

The DNA probes complementary to miR-122 and miR-21 (analyte) for cell experiments had the following sequences respectively:

5’-/Phos/CAAACACCATTTGTCACACTCCA/3AlexF488N/-3’ and

5’-/Phos/TCAACATCAGTCTGATAAGCTA/3AlexF488N/-3’.

Poly (A) DNA probe complementary to miR-21 and poly (T) cholesterol tagged Alexa Fluor 488 labeled probe for serum experiments had the following sequences respectively:

5’-AAAAAAAAAAAAAAAAAAAAATCAACATCAGTCTGATAAGCTA-3’

5’-/5AlexF488N/TTTTTTTTTTTTTTTTTTTTTTT/3CholTEG/-3’.

The DNA probe complementary to miR-16 (analyte) for serum experiments had the following sequence:

5’-CGCCAATATTTACGTGCTGCTA/3AlexF488N/-3’

2.3.2 Hybridization Conditions

The hybridization was carried out in a PCR thermocycler (Mastercycler pro S, Eppendorf, Germany) in the following incubation buffer: 50 mM Tris Acetate, 50 mM NaCl, 10 mM

EDTA, pH 8.1. Temperature was increased to a denaturing 60°C for DNA probe (55°C for RNA probe) and then lowered to 20°C in decrement steps of 1°C every three seconds to allow annealing. To ensure that all miRNA analyte was hybridized prior to injection into the CE, excess of the DNA and RNA probes were used. For very low concentration samples, hybridization was performed at 75°C for 5 min followed by 52°C for 7 h before lowering the temperature to 20°C in decrement steps of 1°C every three seconds. Hybridization conditions were optimized using varying temperatures and times.

2.3.3 Expression and Purification of p19 protein

p19 protein was kindly provided by Dr. John P. Pezacki lab and expression and purification of p19 protein was carried out by them as follows.

Construction of the pTriEx-p19 plasmid encoding the codon-optimized Carnation Italian ring spot virus p19 protein with a C-terminal octa-histidine tag was described previously [261]. Sequence of the resultant DNA plasmid was confirmed by DNA sequencing. Bacterial expression of the His-tagged p19 protein was carried out as previously described [261] with the following modifications. Briefly, *E. Coli* strain BL21 (DE3) cells harbouring the p19 construct were grown at 37°C until an optical density at 600 nm (OD_{600}) of 0.5-0.6 was achieved. Expression of the p19 protein was induced by IPTG at a final concentration of 1 mM. Cultures were then grown for an additional 2-3 h at 28°C or until OD_{600} reaches 1 to 1.5. After harvesting, bacterial pellets were resuspended in lysis buffer (50 mM Tris-HCl, 300 mM NaCl, 10 mM imidazole, 1 mM dithiothreitol (DTT), 1 X Complete Protease Inhibitor Cocktail from Roche, pH 8.0) and lysed by sonication on ice-bath. Cell lysate was then centrifuged at $20,000 \times g$ for 20 min at 4 °C . Soluble lysate fraction containing the His-tagged p19 proteins was loaded on to HisTrap FF nickel affinity column (GE Healthcare, Piscataway, USA). After protein binding, the resin was washed with 10 column volumes of wash buffer (50 mM Tris-HCl, 300 mM NaCl, 50 mM

imidazole, pH 8.0). Elution of the His-tagged p19 proteins was carried out using elution buffer (50 mM Tris-HCl, 300 mM NaCl, 250 mM imidazole, pH 8.0) and 10 mM of DTT was added immediately to the elute. The pooled elutes were concentrated to 0.5 ml using the Amicon Ultra 10-kDa MWCO centrifugal filter device (Millipore, Concord, MA). The concentrated samples were then injected into a size exclusion column (Superdex 200, GE Healthcare, Piscataway, USA) equilibrated with 20 mM Tris, 50 mM NaCl, 1 mM EDTA, 5 mM TCEP, pH 7.4) then eluted at a flow rate of 0.5 mL/min. Fractions containing the desired p19 proteins, as determined by SDS PAGE analysis, were pooled and stored at 4°C for subsequent assays and analyses.

2.3.4 MicroRNA Extraction and Preconcentration from Serum using p19 Bead

MBP-p19-CBD fusion protein bound to magnetic beads was obtained from New England BioLabs (Ipswich, U.S.A.). The p19 has the N-terminal fusion of the maltose binding protein (MBP) and the C-terminal fusion of the chitin binding domain (CBD). The MBP helps in p19 purification, and the CBD allows p19 to bind very tightly to chitin magnetic beads. The p19 beads were resuspended in the p19 binding buffer (20 mM Tris-HCl, 100 mM NaCl, 1 mM EDTA, 1 mM TCEP, 0.02% Tween-20, pH 7.0) mixed with a brief vortex, and 15 μ L of the suspension was placed into a 1.5 mL tube. The concentration of p19 was 0.3 μ g/ μ L in the bead suspension.

After removing the supernatant from beads by a magnetic separation rack (Millipore, U.S.A.), the beads were incubated with the mixture of 1 mL of 100% fetal bovine serum and 200 μ L of the p19 binding buffer spiked with 100 nM masking DNA, 100 nM tRNA, 1 μ L of murine RNase inhibitor (40 units, NEB, no M0314), 0.5 fM to 500 pM miRNA-122, and 1.25 nM miR-122 probe. The binding reaction was then incubated by shaking on a benchtop shaker for 2 hours at 23°C .

Unbound RNA was removed by washing 10 times with 500 μ L of 1 $\times$ wash buffer (20 mM Tris-HCl, 100 mM NaCl, 1 mM EDTA, 100 μ g/mL BSA, pH 7.0) that was preheated to 37°C . For each wash, the beads were shaken for 5 min at room temperature. The bound miRNA was eluted from beads in 10 μ L of 1 $\times$ p19 elution buffer (20 mM of Tris-HCl, 100 mM of NaCl, 1 mM of EDTA, 0.5% SDS, pH 7.0) by shaking for 10 min at 23°C followed by another 10 minutes of incubation at 37°C . The eluted 10 μ L of miRNA was analyzed by capillary electrophoresis.

2.3.5 Cell Culture

The human hepatoma cell lines Huh-7 and Hep-G2 were grown in monolayers on a 25cm² flask in Dulbeccos Modified Eagle Medium (DMEM) (Sigma-Aldrich, Oakville,ON) supplemented with 100 nM nonessential amino acids, 50 U/mL penicillin, 50 μ g/mL streptomycin, and 10% (v/v) heat-inactivate Fetal bovine serum (Sigma-Aldrich, Oakville,ON) in an incubator at 37°C with 5% CO₂. 5% CO₂ is used to maintain the pH of the cell culture media in order to ensure that the buffer works as intended.

2.3.6 RNA Extraction from Cells and Serum Samples

Cellular RNA extraction: Small RNAs were isolated from cells using miRVANA miRNA isolation kit (Ambion, USA) according to manufacturer's protocol. Briefly, cells were first lysed in a denaturing lysis solution. The lysate was then extracted with Acid-Phenol:Chloroform mix, which is a 125:24:1 mixture of phenol:chloroform:isoamyl alcohol with pH 4.3-4.7 (Ambion, P/N 9720). One-third volume of 100% ethanol was then added to the aqueous solution followed by passing through the filter cartridge containing a glass-fiber filter. Two-third of 100% ethanol was then added to the filtrate and passed through the second filter followed by three washes. Small RNAs were then eluted in 100 μ L RNase free water.

Serum-Circulating RNA extraction: Two samples of healthy human serum were thawed on ice. β -Mercaptoethanol was added to the lysis solution (obtained with the RNA purification kit, company proprietor) before mixing with each serum sample to inactivate RNases. Then, the total RNA was isolated from each serum sample using plasma/serum circulating and Exosomal RNA Purification Kit (Norgen BioTek corporation, Canada) according to the manufacturers protocol. Briefly, each sample was mixed with 3.8 mL of lysis solution and 200 μ L of the separation matrix. The mixture was then vortexed for 30 sec followed by incubation at 60°C for 20 min. Next, 3 mL of 100% ethanol added and mixed by vortexing for 30 sec. Afterwards, the mixture was centrifuged using a swinging bucket rotor for 1 min at 300 relative centrifugal force (RCF), and then carefully decanted in order to ensure that the slurry pellet was not dislodged. The binding solution was then added to the pellet, and incubated at 60°C for 10 min. After adding 300 μ L of 100% ethanol, the slurry was loaded onto a mini filter spin column. This was followed by washing the bound RNA three times to remove the remaining proteins and other impurities. The purified total miRNA, obtained from each sample, was then eluted in 60 μ L RNase free water.

2.3.7 Capillary Electrophoresis

CE Separation: CE analyses were performed using a ProteomeLab PA 800 CE system (Beckman-Coulter, Brea, USA) equipped with laser induced fluorescence detection. Fluorescence was excited with a 488 nm line of an Ar-ion laser and emission was monitored at 520 ± 10 nm. Separations were carried out using a bare fused-silica capillary of 30 or 50 cm in total length (20 or 40 cm from the injection to detection point, respectively) with an outer diameter of 365 μ m and an inner diameter of 75 μ m. Hydrodynamic injections of 10 or 140 nL were made by applying a pressure pulse of 20 mbar for 4 sec or 103 mbar for 10 sec for experiments without and with preconcentration, respectively. The separations were conducted by applying an electric field of 400 V/cm (positive charge at the inlet and

ground at the outlet). The temperature of the capillary was 15°C . Data were collected and analyzed using 32 Karat version 8.0 software (Beckman Coulter). The background electrolyte (buffer) was 25 mM sodium tetraborate at pH 9.2. For ProFACE, the background electrolyte (buffer) was supplemented with SSB protein in concentrations ranging from 0 to 100 nM or with the p19 protein in concentrations ranging from 0 to 500 nM. At the start of each run, prior to injection, the capillary was rinsed sequentially with 0.1 M of HCl for 2 min, 0.1 M of NaOH for 2 min, deionized water for 2 min and the run buffer for 4 min.

Limit of Detection and Resolution Mesearments: Different concentrations of miRNA standards (from 1 pM to 20 nM) were hybridized with 50 nM ssDNA or 50 nM ssRNA fluorescent probes. The background electrolyte (buffer) was supplemented with either 1-50 nM SSB or 10-250 nM p19. LOD was defined as the lowest concentration of miRNA when its duplex had the signal to noise ratio of 3. Noise was determined by using the standard deviation of all peaks in the control (blank) sample. Resolution was calculated according the following expression:

$$R = \sqrt{2(\ln 2)} \times \frac{(t_2 - t_1)}{(W_{h1} + W_{h2})} \quad (2.1)$$

where W_{h1} is the width of peak 1 at half its height, W_{h2} is the width of peak 2 at half its height, and t_1 and t_2 are the migration times of peaks 1 and 2, respectively [262].

2.4 Results and Discussion

2.4.1 Developement of ProFACE-LIF Assay of miRNA using SSB

The SSB-mediated ProFACE-LIF assay was investigated with two types of probes. Prior to CE separation, 22 nt miRNA-122 (miR-122) was incubated with an excess of either fluorescently labeled ssDNA probe or fluorescently labeled ssRNA probe. The temperature was increased to 60°C (55°C for the RNA probe) and slowly decreased to 20°C to allow proper hybridization of the probes with its complementary miRNA. Free-zone capillary electrophoresis without SSB showed little separation in 25 mM borax run buffer pH 9.2 or no separation in 50 mM tris-acetate run buffer pH 8 between the probe and miRNA-probe duplex (Figure 2.2 and Figure 2.3, for DNA and RNA probes, respectively). Every nucleotide in DNA or RNA bears a single negative charge. Therefore, the charge-to-size ratio of both DNA and RNA is highly negative and does not depend on their length or hybridization state. As a result, a single-stranded probe and double-stranded duplex have similar electrophoretic mobilities in free solution electrophoresis.

Poor separation between the excess probe and miRNA hybrid does not allow accurate quantification of the target miRNA. Therefore, we added 50 nM SSB into the run buffer to facilitate separation between the probe and the duplex (Figure 2.2 and Figure 2.3, for DNA and RNA probes, respectively). An SSB molecule is composed of 178 amino acids and bears only a small negative charge of -4 at pH 9.0 [263]. Thus, the electrophoretic mobility of SSB is higher than those of a probe and duplex. SSB binds to the probe but does not bind to the duplex. Upon binding to the probe, SSB increases its electrophoretic mobility, whereas the mobility of the duplex is decreased as SSB lowers the electroosmotic flow in a capillary. Apparent mobility of the duplex is decreased as SSB greatly modify the electric double layer and the viscosity in a capillary. The optimum resolution of the

probe-SSB complex and RNA-DNA duplex is achieved when the concentration of SSB is in the range of 10-50 nM. The LOD for the DNA probe without SSB was 210 pM, and with SSB was 561 pM. Addition of SSB increased the width of the peak; nevertheless, the resolution was significantly better, making it easier to calculate the amount of the target miRNA. Thus, we demonstrated that SSB effectively increases the separation of DNA and RNA probes, from their hybridization products with miRNA.

2.4.2 Developement of ProFACE-LIF Assay of MicroRNA using p19

The p19 protein has been shown to bind double-stranded miR-122 with a dissociation constant of ~ 4 nM [251]. For our miRNA hybridization assay, we designed an RNA probe complementary to miR-122 so that the length of the duplex region is still in the optimal range for p19 binding. The probe also contains a phosphate group at the 5' end to ensure high affinity binding. In vitro binding studies of p19 with various small RNAs also suggest that miRNA with the absence of a 2 nt 3' overhang may slightly enhance binding of p19 [261]. Thus, the probe was also optimized for p19 binding so that it forms a blunt-ended duplex when hybridized to the target miRNA.

We applied this natural selectivity of p19 and utilized it as a separation enhancer (Figure 2.4). Protein p19 was a molecular engineered dimer made by linking two monomers with a peptide linker (GGGSGGGGS), connecting the C-terminus of one monomer to the N terminus of another. After adding 10 to 250 nM p19 in the run buffer, the CE resolution between peaks of the duplex and the probe doubled from 7.9 to 15.9. The LOD of miR-122 detection was 1.2 nM from experiments with different concentrations of miRNA and 50 nM RNA probe in the presence of 250 nM p19 in the run buffer (Figure 2.5). The response was linear in the range of 1-200 nM miRNA (Figure 2.6). No separation between miRNA/DNA and DNA probe was observed due to the lack of binding of p19 to the heteroduplex. This

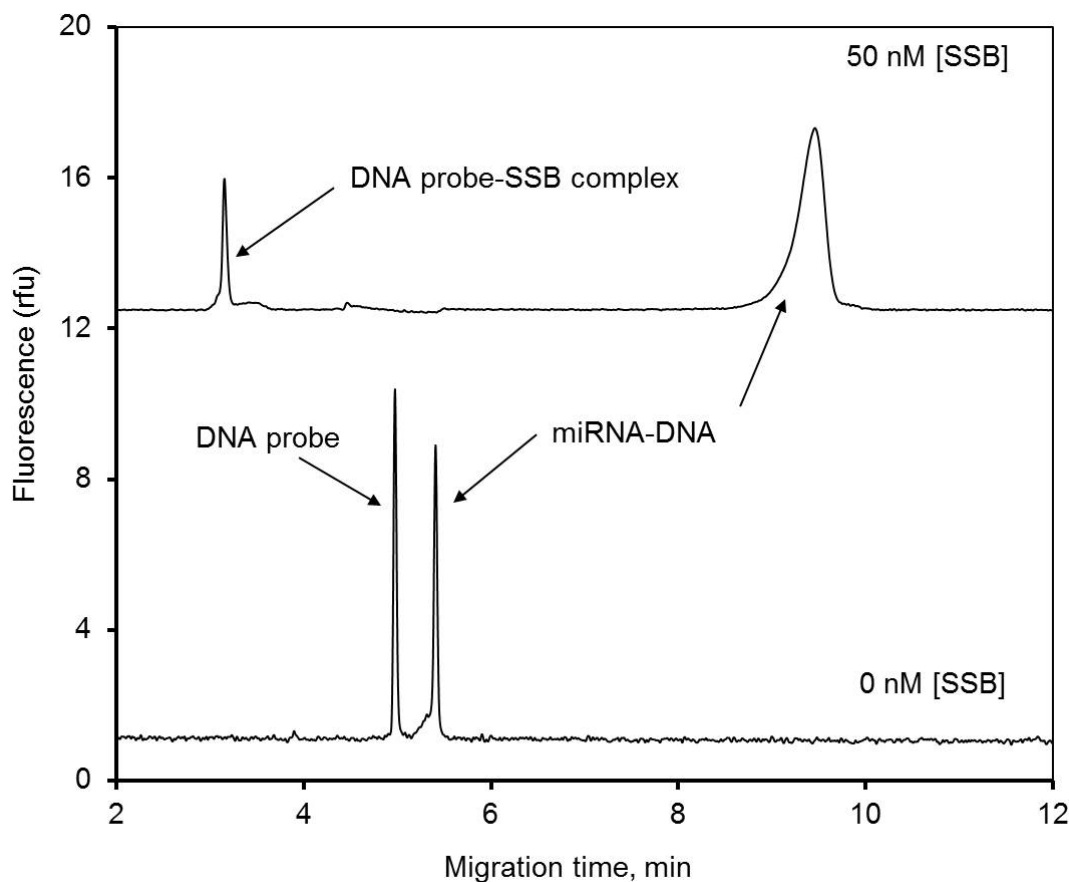


Figure 2.2: Representative electropherograms of ProFACE-LIF assay of miRNA-DNA probe duplex from the excess fluorescent ssRNA probe. Separation of miRNA-DNA probe duplex from the DNA probe without SSB (the bottom electropherogram) and with 50 nM SSB (the top electropherogram) in 25 mM borax pH 9.2 run buffer. Equilibrium mixture prior to injection contained 40 nM miRNA and 90 nM DNA probe. Hydrodynamic injections of 10 nL was made by applying a pressure pulse of 20 mbar for 4 sec for 10 sec for experiments. The separations was conducted by applying an electric field of 400 V/cm. (Reproduced and modified from [172] with permission from ACS publications).

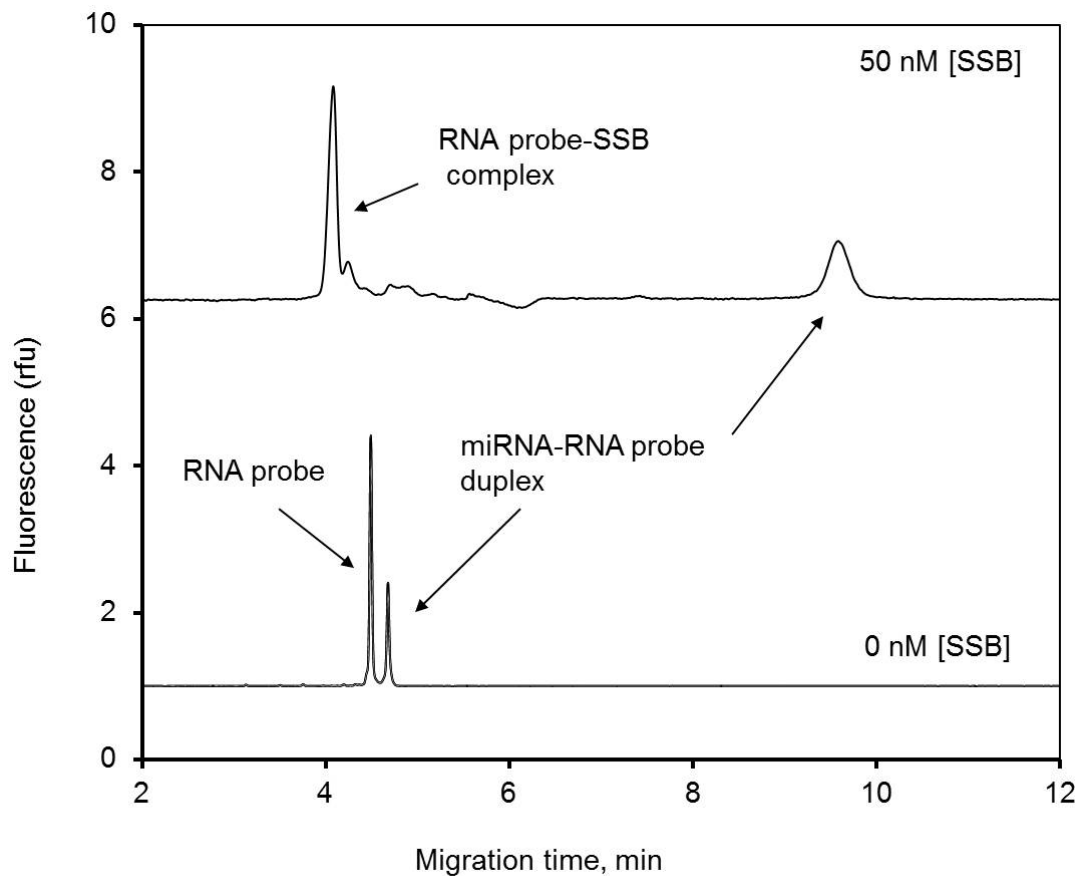


Figure 2.3: Representative electropherograms of ProFACE-LIF assay of miRNA-RNA probe duplex from the excess fluorescent ssDNA probe as a function of SSB concentration. CE separation of miRNA-RNA probe duplex from the RNA probe without SSB (the bottom electropherogram) and with 50 nM SSB (the top electropherogram) in 25 mM borax pH 9.2 run buffer. Equilibrium mixture prior to injection contained 40 nM miRNA and 100 nM RNA probe. Hydrodynamic injections of 10 nL was made by applying a pressure pulse of 20 mbar for 4 sec for 10 sec for experiments. The separations was conducted by applying an electric field of 400 V/cm. (Reproduced and modified from [172] with permission from ACS publications).

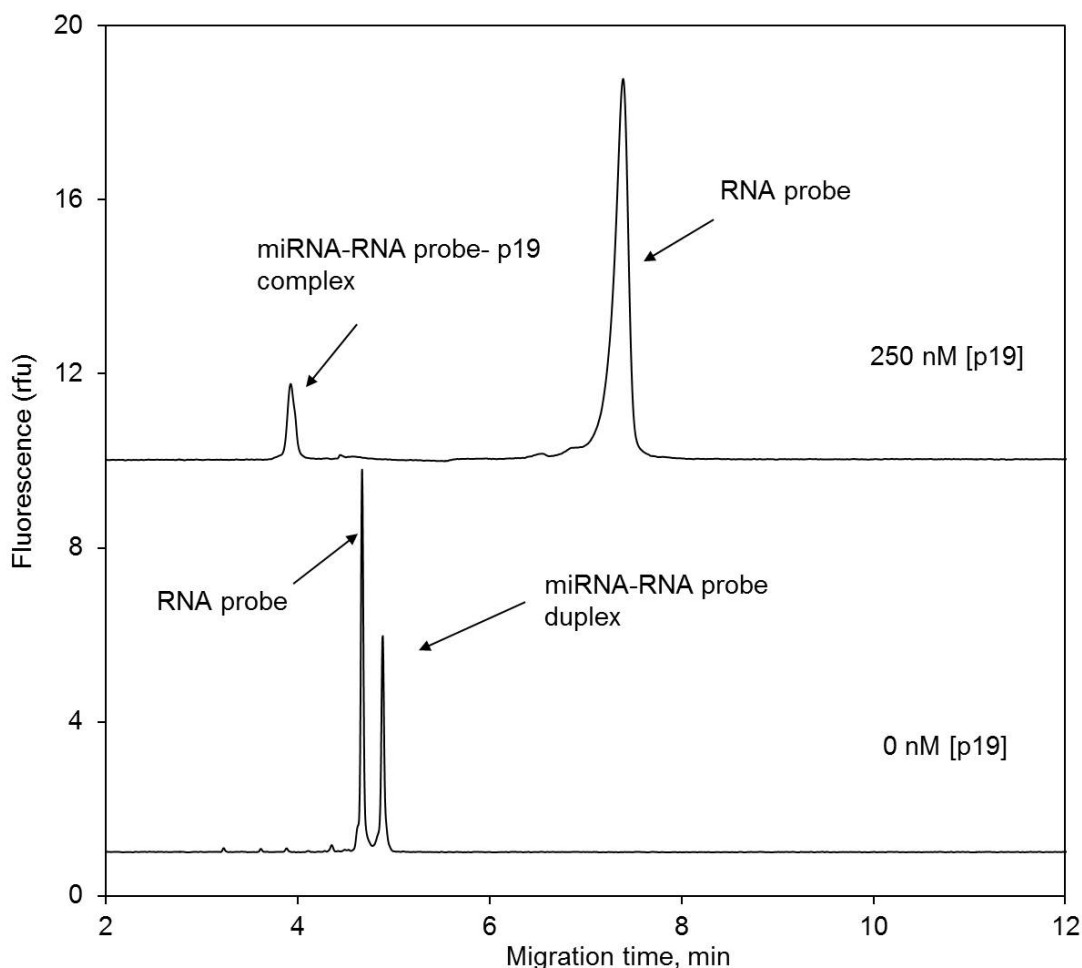


Figure 2.4: Representative electropherograms of ProFACE-LIF assay of miRNA-RNA probe duplex from the excess of a fluorescent hybridization probe using p19. Separation of miRNA-RNA probe duplex from the RNA probe without p19 (the bottom electropherogram) and with 250 nM p19 (the top electropherogram) in 25 mM borax pH 9.2. Equilibrium mixture contained prior to injection 40 nM miRNA and 100 nM RNA probe. Hydrodynamic injections of 10 nL was made by applying a pressure pulse of 20 mbar for 4 sec for 10 sec for experiments. The separations was conducted by applying an electric field of 400 V/cm. (Reproduced and modified from [172] with permission from ACS publications).

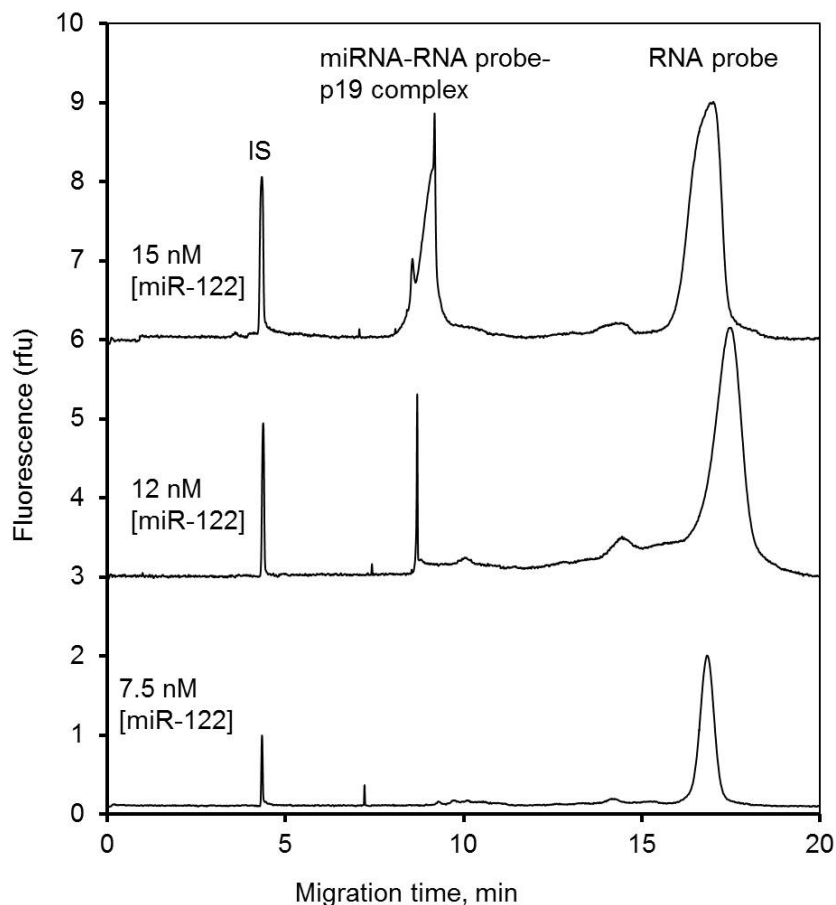


Figure 2.5: Limit of detection (LOD) measurements for miR-122 detection without an additional preconcentration of samples. ProFACE-LIF electropherograms for 7.5, 12, and 15 nM target miRNA and 50 nM RNA probe separated in 25 mM borax pH 9.2 run buffer containing 250 nM p19. BODIPY dye was used as an internal standard (IS). (Reproduced and modified from [172] with permission from ACS publications).

can be explained by the absence of a 2' hydroxyl group in the DNA molecule. The 2' hydroxyl on the RNA sugar phosphate backbone is involved in critical hydrogen bonding and electrostatic interactions with the protein side chains [264]. Moreover, dsRNA has an A-form helix which is wider and approximately 25% shorter than the B-form helix of dsDNA [265]. Therefore, the structure of the duplex formed between the RNA target and the DNA probe may not be in phase with the A-form helix to form proper interaction with the p19 protein.

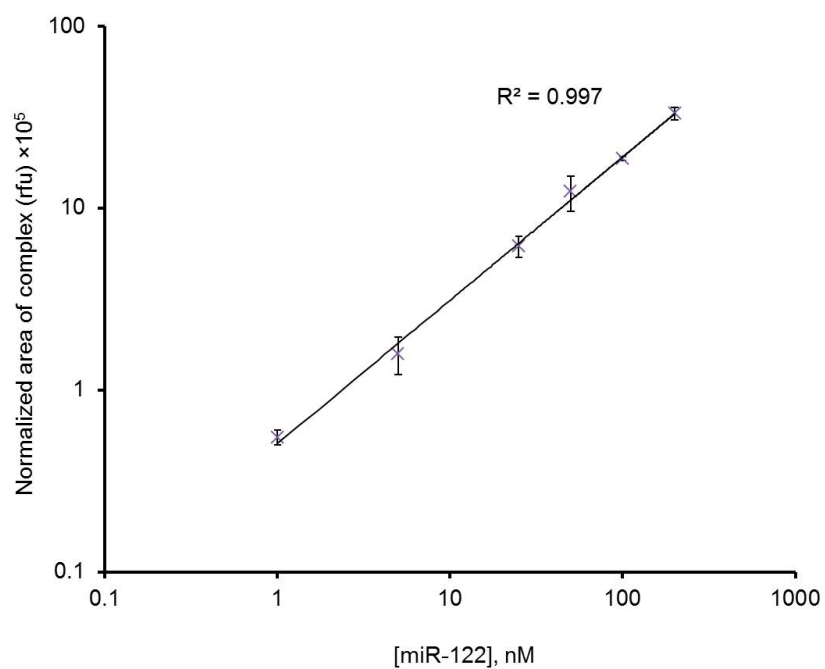


Figure 2.6: Linear regression plot of the relationship between the miRNA concentration and the normalized area (by the migration time) of the detected complex. (Reproduced and modified from [172] with permission from ACS publications).

The use of SSB and p19 proteins enhanced the baseline CE separation and allowed for the measurement of the exact amount of miR-122. Though CE has been used for miRNA detection, previous research has shown a problem of baseline separation of the excess DNA probe from miRNA-DNA probe duplex, making it difficult to accurately quantitate miRNA [167].

2.4.3 Detection of Spiked Synthetic MicroRNA in Serum

Many blood circulating miRNAs are present in stable forms in serum for non-tumor specific miRNAs (miR-19b, miR-16, and miR-24) and for tumor-specific miRNAs (miR-629, miR-660, and miR-141) in the range from 10^2 to 10^{10} miRNA copies/mL [117]. For example, the serum level of miR-141 (a miRNA expressed in prostate cancer) in cancer patients is elevated 46-fold compared with the healthy group.

For the detection of ultra-low amounts of miRNA in blood samples, we applied two preconcentration techniques before CE-LIF analysis: p19-coated magnetic bead precipitation [258] and CE sample stacking [203]. In the first off-line preconcentration procedure, we incubated 15 μ L of p19-coated magnetic beads (4.5 μ g of p19 on the beads) and 1 mL of serum spiked with the synthetic miR-122 (0.5 fM to 500 pM), fluorescent RNA probe (1.25 nM), 100 nM masking DNA (20 nt scrambled synthetic DNA oligonucleotide), 100 nM tRNA from yeast, and 40 units of murine RNase inhibitor. The masking DNA and tRNA prevented probes and miRNA from degradation by nucleases present in serum. After extensive washing of the beads with the p19 washing buffer, miRNA-RNA probe duplex was isolated in 10 μ L of elution buffer and injected into the CE. This was followed by the second online preconcentration, done with the low ionic strength based stacking method. The low ionic strength was achieved by hydrodynamic injections of a large plug of the sample (140 nL) in the p19 elution buffer, followed by a short plug of water (6 nL). The mechanism of sample stacking is described in section 1.5.3.

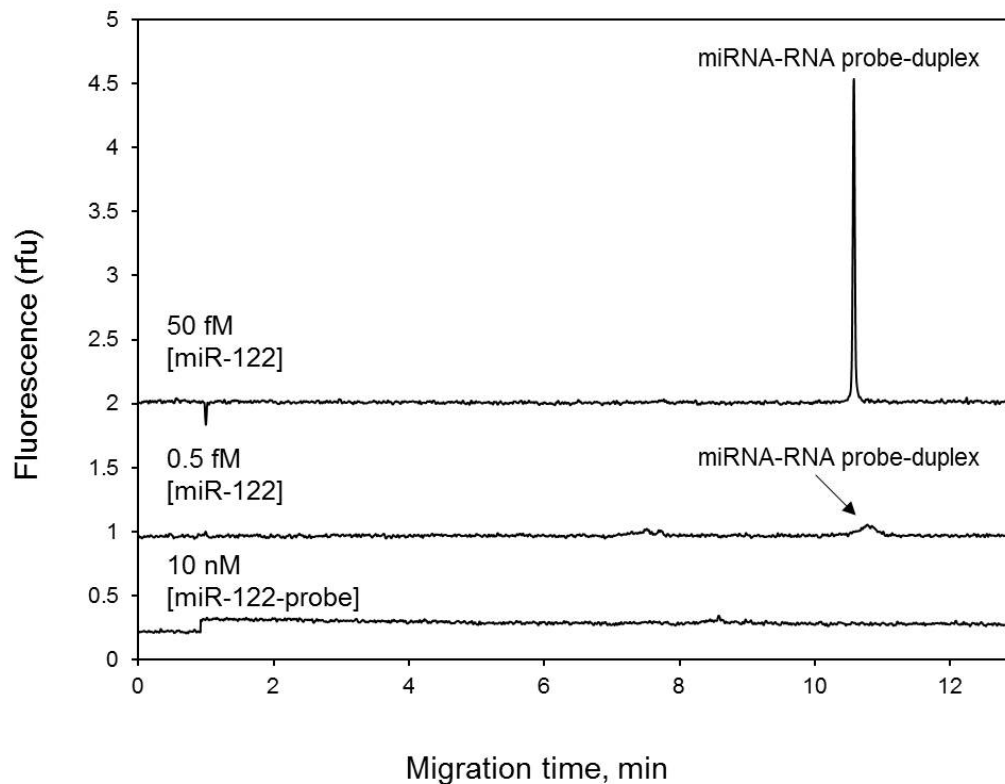


Figure 2.7: CE-LIF electropherograms of serum sample containing 0.5 and 50 fM miRNA with 1.25 nM RNA probe and 10 nM RNA probe alone (no miRNA was added) as a control after p19 bead preconcentration and CE sample stacking. The separations were performed in 25 mM borax pH 9.2 run buffer. (Reproduced and modified from [172] with permission from ACS publications).

The resulting electropherograms showed a major peak for the miRNA-RNA probe duplex and a minor peak for the free RNA probe (Figure 2.7). We think that the RNA probe was co-precipitated with the beads because of nonspecific binding to them. The limit of detection for the miRNA-RNA probe duplex was as low as 0.5 fM or 3×10^5 miR-122 copies in 1 mL of blood serum.

The p19 bead precipitation and CE stacking helped enrich the miRNA greater than 1000-fold from serum compared to our CE experiments without the preconcentration. The use of p19 beads brings two major benefits: miRNA-selective extraction and sample enrichment. The unbound fluorescent probe and other serum components are removed by

washing. The eluted miRNA duplex is then efficiently separated by CE and quantitatively detected by LIF.

2.4.4 Endogenous MicroRNA Detection by CE-LIF

After successfully performing ProFACE assay, where spiked miRNAs in fetal bovine serum was detected, we went on to test if endogenous miRNAs can be detected from human serum and cancer cell lines using CE-LIF with online sample stacking method. Unlike ProFACE where miRNA-selective extraction was performed before electrophoresis, here we implemented commercially available traditional RNA purification kits to prepare our samples for analysis. The goal for this part of the study was to detect endogenous miRNAs from total isolated RNA by CE-LIF without any enzymatic amplification.

Compared to mRNAs, the relatively low copy number (from several to 10^4) of miRNAs in certain cell types, makes it difficult to detect endogenous miRNAs in cell, tissue or serum samples. For the detection of cellular miRNAs, we used human hepatocellular carcinoma cell lines Huh-7 and Hep-G2. It has been shown that Huh-7 cells express miR-122, while Hep-G2 cells do not express this miRNA [260, 266]. On the other hand, both cells express miR-21. Therefore, these are excellent model cell lines for this type of study. Using CE-LIF with on-line sample preconcentration, both endogenous cellular miRNAs, miR-122 and miR-21 were detected in cell extracts. Prior to the CE analysis, cells were lysed and small RNAs were isolated using mirVana miRNA purification kit according to manufacturer's protocol (see section 2.3.6).

For the detection of miR-122, Alexa-488 labeled DNA probes were mixed with total small RNA extracts from Huh-7 cells, in incubation buffer at different concentrations (0.05 nM, 0.1 nM, 0.5 nM, 1 nM, 5 nM and 20 nM). Hep-G2 cells were used as a negative control for these experiments and 0.5 nM DNA probe was mixed with total small RNA extracted from Hep-G2 cells. Each sample mixture was annealed using a PCR thermocycler as

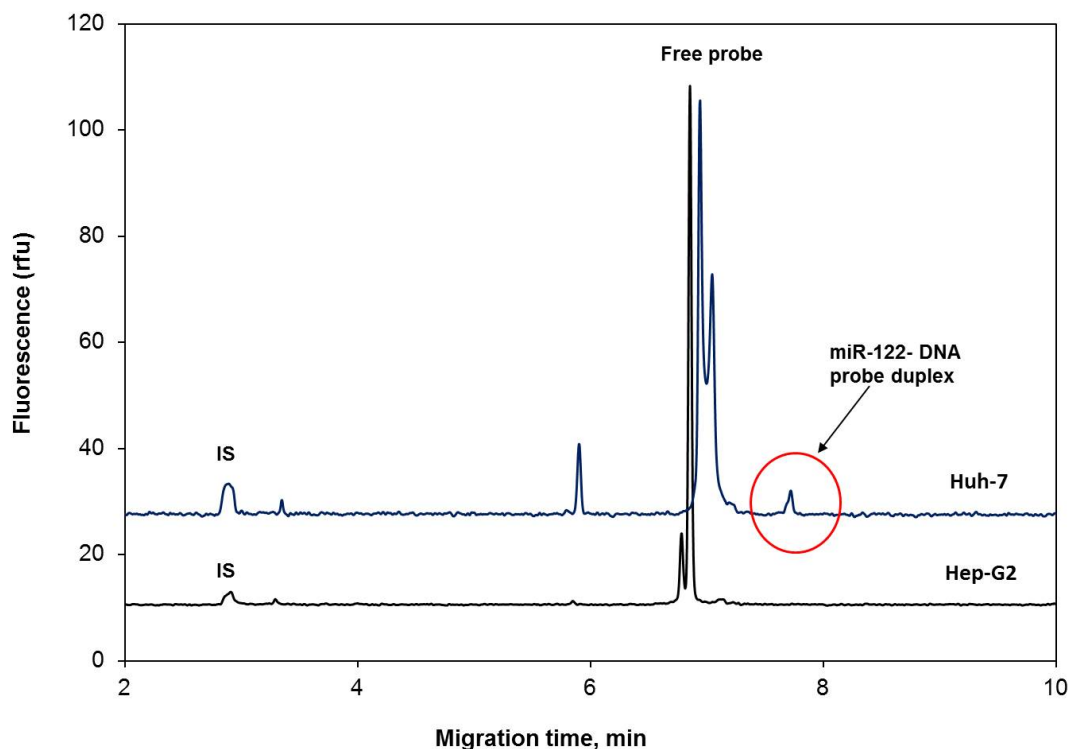


Figure 2.8: CE-LIF detection of endogenous miR-122 from Huh-7 cells. Representative electropherograms of total RNA samples extracted from Huh-7 cells (upper electropherogram) and Hep-G2 cells (bottom electropherogram) containing 0.5 nM DNA probe. Hep-G2 cells are used as a negative control. The separations were performed in 25 mM borax run buffer, pH 9.2. 10 pM BODIPY was used as an internal standard(IS).

described in section 2.3.2. Obtained sample was subjected to electrophoresis with on-line sample stacking as explained in section 2.4.3. The resulting electropherograms (Figure 2.8) show two major peaks: one corresponding to target endogenous miR-122-DNA probe duplex, and a second one for excess DNA probe (Figure 2.8, upper electropherogram). In the bottom electropherogram (Figure 2.8), as expected only one peak corresponding to the DNA probe was observed.

For the detection of miR-21, different concentrations of DNA probes complimentary to miR-21 (0.05 nM, 0.1 nM, 0.5 nM, 1 nM, 5 nM and 20 nM) were mixed with total small RNA extracted from Hep-G2 and Huh-7 cells and were hybridized. Here we used miR-122 as a negative control and 500 pM of DNA probes complimentary to miR-122.

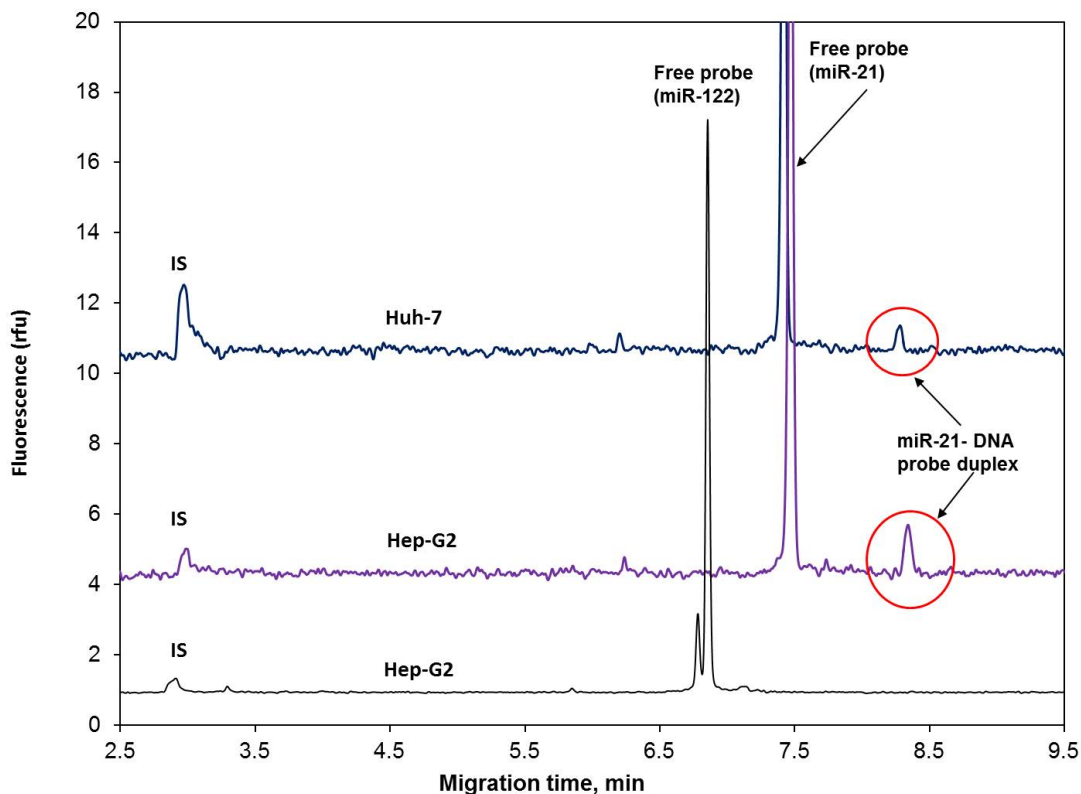


Figure 2.9: CE-LIF detection of endogenous miR-21 from Huh-7 and Hep-G2 cells. Representative electropherograms of Total RNA samples extracted from Huh-7 cells (upper electropherogram) and Hep-G2 cells (middle and bottom electropherograms) containing 500 pM DNA probe. Here miR-122 probes in Hep-G2 cells are used as a negative control. The separations were performed in 25 mM borax run buffer, at pH 9.2. 10 pM BODIPY was used as an internal standard (IS).

The hybridization was performed using the same method as described in section 2.3.2. The resulting electropherograms show two major peaks: one for endogenous miR-21-DNA probe duplex (Figure 2.9, upper and middle electropherogram) in Huh-7 and in Hep-G2 cells respectively, and a second peak for excess DNA probe; no peak was detected for miR-122 (Figure 2.9, bottom electropherogram). We found that the expression of miR-21 in Hep-G2 cells is higher than in Huh-7 cells.

Endogenous miRNAs circulate in human plasma and serum in a remarkably stable form and are contained within vesicles, in protein and lipoprotein complexes [117, 147]. Since serum contains relatively lower levels of miRNA than cells, it is even more challenging

to detect endogenous miRNAs in serum. Here we attempted to detect serum circulating RNAs using CE-LIF with online sample stacking. We targeted two different miRNAs, miR-21 and miR-16, which are known to be abundant in normal human serum [118]. Total RNAs were extracted from 2 mL of serum sample using Norgen Biotek purification kit according to manufacturer's protocol (see section 2.3.6) with some modifications. Different concentrations of DNA probes (0.1 nM, 0.2 nM, 0.5 nM, 0.6 nM and 1 nM) complimentary to miR-21 and miR-16 were incubated with total RNA extract from normal human serum. Hybridization was performed as described in section 2.3.2. Total RNA without probe was used as a negative control. The resulting electropherogram shows a duplex peak at 11.3 min for miR-16 (Figure 2.10, upper electropherogram) and at 11.4 min for mir-21 (Figure 2.10, bottom electropherogram).

Quantification of miR-122 in Huh-7 cells.

The concentration of endogenous miRNAs was calculated according to the following equation 2.2:

$$[miRNA] = \frac{(A_H \times [P]_0)}{(A_H + q_H \times A_P)} \quad (2.2)$$

where A_H is the normalized area of the duplex, $[P]_0$ is the initial concentration of the probe, q_H is the relative quantum yield of the miRNA-DNA probe duplex and A_P is the normalized area of the probe [171].

The quantum yield was determined using the following equation 2.3:

$$q_H = \frac{A_H}{(A_P^{[miRNA]=0} - A_P^{[miRNA] \neq 0})} \quad (2.3)$$

where, A_H is the normalized area of the duplex, $A_P^{[miRNA]=0}$ is the normalized area of the DNA probe in the absence of the target miRNA and $A_P^{[miRNA] \neq 0}$ is the normalized area of the excess DNA probe in the presence of the target miRNA.

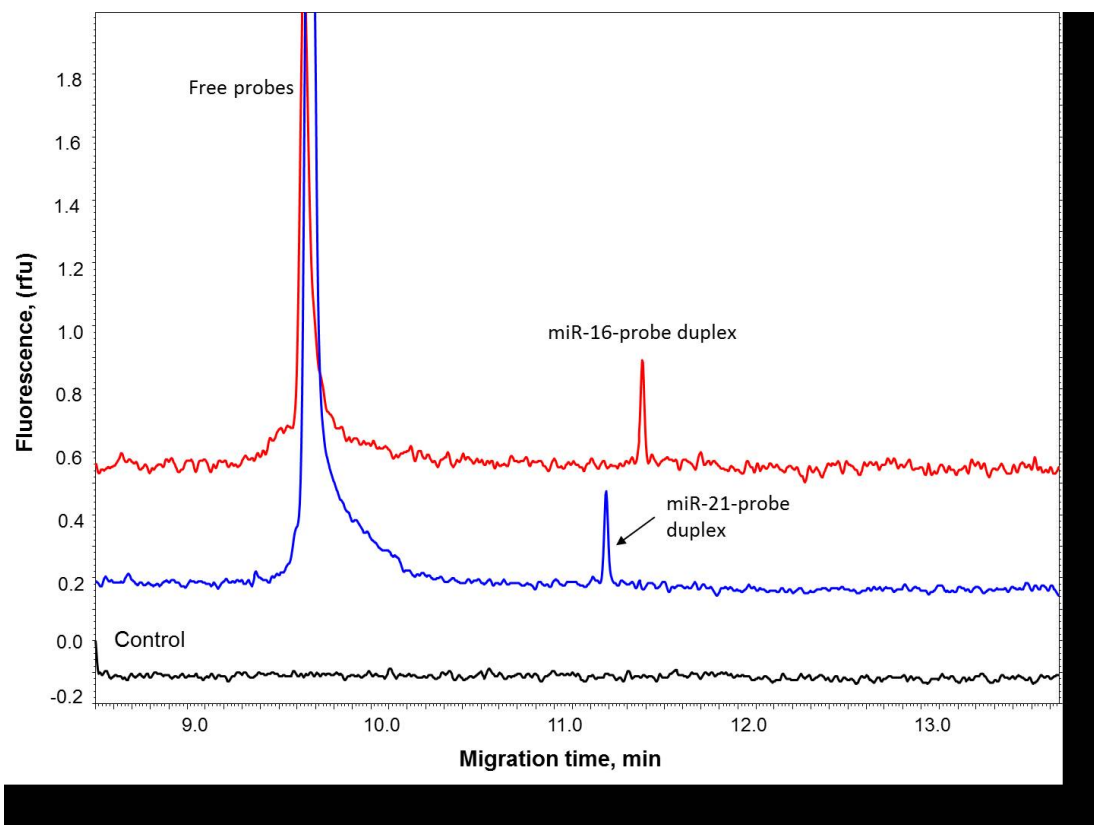


Figure 2.10: CE-LIF detection of endogenous miR-21 and miR-16 from normal human serum. Representative electropherograms of total RNA samples extracted from normal human serum containing 100 pM fluorescently labeled DNA probe complimentary to miR-16 (upper electropherogram) and 100 pM fluorescently labeled DNA probe complimentary to miR-21 (middle electropherogram). Extracted total RNA sample without probes (bottom electropherogram) is used as a negative control. Separations were performed in 25 mM borax run buffer, at pH 9.2.

Quantum yields of DNA probes with respect to when they are bound to miRNA was determined by injecting two samples of 500 nM DNA probe with and without miRNA target. The calculated concentration for miR-122 in Huh-7 was 199 ± 38 pM.

2.4.5 Multiplexed Detection of MicroiRNAs by Capillary Electrophoresis with Laser Induced Fluorescence Detection

MicroRNAs have been identified in normal and malignant cells and it has been found that they are frequently deregulated in cancer cells. Deregulation of several, specific miRNA species, produces a miRNA fingerprint that can distinguish between cancerous and non-cancerous cells. Due to similar miRNA length, it is not possible to separate multiple miRNAs in the same CE run. For multiple miRNA detection by CE-LIF, we designed (see Figure 2.11) complementary DNAs (cDNA) to recognize specific miRNA with 20 nucleotides poly (A) at the 5' end of the sequence. A short fluorescent labeled probe with 20 nucleotide poly (T), was designed to anneal the complementary DNA. Hybridization product may result in trimer triplex with miRNA, partial hybrid with cDNA and free probe. In order to detect two miRNA species simultaneously, the poly T DNA probe was modified at the 3' position with cholesterol (labeled as Tag in Figure 2.11) and the other had no modifications. This modification caused the duplex peak of modified miR-21 to shift by decreasing its migration time. A mixture of 25 nM miR-122 and 25 nM miR-21 was mixed with 50 nM of DNA probe complementary to miR-122, 50 nM poly (T) DNA probe tagged with cholesterol in its 3' position, and 50 nM poly (A) cDNA complementary to miR-21 (Figure 2.11). The mixture was hybridized as described in section 2.3.2. Resulting electropherograms show one miRNA duplex peak for miR-122 and one miRNA triplex peak for miR-21 (see Figure 2.12, (3)), along with partial hybridization product for miR-21 probe and two peaks for free probes. By adding 0.05% triton X-100, a non-ionic surfactant in the run buffer, all peaks containing miR-21 were shifted to the left, leaving behind only

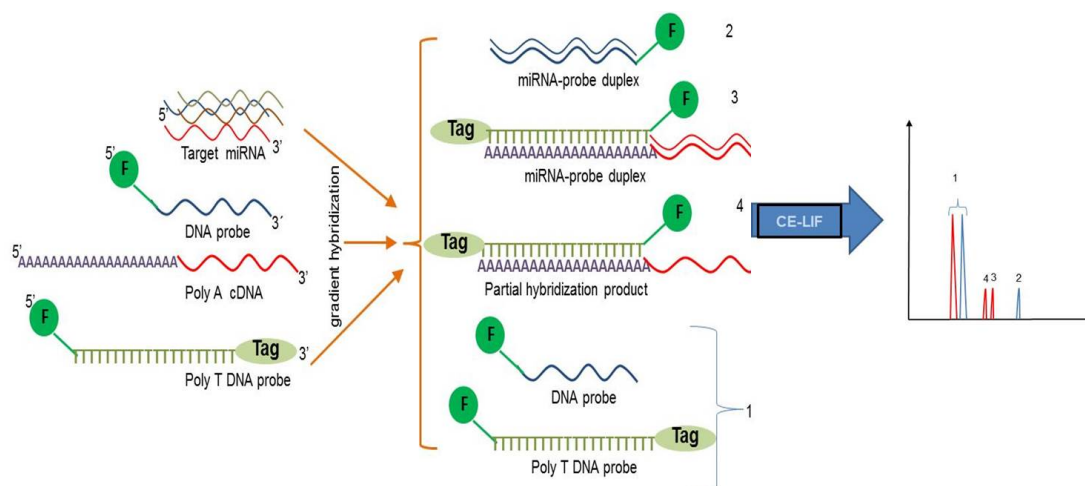


Figure 2.11: Complementary DNA sequences are designed to recognize specific miRNA with 20 nucleotides poly (A) at the 5' end. A short fluorescently labeled probe with 20 nucleotide poly (T), is designed to anneal the cDNA. Hybridization product may contain triplex miRNA, partial hybrid with cDNA or free probe. In order to detect three miRNA species simultaneously, one of the DNA probes is modified at the 3 position with cholesterol. These modifications cause the duplex peak to shift by increasing or decreasing its migration time.

two peaks: miR-122-probe duplex and free probe for miR-122 (Figure 2.12, (4)). The DNA probe for miR-21 was tagged with a cholesterol moiety. This hydrophobic cholesterol moiety has a higher affinity for the hydrophobic core of the micelle (triton X-100). As such, association with the micelle increases greatly the mass of the complex, thereby reducing its anodic electrophoretic mobility. Because EOF is strong at the buffer pH used, the net or apparent mobility is increased and thus alters the oligonucleotide's overall migration time and allows it to elute out of the capillary faster, separating the two miRNAs.

As a proof of principle, we applied these new probes to a more complex matrix to show their potential in biological samples. Cholesterol-tagged DNA probe for miR-21, along with another unmodified DNA probe for miR-16 was incubated with serum-circulating RNA extracted from normal human serum (Figure 2.13). We were able to detect both miRNAs simultaneously in the extracted sample. Due to laser limitations used in this experiment, we were not able to detect miR-16 with better confidence. Taking all of these

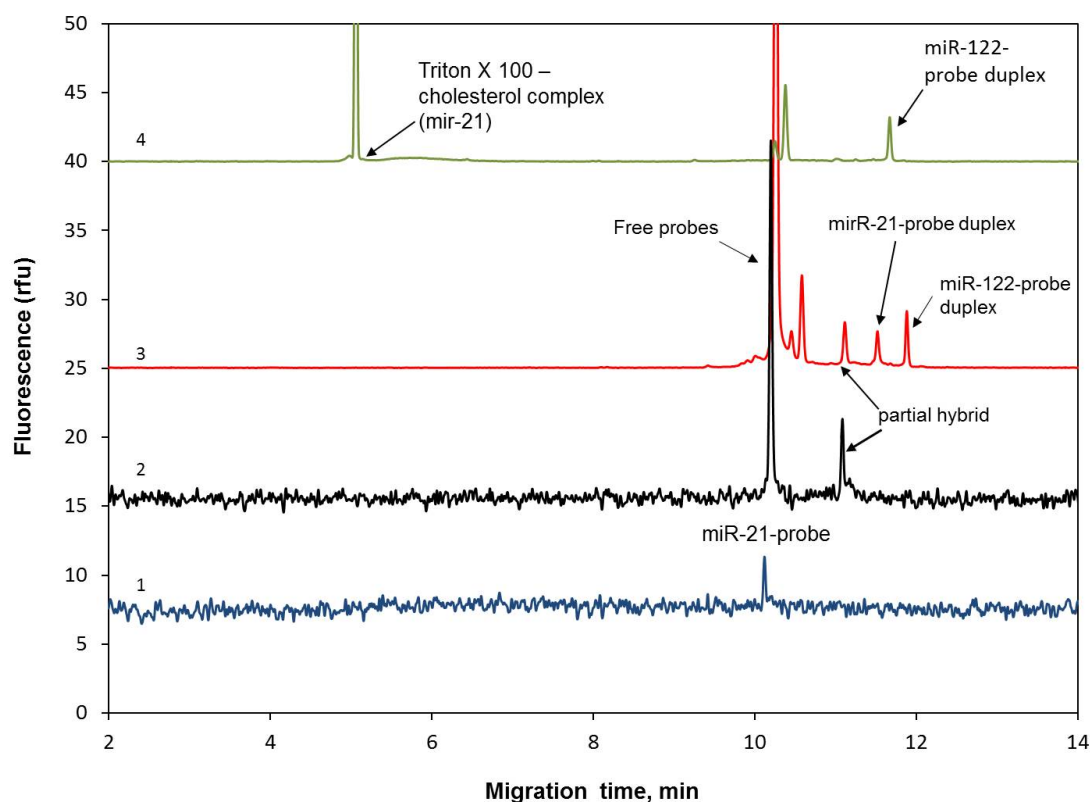


Figure 2.12: Separation of two different miRNA-RNA probe duplexes from DNA probes. Equilibrium mixture contained: (1) 10 nM cholesterol tagged poy (T) DNA probe with 5 nM miR-21; (2) 25 nM cholesterol tagged poly (T) DNA probe with 25 nM poly (A) cDNA to miR-21; (3) mixture of 25 nM miR-122 and 50 nM DNA probe to miR-122, 25 nM miR-21, 50 nM poly (A) cDNA to miR-21 and 50 nM cholesterol tagged poly (T) DNA probe; (4) mixture of 25 nM miR-122 and 50 nM DNA probe to miR-122, 25 nM miR-21, 50 nM poly (A) cDNA to miR-21 and 50 nM cholesterol tagged DNA probe with 0.05% triton X-100 in run buffer.

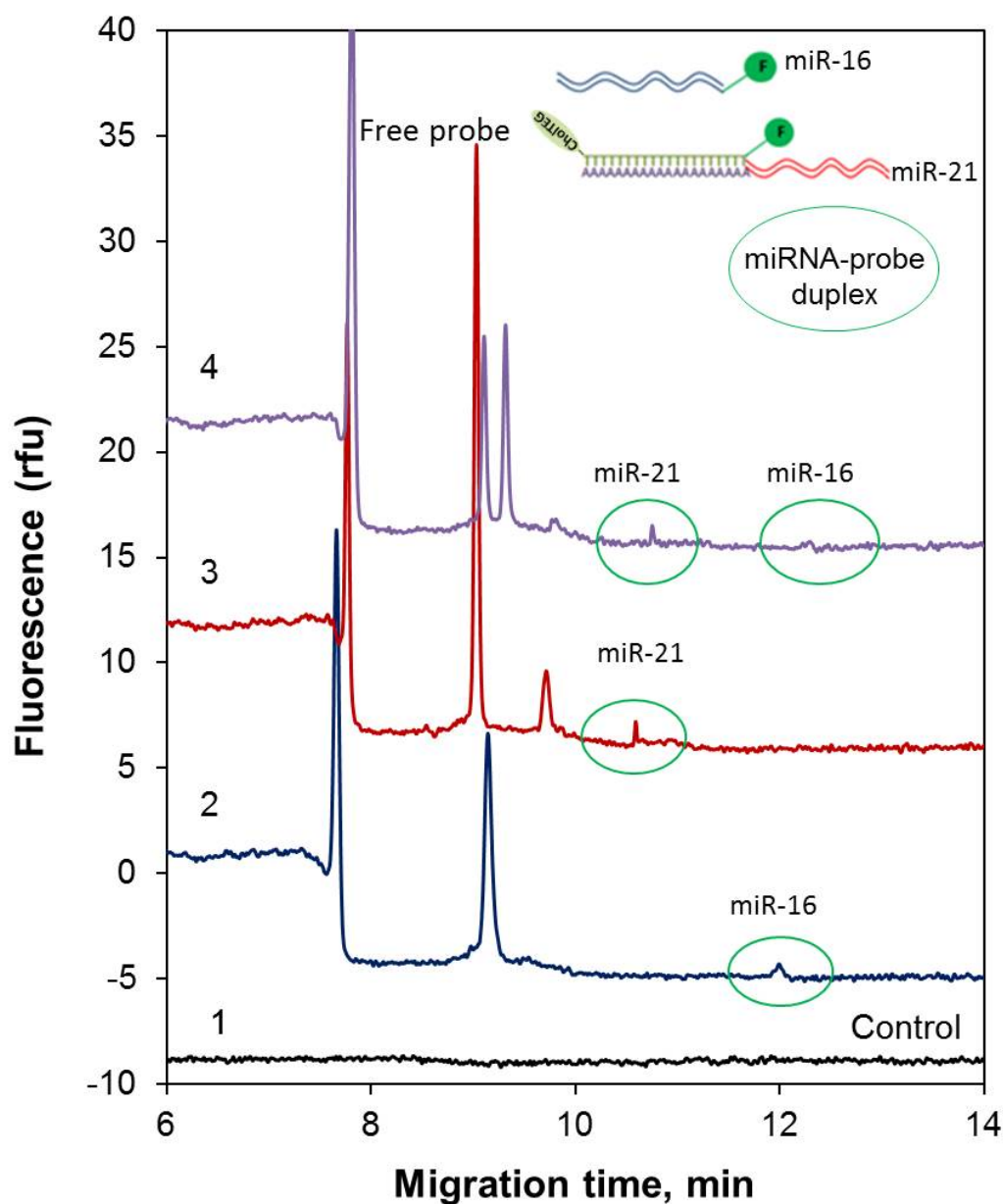


Figure 2.13: CE-LIF detection of endogenous miR-21 and miR-16 from normal human serum. Representative electropherograms of serum-circulating RNA samples. Equilibrium mixture contained: (1) extracted total RNA sample without probes as a negative control; (2) 500 pM DNA probe to miR-16 with total RNA; (3) 500 pM DNA probe to miR-21 with total RNA; and (4) mixture of 500 pM DNA probes to miR-16 and miR-21. Separations were performed in 25 mM borax run buffer, at pH 9.2.

results into account we showed that CE-LIF with online sample stacking is a suitable method for endogenous miRNA detection.

2.5 Conclusion

Several simple techniques for miRNA analysis were developed. We showed that SSB and p19 proteins can work as enhancers of the CE separation. Ultralow amount of spiked microRNA was detected in serum involving four steps: (i) hybridization with a fluorescently labeled RNA probe, (ii) precipitation of miRNA with p19 magnetic beads, (iii) injection with the sample stacking into a capillary, and (iv) CE-LIF based separation and quantitation. Without PCR amplification, as low as 3×10^5 molecules of miRNA in 1 mL of serum can be measured in a 20 min CE-LIF run. The sensitivity of the method is comparable with existing PCR techniques. In addition, endogenous microRNAs miR-122, miR-21 and miR-16 were detected in extracted total RNA of Huh-7 and Hep-G2 cells and human serum by CE-LIF with an online stacking method.

Chapter 3

Evaluation of Different MicroRNA Isolation Methods for Use in Mass Spectrometry Analysis of MicroRNA

3.1 Objective

The objective of this project was to identify the best miRNA purification method for mass spectrometry analysis of miRNAs in cells and serum.

3.2 Introduction

In order to put forward particular miRNA molecules as clinically relevant biomarkers, one should consider important steps: a reliable RNA isolation method, a reproducible recovery from biological samples, and reliable measurements of the isolated RNA. Different sample preparation methods might affect concentration measurements of individual miRNAs. Depending on the extraction method, the amount of miRNA in any given biological sample

will vary. This project was funded by NSERC Engage Grant with in-kind contribution from Norgen Biotek Corporation, a Canadian biotechnology company.

In Chapter 4 of this thesis, I describe a method using CE coupled on-line with MS to analyze endogenous miRNAs in B-cell chronic lymphocytic leukemia (CLL) serum. Due to complex nature of biological matrices, it is difficult to obtain high-quality miRNA samples that are required for a successful analysis of low-abundant microRNAs by MS. Thus, our goal was to determine the most suitable RNA purification method (see Table 3.2) yielding high quality miRNA that is required for MS analysis. To examine this, we used four commercially available RNA purification methods to extract RNAs from normal human serum and Huh-7 cancer cell line. Due to a lower copy number of endogenous miRNAs per cell and in serum, it was necessary to spike synthetic analogs of miRNA into biological samples before miRNA purification. Therefore, we evaluated the RNA extraction methods and their potential to extract the spiked RNA with high purity and considerable yield, which is required for the analysis of miRNA by MS.

3.3 Materials and Methods

3.3.1 Materials

Chemicals and Consumables: Buffer components were purchased from VWR (Mississauga, Canada). Ethanol and chloroform were obtained from Fisher Scientific Company (Ottawa, Canada). The bare fused silica capillary was purchased from Polymicro Technologies (Phoenix, USA). All solutions were made using Milli-Q-quality RNase free water and filtered through a 0.22 μm filter (Millipore, USA).

Cell Culture: The human hepatoma cell line Huh-7 was grown in monolayers on a 25 cm^2 flask in Dulbeccos Modified Eagle Medium (DMEM) (Sigma-Aldrich, Oakville, ON) supplemented with 100 nM non essential amino acids, 50 U/mL penicillin, 50 $\mu\text{g}/\text{mL}$

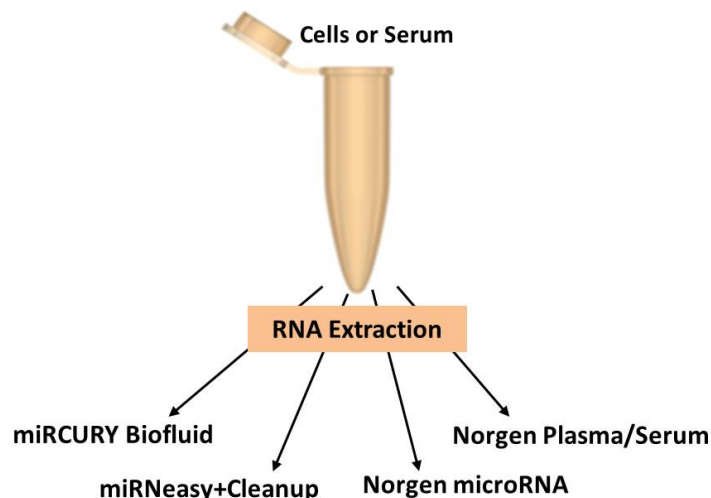


Figure 3.1: Image showing the methods of extraction tested. Cells or serum samples were lysed, and split into eight different fractions that were used in seven miRNA concentrations of spiking (5, 10, 25, 50, 100, 250, 500 nM) and one fraction as a control without spiking.

streptomycin, and 10% (v/v) heat-inactivate fetal bovine serum (Sigma-Aldrich, Oakville, ON) in an incubator at 37°C with 5% CO₂. The cells were harvested and pelleted by centrifugation at 500× g for 5 minutes. For miRNA isolation, for each extraction method (Figure 3.1), 2-4×10⁶ cells were split into eight fractions. In each fraction respective lysis solution was added followed by different concentration of three synthetic miRNA mixtures: fraction 1 - sample without spiking; fraction 2 - 500 nM spike; fraction 3 - 250 nM spike; fraction 4 - 100 nM spike; fraction 5 - 50 nM spike; fraction 6 - 25 nM spike; fraction 7 - 10 nM spike; and fraction 8 - 5 nM spike. Cells were then lysed by vortexing and RNA was purified according to manufacturer's protocol.

Serum Sample: Human serum samples from normal male and female donors (pooled) were purchased from Bioreclamation Incorporation (Hicksville, NY). All samples were kept at −80°C until ready to use. For circulating RNA isolation for each extraction method (Figure 3.1), 2 mL of normal serum was thawed on ice, and was split into eight fractions. In each fraction, respective lysis solution was added followed by different concentration of three synthetic miRNA mixtures: fraction 1 - sample without spiking; fraction 2 - 500

nM spike; fraction 3 - 250 nM spike; fraction 4 - 100 nM spike; fraction 5 - 50 nM spike; fraction 6 - 25 nM spike; fraction 7 - 10 nM spike; and fraction 8 - 5 nM spike. Serum was then lysed by vortexing and RNA was purified according to manufacturer's protocol.

3.3.2 RNA Extraction Methods

RNAs were extracted from cells and serum samples using four different methods: miRNeasy mini kit followed by RNeasy minElute cleanup kit for cells (referred to henceforth as miRNeasy+Cleanup) (Qiagen, Hilden, Germany), microRNA purification kit for cells (referred to henceforth as Norgen microRNA) (Norgen Biotek corporation, Thorold, Canada), miRCURY RNA isolation kit for biofluids (referred to henceforth as miRCURY Biofluid) (Exiqon, Vedbaek, Denmark) and exosomal and plasma/serum circulating RNA purification kit (referred to henceforth as Norgen Plasma/Serum) (Norgen Biotek corporation, Thorold, Canada) (see Table 3.1 and Figure 3.2). The methods miRCURY Biofluid and Norgen microRNA for cells are both column-based methods. The miRNeasy+Cleanup method is a combined phenol/guanidine and column-based method that is followed by small RNA cleanup kit. The Norgen Plasma/Serum method for serum is a column-based technology that uses Norgen's proprietary resin as a binding matrix. Methods were used according to the manufacturers protocol with some modifications. Three to four synthetic miRNAs (see Table 3.2) were added to each sample and were always freshly diluted. Synthetic RNAs were added specifically after denaturation of the sample (i.e. after adding lysis solution) to avoid degradation by endogenous RNases. At that point, following the manufacturer's protocol, the sample was loaded into a spin column, followed by washing steps, and then eluted in 25-50 μ L of RNase free water as depicted in Figure 3.2.

Table 3.1: Names and details of RNA purification methods.

Method name	Isolation kit	Company	Extraction technique
miRCURY Biofluids	miRCURY RNA Isolation kit-Biofluids	Exiqon	Column based technology
miRNeasy+Cleanup	miRNeasy Mini Kit+Cleanup	Qiagen	Combined phenol/guanidine and column based technology
North microRNA	microRNA Purification Kit	Norgen Biotek	Column based technology
Norgen Plasma/Serum	Plasma/Serum Circulating RNA Purification Kit	Norgen Biotek	Column based technology (slurry format)

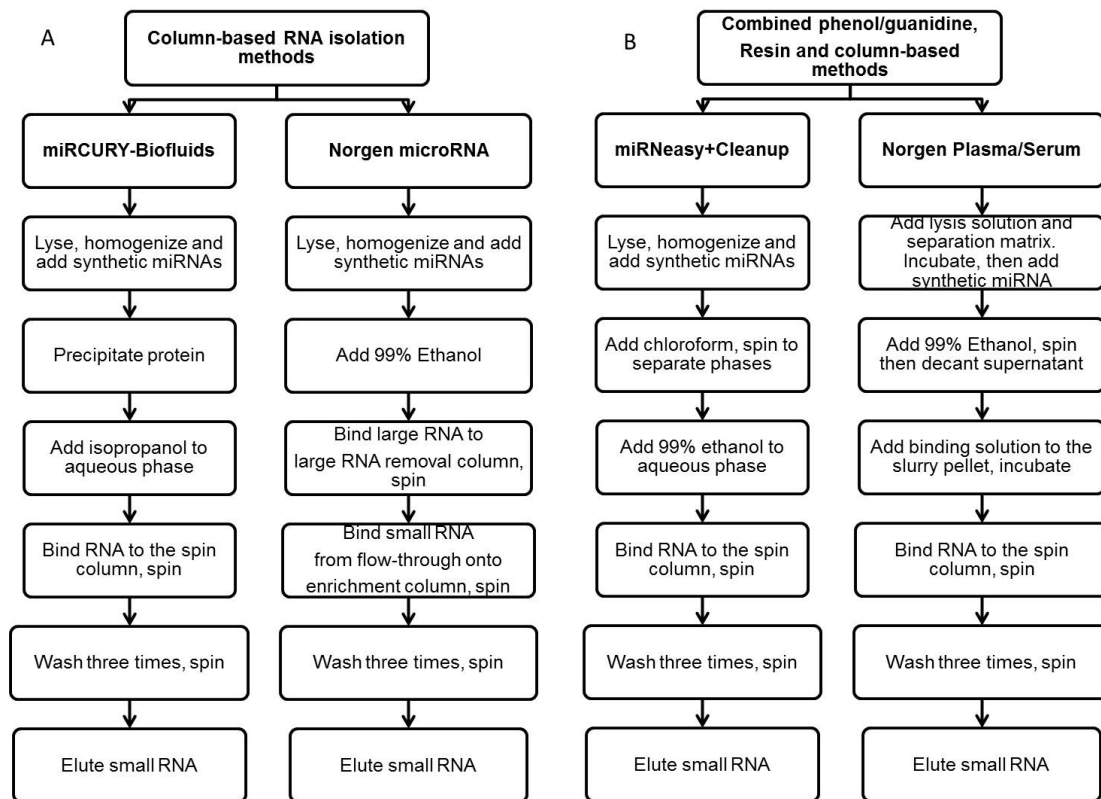


Figure 3.2: Flow charts for different RNA extraction methods. RNA from cells and serum was isolated using (A) pure column based techniques: miRCURY Biofluid, and Norgen microRNA; (B) combined phenol and column based approaches: miRNeasy+Cleanup and combined column and resin based method: Norgen plasma/serum.

Table 3.2: Correlation of spiked miRNA with extraction methods.

miRNA ID	Serum	Huh-7 cell	miRCURY Biofluid	miRNeasy+Cleanup	Norgen Plasma/Serum	Norgen microRNA
	[spiked RNA], nM	[spiked RNA], nM				
hsa-miR-150-5p	1000		x ^a		x ^a	
hsa-miR-16-5p	5-500		x ^a		x ^a	
hsa-miR-223-3p	5-500		x ^a		x ^a	
hsa-miR-21-5p	5-500	10-500	x ^a	x ^a	x ^a	x ^a
hsa-miR-122-5p		10-500		x ^a		x ^a
hsa-miR-32-5p		10-500		x ^a		x ^a
cel-miR-39-3p		1000		x ^a		x ^a

^a X marks presence of indicated miRNAs in samples.

miRNeasy+Cleanup Versus Norgen microRNA Methods for Cells: Total small RNAs were extracted from 250,000 - 300,000 Huh-7 cells using miRNeasy+Cleanup method for cells, and from 500,000 - 600,000 Huh-7 cells in case of Norgen micRoRNA method, both with and without spiking. Prior to extraction, three synthetic miRNAs, miR-21-5p, miR-122-5p and miR-32-3p were mixed together and spiked into the cell lysate at seven different concentrations. Synthetic miRNAs were directly added to the lysate before RNA extraction. Final concentrations of spiked miRNAs were 500 nM, 250 nM, 100 nM, 50 nM, 25 nM, 10 nM and 5 nM, assuming 100% recovery. 1 μ M of *cel*-miR-39 was spiked to all samples as a control (see Table 3.2). One sample contained cell without synthetic RNA spiking. All extractions were performed in triplicate for a total of 24 samples for each extraction method. Manufacturer's protocols were followed for both methods (Figure 3.2), the extracted total small RNAs were measured by CE-MS, and percent recovery was calculated.

miRCURY Biofluid Versus Norgen Plasma/Serum Methods: Serum-circulating RNAs were extracted using miRCURY Biofluid method from 200 μ L of normal serum; for Norgen Plasma/Serum method, 250 μ L of serum was used, both with and without spiking. Prior to extraction, three synthetic miRNAs, miR-21-5p, miR-16-5p and miR-223-3p, were mixed together and spiked into the lysed serum sample at seven different concentrations. Synthetic miRNAs were directly added to the lysate before RNA extraction. Final concentrations of spiked miRNAs were 500 nM, 250 nM, 100 nM, 50 nM, 25 nM, 10 nM and 5 nM, assuming 100% recovery. One μ M of miR-150 was spiked into all samples as a control (see Table 3.2). One sample contained serum without synthetic RNA spiking. All extractions were performed in triplicate for a total of 24 samples for each extraction method. Manufacturer's protocols were followed for both methods (Figure 3.2), the extracted serum-circulating RNAs were measured by CE-MS, and percent recovery was calculated.

3.3.3 MicroRNA Analysis

The quality of extracted RNA was analyzed using CE-ESI-MS. Experiments were performed with a Synapt G2 HDMS mass spectrometer (Waters, UK) that was coupled on-line with a PA 800 plus Pharmaceutical Analysis CE system (Beckman Coulter, USA) through a microvial electrospray CE-MS interface (CMP scientific, USA). CE-MS parameters are described in details in Section 4.3.3. Samples were run for 40 min and the mass spectra were collected from 1000-1600 m/z .

3.4 Results and Discussion

In this work, four different RNA isolation methods, based on different extraction techniques were evaluated. Two extraction methods for serum, and two methods for cell samples were used. Since assessment of different RNA extraction methods requires the use of a standard sample source, all isolations were conducted using a same batch of serum and same batch of cells. We focused on the purity of miRNAs obtained from serum and cells, since for the MS analysis, sample quality is an absolute criterion. Detecting miRNAs by MS is extremely difficult due to the low abundance of miRNAs in biological samples, specifically in biofluids. Moreover, isolation kits have limitations on how much sample can be loaded on a purification column without clogging it. To overcome above mentioned obstacles, synthetic miRNA molecules were spiked into human serum and cell lysate samples for the method assessments using four manufacturer's kits and for method optimization (described in Chapter 4). Purified RNA samples were directly analyzed by CE-ESI-MS.

First, we compared the miRCURY Biofluid method with the Norgen plasma/serum method. Both of these were performed in triplicate with and without spiking. Although both of these methods are column based, Norgen plasma/serum method involves multiple steps, with a long incubation time using separation matrices, whereas miRCURY Biofluid

kit consists only of a protein precipitation step before "RNA binding to the column" step. Therefore, unlike Norgen Plasma/Serum method, the miRCURY Biofluid method is faster to perform, and is most likely exposed to less technical variations. We then compared the miRNeasy+Cleanup method with Norgen microRNA method for cells, both of which were performed in triplicate with and without spiking. miRNeasy+Cleanup method includes an organic phase separation step, whereas Norgen microRNA kit for cells does not (Figure 3.2). Moreover, miRNeasy+Cleanup method also includes a cleanup step to enrich miRNAs, similar to the Norgen microRNA method, where it includes a small RNA binding column after a large RNA removal step. Both of these kits showed similar performances.

3.4.1 Linearity and Limit of Detection

Linearity of the CE-MS instrument:

To assess the linear dynamic range of the CE-MS analysis of pure oligoribonucleotides, eight standards were prepared with the following concentrations of synthetic miRNA in RNase free water: 500 nM, 250 nM, 100 nM, 50 nM, 25 nM, 10 nM, 5 nM and 1 nM. A miRNA with a concentration of 1 μ M was added to each solution as an internal standard. Ten μ L of each solution was used for CE-MS run. Calibration curves for hsa-miR-21-5p, hsa-miR-16-5p and hsa-miR-223-3p for ions having a charge state of $[M-5H]^{5-}$ are shown in Figure 3.3. The highest intensity count for the deprotonated charge state versus the concentration of miRNA is plotted. Regression analysis was performed as represented by best-fit lines and R^2 values. The instrumental response to synthetic solutions of miRNA in water is linear in miRNA concentrations ranging from 1 nM to 500 nM.

Limit of Detection:

The limit of detection (LOD) was assessed by running seven dilutions of serum and cell extract spiked with synthetic miRNAs with the following concentrations: 500 nM, 250 nM, 100 nM, 50 nM, 25 nM, 10 nM, 5 nM and 1 nM. LOD was found to be at 5 nM for

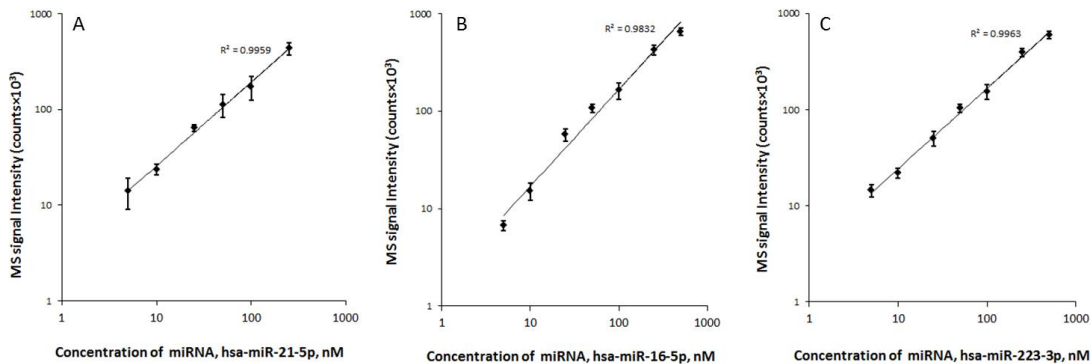


Figure 3.3: Linear regression plot of a relationship between synthetic miRNA concentrations versus intensity of the $[M-5H]^{5-}$ ion in the ESI-MS spectrum without kit isolation. (A) hsa-miR-21-5p; (B) hsa-miR-16-5p; (C) hsa-miR-223-3p. Regression analysis was performed with the R^2 value listed on each curve.

serum samples and 10 nM for cells. The LOD was defined as the lowest spiked miRNA concentration at which miRNA is detected and the charge state can be determined from the signal.

3.4.2 MicroRNA Recovery

Synthetic miRNAs were spiked into the sample before extraction. Analyte recovery (R) of spiked miRNAs was calculated by comparing the signal of a spiked extract from serum and cell samples to a signal of a synthetic miRNA prepared in water using the following equation 3.1. Since a signal of an unspiked sample is below detection limit of the instrument, it was ignored:

$$\%Recovery = \frac{\text{signal from spiked miRNA in cells or serum sample after extraction}}{\text{signal from synthetic miRNA in water}} \times 100\% \quad (3.1)$$

Table 3.3: Percent recovery of miRNAs by direct CE-MS analysis after small RNA isolation

Sample	miRCURY Biofluid	Norgen Plasma/serum	miRNeasy+Cleanup	Norgen microRNA
Spiked RNA, nM	%recovery $\pm SD^a$	% recovery $\pm SD^a$	% recovery $\pm SD^a$	% recovery $\pm SD^a$
500	64 $\pm$ 4	8 $\pm$ 2	28 $\pm$ 5	18 $\pm$ 2
250	57 $\pm$ 4	7 $\pm$ 1	29 $\pm$ 6	15 $\pm$ 3
100	69 $\pm$ 6	6 $\pm$ 1	24 $\pm$ 3	18 $\pm$ 2
50	62 $\pm$ 3	3 $\pm$ 1	26 $\pm$ 3	4 $\pm$ 1
25	46 $\pm$ 15	3 $\pm$ 1	25 $\pm$ 6	10 $\pm$ 3
10	39 $\pm$ 14	5 $\pm$ 1	16 $\pm$ 5	19 $\pm$ 9
5	14 $\pm$ 3	4 $\pm$ 1	-	-

^a Standard deviation (SD) values were obtained from three independent measurements.

Our results show that the miRCURY Biofluid method had the best recovery among the tested methods. This result supports previous findings from other groups [267, 268]. The Norgen Plasma/Serum method showed the lowest percent recovery for serum samples, while miRNeasy+Cleanup method and Norgen microRNA method for cells showed a similar percent recovery (see Table 3.3). The method that showed the lowest percent recovery in serum is the combination of resin and column-based approaches, while the method that showed the highest percent recovery was the pure column-based approach. However, these differences were not seen in samples extracted from cells that were analyzed by these methods. The mass spectra (Figure 3.4 and Figure 3.5) for analyzed RNAs illustrated the substantial difference between samples.

Eldh *et al.* and McAlexander *et al.* [267, 268], compared the miRCURY Biofluid kit with other commercially available kits (however, not the Norgen plasma/serum kit) for serum and plasma endogenous miRNA extraction using a bioanalyzer and qRT-PCR for their method evaluation. Their results also showed that the miRCURY Biofluid kit performed better than the other methods tested. Better performance of the miRCURY Biofluid kit can be explained by the relatively easy to perform procedure that requires a short processing time compared with Norgen Plasma/Serum thus contributing to less pipetting errors and is less prone to RNA degradation. It is also possible that the miRCURY Biofluid kit has a better lysing ability and/or better RNA binding and elution capacities of the column [267].

Both the miRNeasy+Cleanup and Norgen microRNA methods for cells showed similar percent recovery. Although miRNeasy+Cleanup kit showed slightly better performance, which is in agreement with findings by Eldh *et al.* [267] (see Table 3.3 and Figure 3.6), when compared to Norgen microRNA kit for cells. To our knowledge, there are no published data available at this moment to compare the Norgen Plasma/Serum (slurry) or Norgen microRNA kits. However, Li and co-workers [269] did a similar study, where they evaluated three microRNA isolation methods for total RNA extraction from serum. One of the methods they used was total RNA isolation kit (Norgen), similar to the Norgen microRNA

kit [269]. They also found that the quality of RNA isolated with the miRNeasy kit was better than with the Norgen kit.

Our results should be viewed in light of the applicability of these extracted RNAs for CE-MS application since percent recovery was calculated using the MS signal of spiked miRNAs. Analyzing the mass spectra, we found that the majority of miRNA signals fall over the m/z range of 1000-1500. Charge state distribution of -5 to -7 is observed, with the most abundant charge state of -5. At -5 charge state distribution all four miRNAs can be resolved. Mass spectra of samples extracted with the miRNeasy+Cleanup kit and the Norgen microRNA kit for cells have a higher background signal (Figure 3.4, A), dominated by other small RNA peaks in the mass range of 9-12 kDa, as well as low molecular weight species (approx. 1000 Da). Although metal ion adducts (mainly sodiated) are present in both spectra, samples purified with the miRNeasy+Cleanup kit have a higher microRNA signal (Figure 3.4, B), confirming higher miRNA recovery (Figure 3.6, A).

Mass spectra of samples extracted with the Norgen Plasma/Serum kit have a higher background signal (Figure 3.5, A) dominated by short fragments of presumably degraded species of large RNA. Mass spectra of samples extracted with the miRCURY Biofluid kit have a very low background signal. Although a higher signal from deprotonated miRNA peaks is observed with samples extracted with the miRCURY Biofluid kit, a number of metal ion adducts, mainly sodiated adducts, are present when compared to the Norgen Plasma/Serum kit, suggesting that the Norgen Plasma/Serum kit has the best desalting ability out of all of the methods tested. Sodiation (or other metals) spreads the overall ion count over a range of adduct peaks, thus reducing the signal-to-noise ratio (S/N) of the spectrum [270]. Given that samples from the Norgen Plasma/Serum kit produce less sodiated adducts, we should have better spectral quality. However this was not the case. Therefore, we assumed that the sample loss occurs during the extraction procedure. The Norgen Plasma/Serum kit (slurry format that is used for this study) has multiple-step procedures with a long incubation time using a solution containing Norgens proprietary

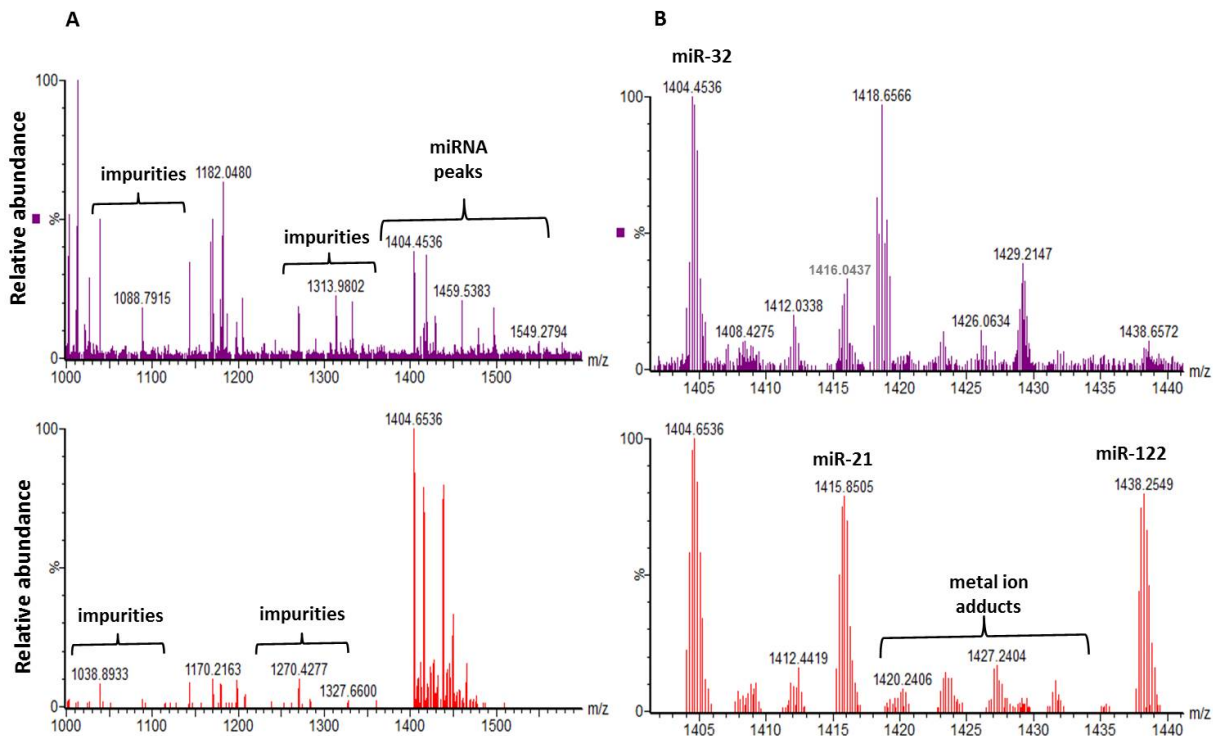


Figure 3.4: Mass spectrum of 500 nM spiked miRNA extracted by (A) Norgen mi-coRNA method for cells (upper spectrum of panel A) and miRNeasy+Cleanup method for cells (bottom spectrum of panel A). (B) Expanded MS view of the charge state of $[M-5H]^{5-}$ extracted with Norgen microRNA kit for cells (upper spectrum of panel B) and miRNeasy+Cleanup kit for cells (bottom spectrum of panel B).

resin serving as the separation matrix before the sample is loaded onto the filter column. This makes the extraction procedure lengthy and is most likely exposed to more technical variations, which could explain the reason for its low extraction efficiency. Moreover, in this method, the resin is loaded into a column containing the membranes, in which RNA would be bound. We believe that the resin may clog the membrane, subsequently leading to the loss of RNA. This may explain the very low recovery of Norgen Plasma/Serum kit.

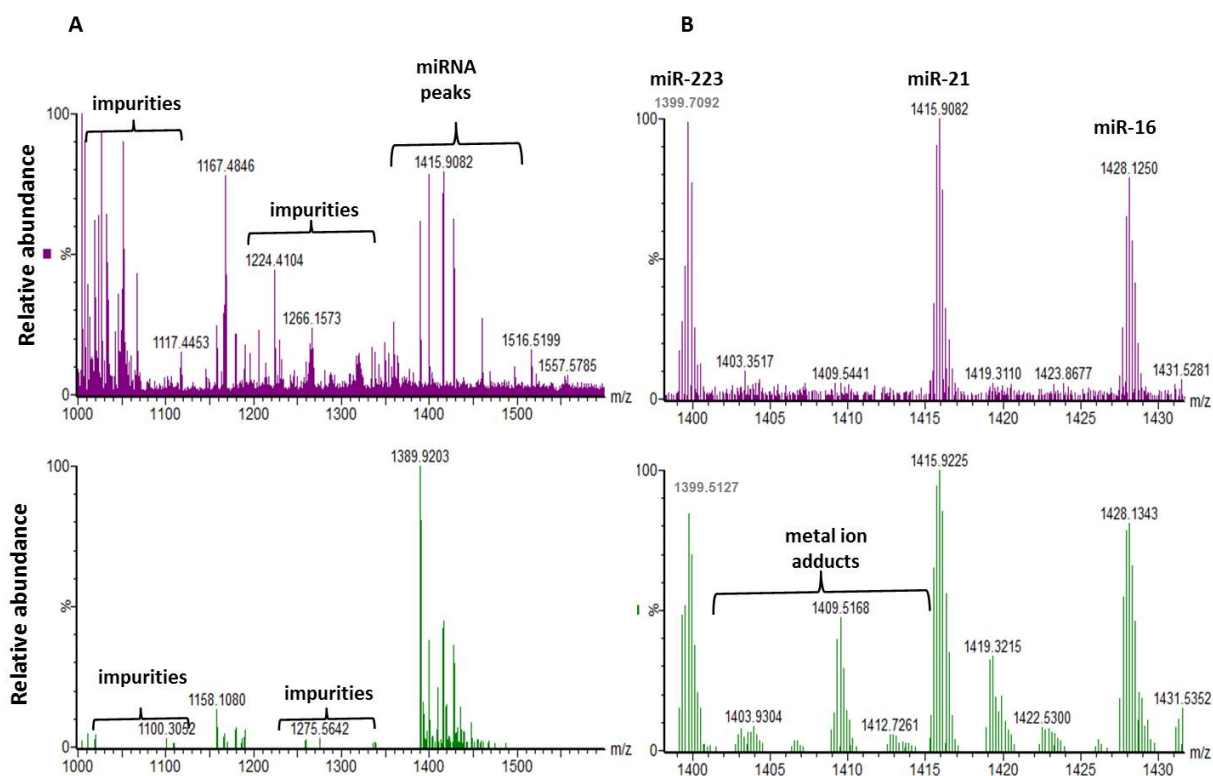


Figure 3.5: Mass spectrum of 500 nM spiked miRNA extracted with (A) Norgen Plasma/Serum kit (upper spectrum of panel A) and miRCURY biofluid kit (bottom spectrum of panel A). (B) Expanded MS view of the charge state of $[M-5H]^{5-}$ extracted with Norgen Plasma/Serum kit (upper spectrum of panel B) and miRCURY biofluid kit (bottom spectrum of panel B).

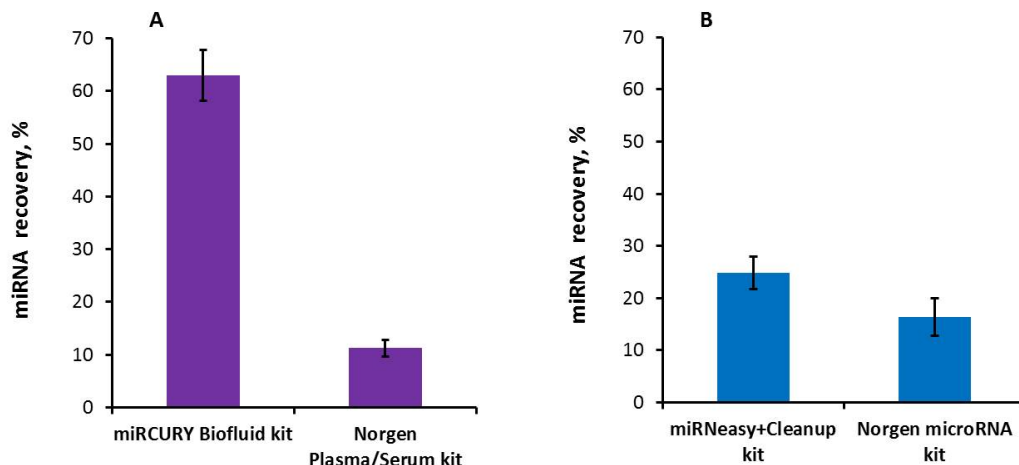


Figure 3.6: Percent recovery of miRNA. Recovery of 500 nM spiked miRNAs (A) from serum and (B) from cell for the different extraction methods. Data is shown as mean values for each method.

3.5 Conclusion

To conclude, in this chapter of the thesis we presented a comparison of four different miRNA purifications methods to select the best-suited method for miRNA sample preparation for CE-MS analysis. The four methods were evaluated for their miRNA extraction potential with a focus on the quality and the yield of RNA. According to our results, all four methods allowed the extraction of high to medium quality RNA making it acceptable for other downstream applications such as qPCR and microarray. On the other hand, for CE-MS analysis, highly pure sample is of great importance.

Our results showed that the relative amount of extracted small RNAs using the miRCURY Biofluid method was higher than with other methods tested. Therefore, the miRCURY Biofluid kit was selected to purify RNA from human serum for further CE-MS analysis. RNA recovery may also be different from one method to another based on RNAs GC content, length or other characteristics, as was shown by Kim *et al.* [271]. However, the low efficiencies and the high variability of some of the kits reported here might be due to a different issue, which is yet to be determined.

To improve the extraction efficiency, modifying the current extraction protocols might be an option. This can be done by changing the ratio of the lysis solution to serum or cell sample [269]. For the extraction methods that use the phenol-chloroform phase separation, performing second phenol-chloroform extraction of the first aqueous layer with RNase-free water could minimize the RNA loss and further remove contaminants [272]. Since different extraction methods yield such a variation of circulating/small RNA, it is important to choose the method carefully, depending on the research question and downstream application. This is particularly important with research involving circulating miRNAs, since quantity of miRNA in plasma/serum is often relatively low.

Chapter 4

Analysis of Serum Circulating RNAs by Capillary Electrophoresis-Mass Spectrometry

4.1 Objective

The objective of this project was to develop a CE-MS method for detection of miRNAs and their modifications directly from patient's blood serum.

4.2 Introduction

Recently, extracellular miRNAs have been found to circulate in the bloodstream in remarkably stable form, protected from endogenous RNase activities [117]. The level of some of these extracellular miRNAs have been associated with different diseases. MicroRNA-expression profiling of human tumors rather than single miRNA levels has identified signatures associated with diagnosis, staging, progression, prognosis and response to treatment.

Moreover, profiling has been exploited to identify miRNA genes that target protein-coding genes involved in cancer [273]. Blood-based biomarkers are attractive for cancer screening due to their minimal invasiveness, relatively low cost and ease of reproducibility.

It has been reported that 3' ends of several miRNAs undergo post-dicing adenylation or uridylation [46, 274–276]. Post-transcriptional addition of nucleotides to the 3' end of miRNAs is a mechanism for regulating miRNA activity. For example, such modification in plants and *C. elegans* influence miRNA stability [52–55]. In humans, effect on stability and on mRNA target repression have both been observed. For instance, adenylation of a mature miRNA, miR-122 by the RNA nucleotidyl transferase GLD-2 in human and mice, resulted in an increase miRNA stability [56]. In contrast, uridylation of miR-26a, had no effect on miRNA stability, but had effect on the ability of miR-26a to inhibit its mRNA target [57]. Modification of miRNA at the 3' position is a physiological and common posttranscriptional event that shows selectivity for specific miRNAs and is observed across species ranging from *C. elegans* to human [45]. Thus, there is a need for a miRNA detection technique which is direct and multiplexed, requiring minimal sample preparation and can provide qualitative information regarding these modifications.

In this work, we developed a multiplexed miRNA detection technique based on capillary electrophoresis-electrospray ionization mass spectrometry (CE-ESI-MS) that offers a convenient platform for label-free, direct analysis of miRNA from biological samples. CE is highly efficient, and versatile, CE separations are fast, relatively inexpensive, and robust, requiring small amounts of sample and reagents [277, 278]. Coupling CE with MS makes it a powerful method for analysis of biomolecules as it combines high-resolution separations with high detection selectivity and sensitivity [279]. Electrospray ionization is a soft ionization technique used in mass spectrometry, for RNA analysis. miRNAs can be directly observed without amplification by this method but are harder to detect than peptides due to their low abundance in biological sample. On-line sample pre-concentration with desalting prior to CE-ESI-MS improves concentration sensitivity for detection of very low

amounts of miRNA in complex biological samples without ionization suppression. Due to the size of mature miRNA molecules (21-23 nt), the effect of nucleotide chain fragmentation can potentially be minimized. Therefore, CE-ESI-MS represents a promising method for endogenous miRNA expression and sequence analyses.

The results for the CE-MS study were validated by conventional SYBR green-based qRT-PCR. For the miRNA identification process, we first conducted a literature search to identify miRNAs that are present in serum of CLL-affected patients, followed by a screening of those miRNAs against observed m/z value. Identified miRNA candidates were further confirmed by qRT-PCR.

4.3 Materials and Methods

4.3.1 Materials

Chemicals and reagents. Buffer components were obtained from VWR (Mississauga, Canada). Isopropanol (Sigma-Aldrich, USA) was of analytical grade and was used as received. All solutions were made using Milli-Q quality deionized water and filtered through a 0.22 μm pore size membrane filters (Millipore, Nepean, ON, Canada). The bare fused silica capillary was purchased from Polymicro Technologies (Phoenix, USA).

Human serum samples. Human serum samples were purchased from Bioreclamation Inc., Hicksville, NY and include: sera from normal male and female donors (pooled) and B cell-chronic lymphocytic leukemia (CLL) sera of a Caucasian, 89 years old, female donor with stable disease stage. No information of treatment outcome was supplied with sample. All samples were kept at -80°C until ready to use.

4.3.2 RNA Isolation

Circulating RNAs were isolated from all samples using miRCURY RNA Isolation kit-Biofluids (Exiqon, Vedbaek, Denmark) according to the manufacturers protocol with the following modifications for the samples used for method optimization and qPCR validation. For method optimization, four synthetic miRNAs based on natural sequences (see Table 4.1) were added to each sample. Synthetic miRNAs were added specifically after denaturation of the sample, to avoid degradation of synthetic miRNAs by endogenous serum RNases. Briefly, 2 mL of normal human serum was thawed on ice, and then split into eight different fractions, 200 μ L each. Sixty μ L of lysis solution was added to each serum fraction. The serum sample was then lysed by vortexing for 1 minute followed by incubating for 3 minutes at room temperature. A mixture of three synthetic oligonucleotides mimicking microRNA sequences of miR-21-5p, miR-16-5p and miR-223-3p (see Table 4.1) was added to each lysate fraction in the following concentrations: 1 μ M, 500 nM, 250 nM, 100 nM, 50 nM, 25 nM, 10 nM and 5 nM separately. One μ M of miR-150-5p (see Table 4.1) was added as an internal control to each lysate fraction, which were then mixed by vortexing. Next, 20 μ L of protein precipitation solution was added and mixed by vortexing followed by incubation for 1 minute at room temperature. The mixture was then centrifuged for 3 min at $11000 \times g$. The supernatant was transferred into a new collection tube. After adding 270 μ L of isopropanol, the sample was loaded onto a microRNA mini-spin column followed by incubation for 2 minutes at room temperature and spun down. Finally, after washing off the bound RNA three times, the RNA was eluted in 50 μ L of RNase free water. Purified RNAs were kept at -80°C until ready to use.

For qPCR experiments, prior to RNA isolation, 7 pM of synthetic *C. elegans* miRNA oligonucleotide *cel*-miR-39-3p based on natural sequences (see Table 4.1) was added to 200 μ L of each normal serum and CLL serum sample to monitor the efficiency of the RNA extraction and to allow for normalization of sample-to-sample variation in RNA isolation.

Table 4.1: Synthetic RNA oligonucleotides used for standard curve and spike-in experiments.

MicroRNA ID	Target miRNA Sequences
hsa-miR-16-5p	UAGCAGCACGUAUAAUUAUUGGCG
hsa-miR-21-5p	UAGCUUAUCAGACUGAUGUUGA
hsa-miR-223-3p	UGUCAGUUUGUCAAAUACCCCA
hsa-miR-150-5p	UCUCCCAACCCUUGUACCAGUG
<i>cel</i> -miR-39-3p	UCACCGGGUGUAAAUCAGCUUG

RNA isolation was then carried out as described above.

For endogenous miRNA detection by CE-MS, 900 μ L of each normal serum and CLL serum was used. No synthetic miRNA oligonucleotide were spiked into the samples before RNA isolation.

4.3.3 Capillary Electrophoresis Coupled to Mass Spectrometry

CE-MS experiments were performed with a Synapt G2 HDMS mass spectrometer (Waters, UK) that was coupled on-line with a PA 800 plus Pharmaceutical Analysis CE system (Beckman Coulter, USA) through a microvial electrospray CE-MS interface (CMP scientific, USA). The capillary was 158 cm long with an inner diameter (i.d.) of 50 μ m and an outer diameter (o.d.) of 360 μ m. The capillary was kept at constant temperature of 15°C and sample storage at 4°C. Sample was injected into the capillary from the inlet end by a pressure pulse of 90 s $\times$ 35 psi. CE separation was performed at 30 kV. Twenty five mM ammonium acetate (pH 6.0) was used as run buffer. Before each sample injection, the capillary was rinsed with 0.1 M NaOH for 2 minutes, deionized water for 2 minutes, and 25 mM ammonium acetate buffer for 3 minutes by a pressure of 75 psi. The sheath liquid

consisting of a 80:20, v/v isopropanol:water with 2 mM ammonium acetate was delivered at a flow rate of 2 $\mu\text{L min}^{-1}$. The mass spectrometer measurement was conducted in the negative-ion mode (ESI-) and the capillary voltage was set at 3.2 kV. Other electrospray parameters under optimum conditions were: sampling cone voltage, 35 V; extraction cone voltage, 3 V; source temperature, 90°C; cone gas, 50 L h⁻¹; and purge gas flow, 0.5 L h⁻¹. Each sample was run for 40 min. The mass spectrum was obtained in the range of m/z 1000-1600.

4.3.4 Quantitative Real-Time RT-PCR of Mature MicroRNAs

A fixed volume of 4 μL of RNA solution from RNA isolation sample was used as input into the reverse transcription (RT) reaction. RNA was reversed transcribed using miRCURY LNA Universal RT microRNA PCR kit and microRNA-specific LNA primer set (Exiqon, Vedbaek, Denmark). Twenty μL RT reaction solution was comprised of 4 μL of 5 $\times$ reaction buffer, 9 μL of nuclease free H₂O, 1 μL of synthetic UniSP6, 2 μL of enzyme mix and 4 μL of input RNA. Nuclease free water was used as a non-template control. Synthetic UniSP6 RNA spike-in oligonucleotide was added to each RT reaction, as a control for the quality of the cDNA synthesis reaction. All components other than the input RNA was prepared as a larger volume master mix. RT reaction was performed using a thermal cycler (Mastercycler pro S, Eppendorf, Germany) at 42°C for 60 minutes, 95°C for 5 minutes followed by immediate cooling at 4°C. Reverse transcription reaction products were diluted 40 times in nuclease free water and 4 μL was combined with 5 μL of SYBR Green master mix and 1 μL of PCR primer mix (Exiqon) to generate a PCR reaction solution of 10 μL . Real-time PCR was carried out in a Bio-Rad CFX thermocycler at 95°C for 10 minutes, followed by 40 cycles of 95°C for 10 second and 60°C for 1 minute. An additional step in the qRT-PCR analysis was performed to evaluate the specificity of the assays by generating a melting curve for each reaction. Obtained data were analyzed using CFX manager software version 3 (Bio-Rad), with the automated assay-specific baseline

and threshold settings.

4.4 Results and Discussion

4.4.1 Optimization of Electrophoretic Separation and Mass Spectrometric Detection

The low abundance of miRNA in serum and the associated salt adducts makes mass spectrometric detection of endogenous circulating miRNAs challenging. We used a CE-ESI-MS and applied an on-line sample pre-concentration method, namely low ionic strength based sample stacking, for detecting very low amounts of miRNA in serum samples. Prior to CE-MS analysis, miRNA was extracted from blood serum. For method optimization, synthetic miRNA based on natural sequences were spiked into the serum before miRNA extraction to mimic the complexity of such samples rather than using synthetic RNA in buffer. Considering their significance as molecular markers for different types of malignancies, we used synthetic RNAs based on natural sequences, mimicking the following microRNAs: miR-21-5p, miR-16-5p, miR-223-3p and miR-150-5p (see Table 4.1). For RNA isolation we used miRCURY RNA Isolation kit, the method that was evaluated to be the best for MS sample preparation as described in Chapter 3. Purified RNA was then directly analyzed by CE-MS with on-line sample pre-concentration (sample stacking) technique.

During CE-MS, sample stacking was achieved by dissolving the sample in a low conductivity matrix, such as water. The capillary was pre-filled with 25 mM of ammonium acetate run buffer and a long plug of sample was hydrodynamically injected followed by a short plug of run buffer. A separation voltage was then applied across the capillary. The sample stacking mechanism is explained in section 1.5.3 [172].

On-line coupling of CE to MS can be done using ESI and either sheath-less or sheath-flow interface. Different experiments were conducted using both types of interface. We

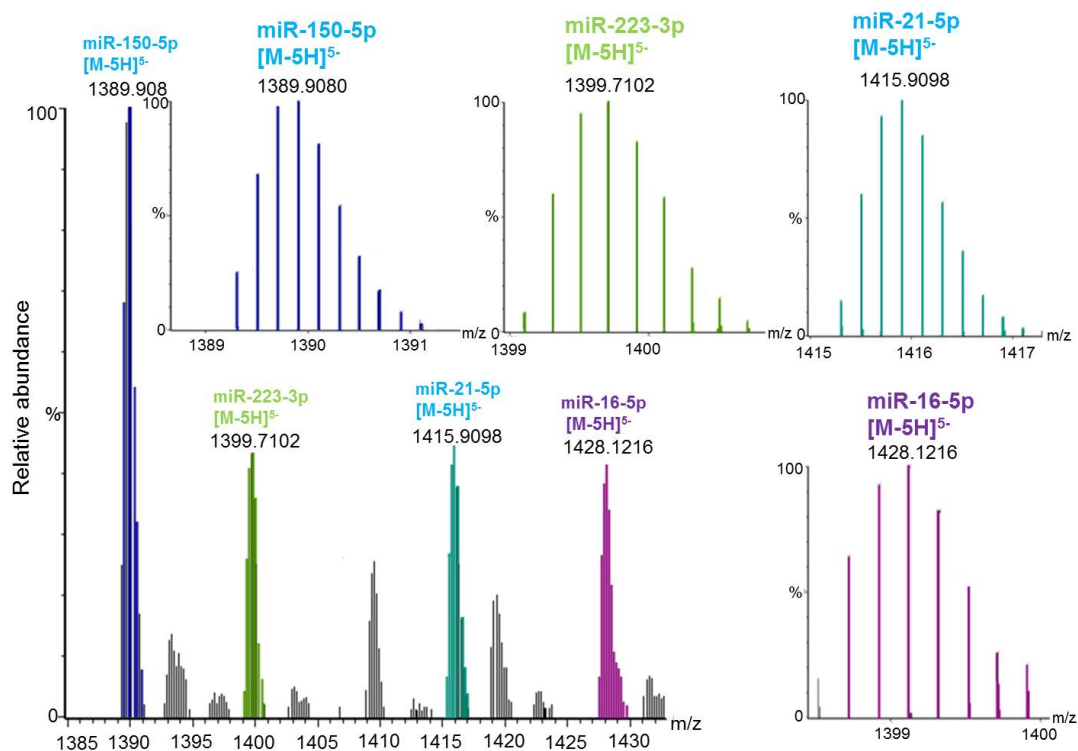


Figure 4.1: CE-MS spectra of the mixture of four miRNAs that were spiked into the serum sample. The concentration of miR-150-5p (blue) is 1 μ M, and for miR-223-3p (green), miR-21-5p (turquoise) and miR-16-5p (purple) is 500 nM. The most abundant signal for all four miRNAs is the fivefold negative charge. A series of isotopic ions of $[M-5H]^{5-}$ are shown in the magnified graphs. The mass spectrum was obtained in the range of m/z 1000-1600.

found that sheath-less interface worked well only for positive ions and in acidic pH of the separation buffer. For negative ions though ionization was not stable. Sheath flow interface on the other hand is robust and created a stable spray allowing for independent optimization of separation buffers. Figure 4.1 shows the mass spectra of purified miRNAs that were spiked into the serum sample after negative ionization. Additives such as triethylamine and ammonium acetate were used for measurement of miRNAs in order to decrease the cation adduct formation and to increase the sensitivity of the detection. Different concentrations and combinations of additives were added to the sheath liquid and tested. The most sensitive sheath mixture yielding the fewest adducts was used for further miRNA analysis from serum samples. Figure 4.1 exhibits the mass spectrum with mass to charge ratio (m/z) of all four deprotonated miRNAs with a negative charge state of $[M-5H]^{5-}$ corresponding to the mass of expected miRNAs (see Table 4.2). The limit of detection (LOD) was found to be 5 nM. The LOD was determined as the lowest spiked miRNA concentration at which miRNA is detected and at which the charge state was being calculated from the signals. The response was linear in the range of 10 nM to 500 nM spiked miRNA (5 nM is outside the linear region) (Figure 4.2).

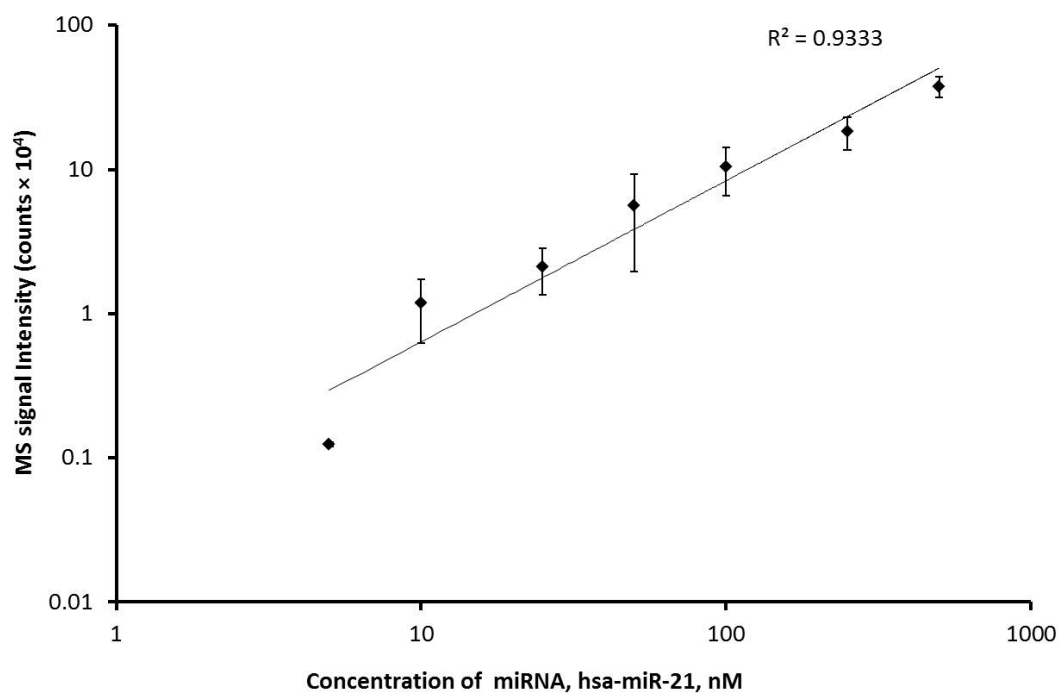


Figure 4.2: Calibration curve between synthetic miRNA concentration and the intensity of $[M-5H]^{5-}$ ions in CE-MS experiment was generated by spiking a dilution series of known input amounts of three synthetic miRNAs into the serum before RNA extraction. A representative regression plot with R^2 value is shown here. All measurements were performed in triplicates.

Table 4.2: Spiked miRNAs and their exact masses

MicroRNA ID	Sequences	m over z			Total mass		
		Theoretical	Observed	Error	Theoretical	Observed	Error
		m/z (Da)	m/z (Da)	(Da) (ppm)	mass (Da)	mass (Da)	(Da) (ppm)
hsa-miR-16-5p	UAGCAGCACGUAAAUAUUGGCG	1428.0478	1428.126	0.07 (54.8)	7145.278	7145.648	0.37 (51.7)
hsa-miR-21-5p	UAGCUUAUCAGACUGAUGUUGA	1415.828	1415.910	0.08 (58.0)	7084.178	7084.589	0.41 (57.9)
hsa-miR-223-3p	UGUCAGUUUGUCAAUACCCCA	1399.628	1399.710	0.08 (58.9)	7003.178	7003.591	0.41 (58.9)
hsa-miR-150-5p	UCUCCCAACCCUUGUACCAGUG	1389.812	1389.908	0.09 (69.0)	6954.078	6954.580	0.50 (72.1)

4.4.2 MicroRNA Detection in Cancer Serum

B-cell chronic lymphocytic leukemia (CLL) is a cancer of the B-lymphocytes and is the most common type of leukemia in adults. CLL derives from a combination of genetic (chromosomal abnormalities, gene mutations) as well as epigenetic (altered microRNA expression, DNA methylation) modifications [280]. Despite recent advances in treatment for CLL, it is still an incurable disease with underlying mechanisms not fully understood. While early stage detection and treatment can control diseases progression, late stage patients often do not respond to treatments [281]. It has been recently shown that extracellular miRNAs circulate in the blood stream and that miRNA levels are associated with different diseases. Previous studies have suggested that miRNA may provide important information about the type of the disease; hence improve its prognosis [282]. In an attempt to acquire informative diagnostic markers with better clinical significance for CLL, Moussay *et al.* investigated changes of extracellular miRNA spectra in CLL plasma sample. The authors suggested that specific plasma miRNA signatures are linked to CLL and can be used as new and informative biomarkers in CLL diagnosis [283].

Existing methods for identifying miRNA rely on using labeled probes to generate a detection signal, i.e obtained signals are not directly attained from miRNA. Our newly developed CE-MS method is direct and can provide higher accuracy than a probe based method. Additionally, the direct mass spectrometric measurements of miRNAs can also be used to detect miRNA modifications, such as nucleotide addition or deletion and methylation.

Here, we used CE-MS with on-line sample pre-concentration as developed by our group, for analyzing miRNAs in biological samples. Due to sample limitation, in our current study we analyzed blood-based miRNA biomarker candidates for CLL based on miRNA expression profiling data reported by Moussay and co-workers. Since serum samples are more plentiful than plasma samples, we used serum sample for our miRNA profiling considering

that both serum and plasma samples are suitable [117]. Small RNAs were extracted from 900 μL of CLL and 900 μL of normal serum (control) without spiking. RNA purification was performed using miRCURY Biofluid method according to the procedure described in Section 4.3.2. Purified RNA was then directly analyzed by the developed CE-MS method with on-line sample pre-concentration with negative mode electrospray ionization. By measuring the exact mass, by time of flight mass spectrometry, we have detected multiply charged negative ions derived from two distinct deprotonated endogenous miRNAs in the mass spectrum of CLL serum (Figure 4.4). Negatively charged small RNA molecules are separated from other impurities, as it can be seen in the total ion chromatogram (see Figure 4.4, A), however both miRNAs migrate at 33.14 min (Figure 4.4, B). Large injection volumes used in stacking, decreases effective separation length of the capillary, which in turn, contributes to reduced resolution.

4.4.3 MicroRNA Identification from the Mass Spectrum

Numerous approaches can be taken to confirm the identity of obtained miRNAs. To identify the miRNA species in the CLL mass spectrum we have performed an initial screening of 45 miRNA species that are reported to be most abundant in different CLL plasma samples (see Table 5.1 Annexe) [283]. In the first round, we screened not only these sequences but also all the possible modifications of each miRNA such as 3'-adenylation, 3'-uridylation and 3'-terminal nucleotide deletion followed by addition of adenine or uridine. Examining these miRNA sequences with their calculated m/z against the observed m/z in our mass spectrum, we found that only a few miRNAs had matching molecular mass with errors in the range of ± 1 Da compared to their calculated molecular mass (see Table 4.3). By process of elimination, for our next step of identification, we had only six possible miRNA candidates.

We further analyzed these miRNA candidates by real time RT-PCR to confirm their

presence in the CLL sample. For qRT-PCR experiments we used the same CLL serum sample and healthy pooled control samples, as for MS analysis. We took two serum aliquots of the CLL and control respectively. Circulating RNAs were isolated from both samples using miRCURY RNA isolation kit-Biofluids (Exiqon, Vedbaek, Denmark) according to the procedure described in Section 4.3.2 with one modification; 7 fM of synthetic *C. elegans* miRNA oligonucleotide based on natural sequences (see Table 4.1) was added to each sample to monitor the efficiency of the RNA extraction and to allow for normalization of sample-to-sample variation in RNA isolation. Real time PCR was performed using ExiLent SYBR Green qRT-PCR assays according to the protocol described in Section 4.3.4. Results of this screening indicated an increased expression level of four of these candidate miRNAs of varying degree, in the CLL serum, compared with the healthy control serum (Figure 4.3). miRNA levels of miR-17-5p and miR-155-5p in CLL samples were the lowest and in normal serum, they were below the limit of detection of the assay. Hence, we excluded miR-17-5p and miR-155-5p from our miRNA candidates list. The level of miR-146a-5p and miR-378a-3p is somewhat higher but lower than that of miR-21-5p and miR-16-5p (Figure 4.3). At the end of the second round of screening we had four possible miRNA candidates that were further analyzed to identify the two peaks observed in the mass spectrum.

By examining the m/z corresponding to the exact mass of these four miRNA sequences and their modifications, we found that 3'-adenylated and 5'-phosphorylated both miR-146a-5p and miR-378a-3p have the calculated m/z corresponding to the exact mass that is similar to the mass observed in the mass spectrum (see Table 4.3) with a mass error of 1.69 Da and 1.09 Da respectively. On the other hand, 5'-phosphorylated miR-21-5p has the calculated m/z corresponding to the exact mass that is similar to the observed mass with a mass error of 0.62 Da and for miR-16-5p with 3'-uridylation and 5'-phosphorylation with a mass error of 0.68 Da (Table 4.3) which is within acceptable range. The mass errors for both miR-146a-5p and miR-378a-3p are higher and their levels in CLL serum are lower

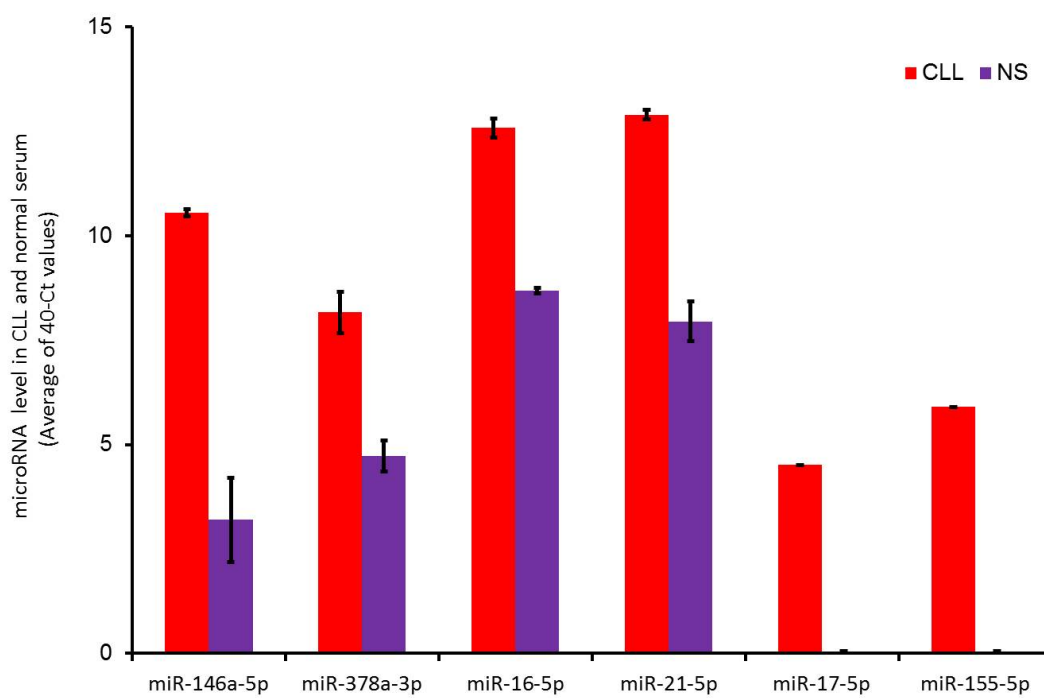


Figure 4.3: MicroRNA level in human normal serum (NS) and CLL serum sample obtained by qRT-PCR. The standard deviation values were obtained from three independent measurements.

than miR-21-5p and miR-16-5p levels, we therefore eliminated these miRNAs as possible miRNA candidates. This leaves us with only two other miRNA candidates. miR-21-5p is a representative oncogenic miRNA [284] and its overexpression in CLL as well as that of miR-16-5p has been reported in several studies [95, 285, 286]. Therefore, it is highly likely that miR-21-5p and miR-16-5p are the miRNAs that are present in our CLL sample.

Table 4.3: MicroRNA candidates used for identification

MicroRNA ID	Sequences	Modifications	Theoretical mass (Da)	Observed mass (Da)	Error (Da) (ppm)
hsa-miR-146a-5p	UGAGAACUGAAUCCAUUGGGUU	23-nt, 5' phos, 3'-A, isomiR	7452.488	7450.797	1.69 (227)
hsa-miR-378a-3p	ACUGGACUUGGAGUCAGAAGGC	23-nt, 3'-A, isomiR	7449.706	7450.797	1.09 (146)
hsa-miR-17-5p	CAAAGUGCUUACAGUGCAGGUAG	23-nt, 5' phos, 3'-U (-G), isomiR	7451.467	7450.797	0.67 (89.9)
hsa-miR-16-5p	UAGCAGCACGUAAAUAUUGGCG	23-nt, 5' phos, 3'-U, isomiR	7451.478	7450.797	0.68 (91.4)
hsa-miR-21-5p	UAGCUUAUCAGACUGAUGUUGA	22-nt, 5' phos	7084.178	7084.799	0.62 (87.6)
hsa-miR-155-5p	UUAAUGC UAAUCGUGAUAGGGGU	22-nt, 3'-de U, isomiR	7083.526	7084.799	1.27 (179)

Finally, taking the exact mass measured by time of flight mass spectrometry and qRT-PCR data together, we confirmed that the peak with a m/z of 1415.952 ± 0.62 Da corresponding to the exact mass of 7084.799 Da represents 22 nucleotide long 5'-phosphorylated miR-21-5p and the peak with a m/z of 1489.151 ± 0.68 Da corresponding to the exact mass of 7450.797 Da represents 23 nucleotide long 5'-phosphorylated miR-16-5p with 3'-uridylation (iso-miR-16-5p) (Figure 4.4 C, Table 4.4). Additionally, these findings were confirmed by performing CE-MS of the synthetic miRNAs based on natural sequences of these two miRNAs (Figure 4.4 D). Briefly, the synthetic miR-21-5p and iso-miR-16-5p were spiked into CLL serum prior to miRNA extraction as described in subsection 4.3.2. Purified RNA sample was then analyzed by CE-MS. Obtained mass spectrum (Figure 4.4 D) shows similar profile with two dominant peaks representing m/z of 1415.935 Da and 1489.156 Da which correspond to the mass of canonical miR-21-5p with mass error of 0.54 Da and iso-miR-16-5p with a mass error of 0.66 Da respectively. Thus, this data supports the identity of the two peaks in the mass spectrum. Furthermore validation by qRT-PCR (Figure 4.5) showed higher abundance of these two miRNAs in CLL serum compared to normal serum (figure 4.5 B). No detectable peaks at this two m/z were observed in normal serum (NS) sample (Figure 4.5, bottom panel of A).

The appropriate selection of miRNA extraction methods for serum sample, on-line sample preconcentration and carefully optimized CE-MS condition helped enrich the miRNA detection by CE-MS. Although the method developed here is able to detect endogenous miRNAs and their modification in serum with mass accuracy in low ppm range, performing MS/MS using high resolution mass spectrometry would additionally confirm the identity of miRNAs.

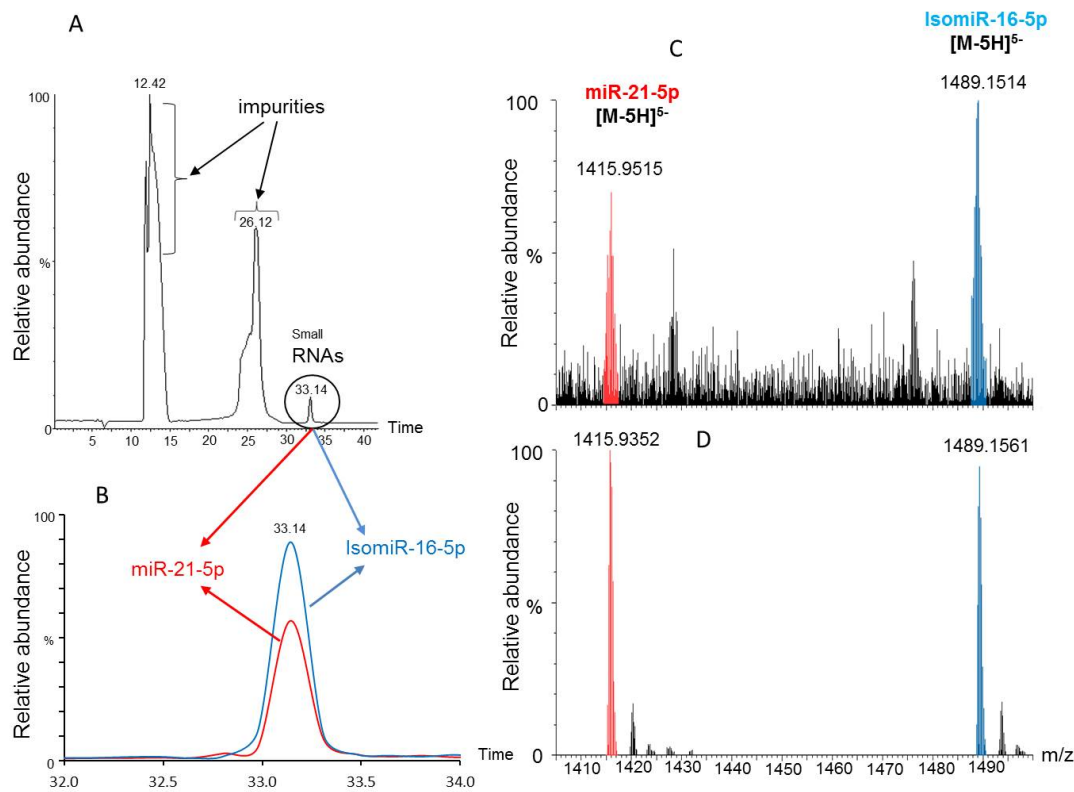


Figure 4.4: CE-MS results of miRNAs isolated from CLL serum. (A) Total ion electropherogram that includes background noise, impurities as well as sample components. (B) Extracted ion electropherogram showing $[M-5H]^{5-}$ of miR-21-5p and iso-miR-16-5p. (C) Negative ion mass spectrum of miRNAs isolated from CLL serum. (D) Negative ion mass spectrum of CLL serum spiked with 1 μ M each miR-21-5p and iso-miR-16-5p.

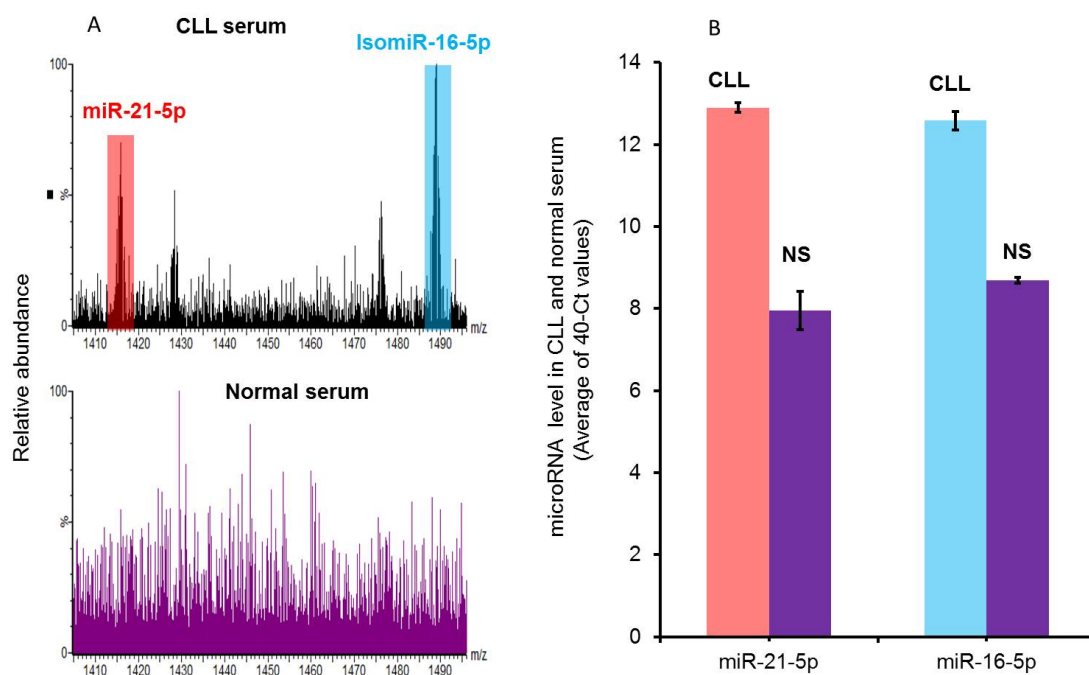


Figure 4.5: Validation of detected microRNAs by qRT-PCR. (A) Negative ion mass spectrum of small RNAs isolated from CLL serum (upper panel) and normal serum (bottom panel) (B) Level of miRNAs miR-21-5p and miR-16-5p in human normal serum (NS) and CLL serum sample obtained by qRT-PCR. The standard deviation values were obtained from three independent measurements.

Table 4.4: Detected miRNAs with their exact masses

MicroRNA ID	Sequences	Modifications	Theoretical mass (Da)	Observed mass (Da)	Error (Da) (ppm)
hsa-miR-21-5p	UAGCUUAUCAGACUGAUGUUGA	22-nt, 5' phos	7084.178	7084.799	0.62 (87.6)
hsa-miR-16-5p	UAGCAGCACGUAAAUAUUGGCGU	23-nt, 5' phos, 3'-U, isomiR	7451.478	7450.797	0.68 (91.5)

4.5 Conclusion

A 23 nucleotide long 5'-phosphorylated miRNA with 3'-uridylation, along with a 22 nucleotide long 5'-phosphorylated canonical miRNA, were detected from only 900 μ L of B-cell chronic lymphocytic leukemia serum sample. We have developed a detection method using capillary electrophoresis coupled on-line to mass spectrometry with electrospray ionization that provides sensitive and reliable detection of low amounts of multiple miRNAs directly from blood serum without amplification. This method applies an on-line sample preconcentration technique as a means of increasing sensitivity by using low ionic strength stacking. Optimized conditions for a large sample volume injection (35 psi, 90 s) were used to achieve up to 100-fold enhancement of sensitivity. Unlike common miRNA detection methods (e.g., qPCR and microarrays) CE-MS is label free, multiplex, fast and easy to perform. The information obtained by CE-MS can complement the data obtained by conventional miRNA analysis methods. This method has the potential to be adapted in high resolution mass spectrometry, which would offer de novo sequencing, enabling identification of existing and novel miRNAs as well as confirming their post transcriptional modifications.

Chapter 5

Conclusion

In this doctoral thesis I discussed some of the challenges for the detection and analysis of endogenous miRNA, provided novel approaches to overcome these challenges, and illustrated the usefulness of these novel methods. In this chapter, I provide a brief summary of this work's contributions as well as discuss future directions.

5.1 Summary of the Thesis

The first objective of this thesis was to develop a simple technique for miRNA analysis in blood serum. The use of traditional techniques, such as northern blotting, qRT-PCR and microarrays are advantageous for the screening of miRNAs, but they have their limitations. Although microarrays allow for massive parallel and relative measurement of all known miRNAs, they lack sensitivity. Some miRNA species have very low copy numbers per cell making it difficult to detect. Moreover, with minute differences in miRNA expression between normal and cancerous cells, high sensitivity is essential. Thus, sensitive and selective diagnostic methods are needed for miRNA detection.

To this end, we developed a protein-facilitated affinity capillary electrophoresis (ProFACE) assay of hybridization products (i.e. duplex of miRNA and complementary probe

specific to miRNA) for sensitive and quantitative detection of small RNAs. In CE, molecules are separated based on their charge-to-size ratios. It is very difficult to separate non-hybridized probes and duplexes without altering their size-to-charge ratios. To overcome this problem, we used two proteins, single-stranded DNA binding protein (SSB) and an RNA binding protein p19, as tools to enhance the separation between fluorescently labeled ssDNA or ssRNA probes and the miRNA probe duplex. Although addition of SSB or p19 increases the peak width, resolution was significantly better (Figure 2.2, 2.3, and 2.4), making it easier to calculate the amount of target miRNA. We showed that SSB and p19 effectively induce the separation of DNA and RNA probes and their hybridization product.

Prior studies have suggested that miRNAs are present in clinical samples of plasma and serum in a remarkably stable form and can serve as cancer biomarkers. These circulating RNAs are present in the range from 100 to 10^{10} miRNA copies/mL [117,147]. For example, the serum level of miR-141 (a miRNA expressed in prostate cancer) shows a 46-fold elevation compared to the healthy group, as reported by Michelle and co worker [117]. We used two strategies for the detection of ultralow amounts of miRNA in blood samples: off-line pre-concentration of miRNA by p19-coated magnetic beads and online sample stacking. Our developed ProFACE miRNA detection method was applied to a liver-specific miRNA, miR-122, expression of which was shown to be repressed in liver cancer [287]. Without PCR amplification, we detected as little as 3×10^5 molecules of miR-122 spiked in 1 mL of serum. The p19 bead precipitation and CE stacking helped to enrich the miRNA from serum by more than 1000-fold when compared to our CE experiments without the pre-concentration. The use of p19 beads brings two major benefits: miRNA-selective extraction and sample enrichment. This separation-based method is applicable to *in vitro* detection of endogenous miRNAs with picomolar and femtomolar sensitivity without and with sample pre-concentration, respectively.

Additionally, I also presented data to show that CE-LIF with on-line sample stacking can be applied to detect endogenous miRNAs. Total RNA was extracted from human

hepatoma cell lines Huh-7 and Hep-G2 (one million cells per sample). It is reported that Huh-7 cells express miR-122, while Hep-G2 cells do not express this miRNA. On the other hand, both cells express miR-21 [266,288]. Endogenous miR-122 and miR-21 were detected with miRNA-specific fluorescently-labeled probes (Figure 2.8 and 2.9) from as little as 10 μ L of Huh-7 or Hep-G2 cell extract. The expression level of miR-122 was calculated to be 199 ± 38 pM in the sample. We showed that CE-LIF with on-line sample stacking is a robust and sensitive method and has potential for use in diagnostics.

Deregulation of several specific miRNA species produces a miRNA fingerprint that can distinguish between cancerous and non-cancerous cells. Profiling of miRNA expression, rather than single miRNA levels, has identified signatures associated with diagnosis, staging, progression, prognosis and response to treatment [273]. Therefore, we moved on to develop a method for detection of multiplexed miRNA using CE-LIF. CE-LIF has been previously used for detection of multiple miRNAs [171,289]. Authors of these articles have achieved separation through attachment of "drag tags" to the labeling probes with an LOD of 100 pM. Similarly, we designed a complementary DNA probe with a modification that causes the duplex peak of the modified miRNA to shift by decreasing its migration time (Figure 2.12). In an experiment with normal human serum using two probes, one modified and another unmodified, two endogenous miRNAs were detected simultaneously (Figure 2.13). MicroRNAs miR-21 and miR-16 are known to be abundant in normal human serum [118]. The result of this proof-of-principle shows that a modified probe enables a simultaneous detection of several miRNAs in normal human serum extract. We think that further investigation is required to optimize this method.

Although other studies [170,171,290] and our CE-LIF study demonstrate the usefulness of the CE-LIF method for miRNA analysis in biological samples with a relatively low detection limit, the use of probes requires one to know the sequence of the target miRNA a priori. Furthermore, multiplexing is still challenging with CE-LIF, considering that the probe design for each target miRNA is complex and costly, among other things. Further-

more, miRNAs from the same family have similar sequences, differing from each other by only one nucleotide. Moreover, post-transcriptional addition of nucleotides to the 3' end of miRNAs is widely observed and has been shown to have functional consequences in plants and animals [7, 52–55, 57]. However, very little is known about these modifications in human samples. Using currently available miRNA profiling methods such as qRT-PCR, microarray and high-throughput sequencing (RNA-seq), it is difficult to measure these miRNAs due to the low sensitivity and high cost. Recently, MS has been used to screen siRNA in plasma samples for doping control purposes [213, 214] with reported LOD of 250 nM, although no endogenous small RNAs were detected with this method. In a recent work, Kullolli and co-worker investigated the utility of high resolution mass spectrometry for the analysis of synthetic miRNAs [291] and LOD was reported to be 1 nM. Therefore, the second objective of this thesis was to develop a multiplexed miRNA detection technique based on capillary electrophoresis- electrospray ionization mass spectrometry (CE-ESI-MS). This technique offers a convenient platform for label-free, easy to perform, direct analysis of multiple miRNAs from biological samples, as well as the identification of post-transcriptional modifications, therefore contributing to better understanding of the miRNA function.

Due to the complex nature of biological matrices, it is difficult to obtain relatively pure miRNA samples required for a successful analysis by mass spectrometry of serum microRNAs that are low in abundance. Furthermore, it is important to have an efficient sample processing that will provide maximal recovery of RNA from a low sample volume.

Thus, in Chapter 3 of this thesis we evaluated four commercially available RNA purification methods using CE-ESI-MS. Extraction methods were analyzed for their miRNA extraction potential with a focus on the purity and the yield of RNA. Our results demonstrated that all of the tested methods allowed the extraction of high to medium quality spiked-in RNA with a large variations in the recovery. Column based miRCURY Biofluid method performed the best, giving us a better percent recovery when compared to the Nor-

gen plasma/serum method (slurry) (Table 3.3 and Figure 3.6). Our finding is in agreement with previous reports [267, 268], where these authors compared the miRCURY Biofluid method with other commercially available kits (however not the Norgen plasma/serum kit) for endogenous miRNA purification using the bioanalyzer and the qRT-PCR methods respectively. They also found that the miRCURY Biofluid method outperformed the other methods tested. We think that its superior performance is due to the fact that it is relatively easy to perform, and requires a short processing time, leaving less space for pipetting errors and less time for degradation. Higher percent recovery of the miRCURY Biofluid method may also suggest better lysing ability and/or RNA binding and elution capacity of the column [267]. Additionally, in case of the Norgen plasma/serum method (slurry), loss of sample can occur during the long binding step with the separation matrix, contributing to a lower miRNA signal and consequently, lower percent recovery. Both miRNeasy+Cleanup and Norgen microRNA methods showed similar performances, miRNeasy+Cleanup method showing a slightly higher percent recovery [267] (Table 3.3 and Figure 3.6). To the best of our knowledge, no data was available to compare the Norgen Plasma/Serum kit (slurry) and Norgen microRNA kit at this moment. However, Li and co-worker did evaluate three commonly used commercial microRNA isolation kits, one being the total RNA isolation kit (Norgen), similar to the Norgen microRNA method [269]. Authors of this study analyzed the total RNA extraction from serum. They also reported that the RNA isolated with the miRNeasy method was of a better quality than with the Norgen method, as it was the case reported by Eldh *et al.* [267] and our group.

One of the ways to improve the extraction efficiency might be by modifying current extraction protocols by changing the ratio of the lysis solution to serum and cells [269] and, in case of the phenol-chloroform extraction, performing a second extraction of the first aqueous layer with RNase-free water might minimize the RNA loss and remove contaminants [272]. However, the purpose of this study was to identify a method that would provide a relatively better miRNA recovery and a good-quality sample for mass spectrom-

etry analysis. Our results showed that the relative amount of small RNA extracted using the miRCURY Biofluid kit was higher than with other methods tested. Therefore, we selected this method to purify RNA from human serum for further MS analysis.

Coupling of CE with MS is a powerful method for the analysis of biomolecules as it combines a high-resolution separation with a high detection selectivity and sensitivity [292]. On-line sample pre-concentration with desalting by CE-ESI-MS improved the sensitivity without ionization suppression for detection of very low amounts of miRNA in complex biological samples. The instrumental dynamic range for detection of synthetic solutions of miRNA in water was found to be 1 nM to 500 nM. The limit of detection was 10 nM for the spiked synthetic miRNA in cells and 5 nM in serum. We showed that the extract of only 900 μ L of CLL serum was enough to detect two endogenous miRNAs, canonical miR-21-5p and isomiR miR-16-5p, with a mass accuracy in the moderate ppm range. An average of 1.3 ppm mass accuracy was reported by [291]. These findings were validated by qRT-PCR experiments, which showed a higher abundance of these two miRNAs in CLL serum compared to normal serum (Figure 4.5, B). MicroRNA miR-21-5p is a representative oncogenic miRNA [293] and its overexpression in CLL, as well as that of miR-16-5p, has been reported in several studies [95, 285, 286]. We showed here that the appropriate selection of miRNA extraction method, on-line sample pre-concentration, and carefully optimized CE-MS conditions helped to improve the miRNA detection by CE-MS. Furthermore, this method has the ability to discriminate variants of a single nucleotide. The developed CE-ESI-MS method is also robust enough to adapt in high-resolution tandem mass spectrometry, providing miRNA sequence information, ultimately eliminating the need for using the qRT-PCR confirmation step. Moreover, this technique is robust and can be used to analyze miRNAs in different biological samples, as it was shown in Chapter 3, where we analyzed spiked miRNAs in serum as well as Huh-7 cell extract.

Finally, regardless of the limitations of this thesis, we developed simple techniques for miRNA analysis in biological samples using commercially available CE-LIF and CE-ESI-

MS systems. These methods are robust and efficient, and can complement conventional miRNA analysis methods. To the best of our knowledge, the developed CE-ESI-MS method is the first to detect endogenous serum-circulating RNAs. This method also has the potential to study miRNA modifications systematically and identify miRNAs that are modified in diseased samples.

5.2 Future Work

This thesis made a significant contribution in the field of miRNA analysis in human serum. However, some improvements in the field are still necessary and we wish to explore that in the future.

The microRNA extraction kit evaluation study performed here has numerous limitations, which could potentially be improved. We measured only several synthetic miRNAs with only one CE-MS method here. It is highly possible that miRNA recovery differs for one method to another and can vary from miRNA to miRNA, as it was reported by Kim *et al.* [271]. A miRNome-wide evaluation of extracted methods using different techniques would be highly beneficial.

Although the CE-MS method developed here enables the detection of endogenous miRNAs and their modification in serum with a mass accuracy in the moderate ppm range, performing MS/MS would additionally confirm the identity of these miRNAs. Currently, due to the limitation of our mass spectrometry instrument, tandem MS was not performed. However, the use tandem MS would enable us to acquire miRNA sequence information and further improve the mass accuracy. High mass accuracy in combination with the charge state identity will enable us to identify fragment ions and base composition. Thus, our developed method can be adapted to high-resolution tandem mass spectrometry, for de novo sequencing and analysis miRNAs in biological sample.

We would also like to explore and develop other innovative and easy to perform methods

to detect miRNAs in clinical samples that could be easily adaptable in routine clinical settings for diagnostic purposes. For instance, bead based miRNA detection using flow cytometry will enable us to detect multiple miRNAs from the same serum sample. For this purpose, biotinylated capture probes complimentary to target miRNAs could be coupled with fluorescent streptavidin beads. Theoretically, bead based hybridization might provide a greater specificity, as was observed by Lu *et al.* [92] where they profiled 217 miRNAs from mammalian cell extracts, that was amplified by PCR prior to performing bead based flow cytometry. Therefore, the bead based miRNA detection using multicolor flow cytometry has the potential to be developed as a high throughput and efficient miRNA profiling method.

List of Publications

1. **Khan, N.**, Mironov, G.G., Berezovski, M.V. (2015) Direct detection of endogenous microRNAs and their modifications in cancer serum by Capillary Electrophoresis-Mass Spectrometry. Submitted to Analytical and Bioanalytical Chemistry.

Statement of contribution: I planned and carried out all the experimental work. I was also the principal writer of the paper.

2. Labib, M., **Khan, N.**, Berezovski, M.V. (2015) Protein Electrocatalysis for Direct Sensing of Circulating MicroRNAs. Analytical Chemistry. 87 - 2: 1395 - 1403.

Statement of contribution: I contributed significantly to this co-authored paper. I performed all miRNA purifications from human serum, and conducted all qRT-PCR experiments to validate the data obtained from the biosensor experiments. Finally, while I was not the primary contributor to writing of this manuscript, I contributed to writing the introduction regarding miRNAs in Chronic Lymphocytic Leukemia, qPCR data analysis, the formulation of ideas expressed in the discussion and to the final editing of this manuscript.

3. Mironov, G.G., Okhonin, V., **Khan, N.**, Clouthier, C.M., Berezovski, M.V. (2014) Conformational Dynamics of DNA G-Quadruplex in Solution Studied by Kinetic Capillary Electrophoresis Coupled On-line with Mass Spectrometry. Chemistry Open, 00, 1 - 8.

Statement of contribution: My contribution to this co-authored paper was to carry out sample preparation and perform several CE-UV and CE-MS experiments.

4. Labib, M., Ghobadloo, S.M, **Khan, N.**, Kolpashchikov, D.M., and Berezovski, M.V. (2013) Four-Way Junction Formation Promoting Ultrasensitive Electrochemical Detection of MicroRNA. Analytical Chemistry (Letter), 85 - 20: 9422 - 9427.

Statement of contribution: My contribution to this co-authored paper includes performing miRNA purifications from human serum, and conducting qRT-PCR experiments

to validate the data obtained from the biosensor experiments. I contributed to writing the introduction and qRT-PCR data analysis.

5. Labib, M., **Khan, N.**, Ghobadloo, S.M., Cheng, J., Pezacki, J.P., Berezovski, M.V. (2013) Three-Mode Electrochemical Sensing of Ultra-Low MicroRNA Levels. *Journal of the American Chemical Society*, 135 - 8: 3027 - 3038.

Statement of contribution:

I contributed significantly to this co-authored paper. I performed miRNA purifications from human serum, and conducted qRT-PCR experiments to validate the data obtained from the biosensor experiments. Finally, while I was not the primary contributor to writing of this manuscript, I contributed to data analysis and the formulation of ideas expressed in the discussion.

6. **Khan, N.**, Cheng, J., Pezacki, J.P., and Berezovski, M.V. (2011) Quantitative Analysis of MicroRNA in Blood Serum with Protein-Facilitated Affinity Capillary Electrophoresis. *Analytical Chemistry*. 83: 6196 - 6201.

Statement of contribution: I planned and carried out all the experimental work. I was also the principal writer of the paper.

Book chapters

1. Berezovski, M.V., and **Khan, N.** (2013). Quantitative Analysis of microRNA in blood serum by Protein-Facilitated Affinity Capillary Electrophoresis. *Methods in Molecular Biology, Nucleic Acid Detection Methods and Protocols*. Humana press. 1039: 245 - 259.

Statement of contribution: I was the principal writer of this book chapter.

Appendix

Table 5.1: MicroRNA sequences used for CE-MS screening

MicroRNA ID	Target miRNA Sequence (5'-3')	Length, nt
hsa-miR-146a-5p	UGAGAACUGAAUCCAUGGGUU	22
hsa-miR-155-5p	UUA AUGCUAAUCGUGAUAGGGGU	23
hsa-miR-195-5p	UAGCAGCACAGAAAUAUUGGC	21
hsa-miR-221-3p	AGCUACA UUGUCUGCUGGGUUUC	23
hsa-miR-23b-3p	AUCACA UUGCCAGGGAUUACC	21
hsa-miR-21-5p	UAGCUUAUCAGACUGAUGUUGA	22
hsa-miR-122-5p	UGGAGUGUGACAAUGGUGUUUG	22
hsa-miR-122-3p	AACGCCAUUAUCACACUAAAUA	22
hsa-miR-223-5p	CGUGUAUUUGACAAGCUGAGUU	22
hsa-miR-223-3p	UGUCAGUUUGUCAAUACCCCA	22
hsa-miR-150-5p	UCUCCCAACCCUUGUACCAGUG	22
hsa-miR-19b-3p	UGUGCAAAUCCAUGCAAAACUGA	23
hsa-miR-92a-3p	UAUUGCACUUGUCCCGGCCUGU	22
hsa-miR-320a	AAAAGCUGGGUUGAGAGGGCGA	22
hsa-miR-484	UCAGGCUCAGUCCCCUCCCGAU	22
hsa-miR-17-5p	CAAAGUGCUUACAGUGCAGGUAG	23
hsa-miR-16-5p	UAGCAGCACGUAAAUAUUGGCG	22
hsa-miR-646	AAGCAGCUGCCUCUGAGGC	19
hsa-miR-618	AAACUCUACUUGUCCUUCUGAGU	23
hsa-miR-376a-3p	AUCAUAGAGGAAAAUCCACGU	21
hsa-miR-518f-3p	GAAAGCGCUUCUCUUUAGAGG	21
hsa-miR-194-5p	UGUAACAGCAACUCCAUGUGGA	22

hsa-miR-628-5p	AUGCUGACAUUUUACUAGAGG	22
hsa-miR-222	AGCUACAUCUGGCUACUGGGU	21
hsa-miR-520c-3p	AAAGUGCUUCCUUUUAGAGGGU	22
hsa-miR-34a	UGGCAGUGUCUUAGCUGGUUGU	22
hsa-miR-106a-5p	AAAAGUGCUUACAGUGCAGGUAG	23
hsa-miR-20a-5p	UAAAGUGCUUAUAGUGCAGGUAG	23
hsa-miR-190b	UGAUAUGUUUGAUAUUGGGUU	21
hsa-miR-342-3p	UCUCACACAGAAAUCGCACCCGU	23
hsa-miR-25-3p	CAUUGCACUUGUCUCGGUCUGA	22
hsa-miR-30a-5p	UGUAAACAUCCUCGACUGGAAG	22
hsa-miR-601	UGGUCUAGGAUUGUUGGAGGAG	22
hsa-miR-151a-3p	CUAGACUGAAGCUCCUUGAGG	21
hsa-miR-451a	AAACCGUUACCAUACUGAGUU	22
hsa-miR-24-3p	UGGCUCAGUUCAGCAGGAACAG	22
hsa-miR-645	UCUAGGCUGGUACUGCUGA	19
hsa-miR-486-5p	UCCUGUACUGAGCUGCCCCGAG	22
hsa-miR-191-5p	CAACGGAAUCCCCAAAAGCAGCUG	23
hsa-miR-875-5p	UAUACCUCAGUUUUAUCAGGUG	22
hsa-miR-378a-5p	CUCCUGACUCCAGGUCCUGUGU	22
hsa-miR-378a-3p	ACUGGACUUGGAGUCAGAAGGC	22
hsa-miR-483-5p	AUCAUAGAGGAAAAUCCACGU	22
hsa-miR-892b	CACUGGCUCCUUUCUGGGUAGA	22
hsa-miR-32-5p	UAUUGCACAUAUAAGUUGCA	22

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