SEPARATION OF MULTIPLE miRNAs USING MICELLAR ELECTROKINETIC CHROMATOGRAPHY MODIFIED CAPILLARY ELECTROPHORESIS WITH LASER INDUCED FLUORESCENCE DETECTION.

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Abstract

MicroRNAs (miRNAs) are short, non-coding, 22-nucleotide regulatory RNA sequences. They are known to induce post-transcriptional gene silencing by binding with imperfect complementarity to target mRNAs. Deregulation in the expression of various miRNAs has been observed in disorders ranging form hepatitis, rheumatoid arthritis, acute myocardial infraction and many cancer types. The potential use of miRNAs as biomarkers for prognosis of such diseases is limited by the low quantitative accuracy and inefficiency of current technologies. Recently, capillary electrophoresis with laser induced fluorescence detection (CE-LIF) has been used to identify miRNAs at low concentrations without the need for amplification. Here we present a novel method to identify and resolve multiple miRNAs using micellar electrokinetic chromatography (MEKC) modified CE-LIF. MiRNA-122 and miRNA-21, two miRNAs know to be up regulated in breast carcinoma were identified using fluorescently labeled, synthetic complementary DNA (cDNA) probes. Once identified, miRNA-122 and miRNA-21 probe hybrids were successfully and efficiently resolved using MEKC modified CE-LIF.

Acknowledgements

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I also wish to thank Marta Janczarski and Danny Pau for their relentless moral support and creatively constructive discussions throughout the year. Without your support my productivity would have been questionable.

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Introduction

Mediation of gene expression is achieved in many ways and is thought to be a crucial advance in the evolution of eukaryotes and the development of higher organisms.[1] We focus here specifically on RNA interference in gene expression, or post-transcriptional gene silencing.[2] In 1998, Fire et al. first reported that introduction of selected double stranded RNA (dsRNA) to nematode (*Caenorhabditis elegans*) embryos would induce systemic silencing of certain genes. The silenced gene would always, to a certain degree be homologous the dsRNA used. The team also noted a significant lack in mRNA relating to the gene that was silenced, hence coining the term RNA interference.[3] When dsRNA is introduced and recognized in a target cell, it is spliced by an RNase III endoribonuclease called *Dicer*. It is spliced to form a double strand segment of small interfering RNA (siRNA).[4] This 21-23 nucleotide siRNA segment is homologous to its mRNA target. When the siRNA is expressed in the cell, it will bind to its target mRNA and induce degradation.

Like siRNAs, small temporal RNAs (stRNA) are non-coding RNA segments ~70 nucleotide in length that are encoded in the cellular genome. When transcribed, the stRNA will form a characteristic hairpin loop conformations.[5] While in this conformation, the stRNA will be spliced in a similar way to dsRNA. The endoribonuclease *Dicer* will splice the stRNA to form small RNA segments called microRNA (miRNA) roughly 22nt in length. Unlike the complete homology necessary for siRNAs to binds its target mRNA, a miRNA will display imperfect

complementarity to its target. This incomplete homology allows a single miRNA wider regulatory scope by targeting multiple mRNAs.[6] [7] [8]

It is estimated that the human genome has encoded up to a thousand unique miRNAs, of which only a fifth have been identified to date.[9] RNA mediated gene silencing has been shown to play an important role in numerous cellular processes as diverse as cell growth, differentiation and death.[10] Abnormal levels of expressed miRNAs have been associated with a variety of medical disorders. Diseases ranging from hepatitis,[11] rheumatoid arthritis,[12] and acute myocardial infraction,[13] to thyroid,[14] ovarian,[15] colon,[16] and breast cancers.[17] MiRNAs are currently under investigation and being considered for use as biomarkers to aid in the accurate detection of such diseases.[18] Early prognosis is often imperative in providing prompt, appropriate and effective therapy to patients.

It is fairly common in cancer cells that more then one miRNA is abnormally expressed. In the case if certain cancers, the normal expression of up to 30 miRNAs can be altered.[19] [16] The identification and quantification of these irregularities is required to be beneficial in diagnosis. Many RNA identification techniques such as northern blotting, microarrays and reverse transcription polymerase chain reaction (RT-PCR) are currently used. These techniques however often lack the sensitivity required for dependable, accurate and fast quantitative analysis. Recently, targeting specific miRNA with complementary

DNA (cDNA) probes followed by electrophoretic separation has been studied as a method to overcome these shortfalls.[20] [21] [22]

Many technologies have been developed for the purpose of separating biological macromolecules. The most widely used techniques presently in use have been established on the principles of electrophoresis. This technique, first optimized by Arne Tiselius involves the separation of macromolecule based on their charge. When a uniform electric field is applied through an ionic buffer, its was observed that positively charged ions (cations) migrated to the negative pole of the field while negatively charged ions (anions) migrated to the positive pole of the filed. By suspending a protein sample between two electrodes in this electrophoresis buffer and applying to it a direct current, an electrical field is produced and ions in the buffer migrate according to their respective charges. The difference in distance traveled by each component of the sample could then be recorded using various imaging techniques. [23]

Smithies et al. revolutionized the technique when they developed what has become known as capillary zone electrophoresis. A neutral medium was added to the electrophoresis buffer to contain its free-floating ions. Using a porous, semi-solid and non-charged matrix provides support for the analyte and improved resolution of component bands. Initially, starch was used [24] as a matrix but subsequently the use of polyacrylamide or agarose gel slabs have become the norm. By modifying pore size of the semi-solid, it was found that analytes could

further be resolved by their size. This was found to have extraordinary applications in the analysis of negatively charged DNA/RNA.

Although gel electrophoresis has allowed for unprecedented analysis of biological macromolecules, it still presents many limitations. The process is quite time and resource consuming; often requiring several microliters of sample. Qualitative analysis of gel data often requires the use of fairly toxic fluorescent dyes such as Ethidium Bromide or Coomassie Blue.[25] Although qualitative analysis can be achieved fairly easily today with such dyes, quantitative analysis becomes more problematic. Peak broadening and smearing caused by resistance to mass-transfer by the analyte during separation severely impedes attempts at quantitative analysis. Capillary electrophoresis (CE) was developed and as an effective high-resolution alternative to gel electrophoresis. By using a fused silica capillary to contain the electrophoresis buffer, many of the shortfalls present in gel electrophoresis could be overcome. Replacing the porous matrix with a narrow-bore capillary all but eliminates mass-transfer resistance during migration of analytes. Sianol groups on the capillary lumen are reduced to the their negatively charged silanoate form using a basic pre-run wash buffer. A layer of positive buffer ions accumulates along the capillary wall and when voltage is applied, migrates towards the negatively charged cathode. This flow of ions, known as the electroosmotic flow (EOF), carries analyte and run buffer towards the cathode. Although all analytes are transported to the cathode by the EOF regardless of charge, separation occurs based on a molecules size-to-charge

ratio. Analytes with a more positive charge ratio will migrate to the cathode faster compared to more negative species, which display futile electrophoretic mobility opposing the EOF.

The small internal diameter of the capillary (typically of 25-100 μm) allows for exceptional separation using only nanoliter volumes of sample. The comparatively small internal volume of the capillary also minimizes power dissipation upon the application of current. The subsequent increase in voltage capacity (up to 400 V/cm) translates as a stronger electric field and consequentially faster separation.[26]

Sample components are recorded as they pass a point near the end of the capillary. Samples can be detected here by spectrometric methods such fluorescence and absorbance as well as by electrochemical methods such as potentiometry. Unlike with gel electrophoresis, samples run using CE are easily analyzed in a non-destructive manner and will exit the capillary shortly after detection. This allows CE to be coupled in tandem with more complex detection methods such as mass spectrometry.[27]

Micellar Electrokinetic Capillary Chromatography (MEKC) was first described by Terabe et al. in 1983. They describe a method in which sodium dodecyl sulphate (SDS), a surfactant, is added to the electrophoresis buffer above it's critical micelle concentration (CMC) during separation of various phenols.[28] A micelle is

formed when surfactant monomers accumulate in sufficient quantity that they collectively take on a spherical geometry. The monomers are oriented such that their polar head groups remain at the surface of the micelle and hydrocarbon tails make up its core. The collective outer charge of the micelle (typically negative) dictates its electrophoretic mobility. It was demonstrated that the negatively charged micelles would move much slower compared to the EOF (lower apparent mobility). Depending on their hydrophobic character, sample species will be distributed between the hydrophobic interior of the micelles and the aqueous run buffer. Samples with higher hydrophobic character will spend more time incorporated in the micelle. As the micelles have lower apparent mobility, the more hydrophobic species will have a decreased apparent mobility aswell and are resolved.

By designing DNA probes of varying hydrophobicity, we hypothesize that these probes can be resolved using MEKC modified CE-LIF. Should these probes hybridize with target miRNAs these hybrids could be resolved and quantified aswell. Figure 1 illustrates two synthetic DNA probes when hybridized to miRNA-122 and miRNA-21 respectively. These two miRNAs are shown to be up-regulated in samples of breast carcinoma. The early detection of such miRNAs may be used as a potential biomarker for these cancers and other diseases. Capillary electrophoresis with laser-induced fluorescence has been used to identify unique miRNAs at picomolar sensitivity.[20] [21]

Materials & Methods

Chemicals, miRNA & Probes.

Hydrochloric acid, Acetic Acid, Sodium Hydroxide, Tris, Triton X-100, BODIPY 505/515 and Fluorescein internal standards, were purchased from Sigma Aldrich (Oakville, Canada). Sodium tertraborate decahydrate was purchased from EMD Inc. (Mississauga, Canada). Synthetic miRNA-21, miRNA-122 and their complementary DNA probes were bought from IDT DNA Technologies (Coralville, U.S.A.). miRNA-21 had sequence: 5'-/Phos/ TAGCTTATCAGACTGATGTTGA-3', miRNA-122 had sequence: 5'-/Phos/ UGGAGUGUGACAAUGGUGUUUG-3', cDNA-21 had sequence: 5'-/ AAAAAAAAAAAAAAAAAAAATCAACATCAGTCTGATAAGCTA-3', cDNA-122 had sequence: 5'-/Phos/CAAACACCATTGTCACACTCCA/6FAM/-3', Cholesterol probe had sequence: 5'-/Alex488/TTTTTTTTTTTTTTTTTTTT/CholTEG/-3'

Hybridization Conditions.

All miRNAs underwent a hybridization cycle prior to CE analysis. Samples were hybridized in incubation buffer (50mM Tris-Ac, 50mM NaCl, 10mM EDTA, pH 8.1) using a PCR thermocycler (Mastercycler pro S, Eppendorf, Germany). One of two thermocycle methods was used. For all samples containing miRNA-21 with cDNA-21 and Cholesterol probe, sample temperature was increased to and kept at 65 °C for 300 seconds before lowered 1 °C every 3 second to 45 °C. Temperature was kept at 45 °C for 3600 seconds then lowered 1 °C every 3 seconds to 20 °C. Temperature was kept at 20 °C for 1200 seconds before being

lowered to 4°C. For samples containing only miRNA-122 with cDNA-122, sample temperature was increased to 60 °C and gradually decreased to 20 °C by 1 °C increments every 3 s.

Capillary Electrophoresis.

Analysis by Capillary Electrophoresis was performed on a P/ACE MDQ capillary electrophoresis system (Beckman-Coulter, Brea, U.S.A.) with laser-induced fluorescence detection. Bare fused-silica capillary (which had an o.d. of 365 μm and i.d. of 75 μm) was purchased from Polymicro Technologies (Phoenix, U.S.A.) [manufacture]. 60cm capillaries (50cm from injection point to detection window) were manually prepared. Sample was loaded by 40 nL or 78 nL hydrodynamic injection. Sample plugs were injection by pressure or vacuum pulse as needed. The loaded capillary was maintained at a temperature of 15 °C and sample separation was performed by applying to it a 400 V/cm electric field. The electric field was oriented with positive charge at the capillary inlet and negative ground at the outlet.

Prior to each run, the capillary was washed with 0.1 M HCl for 2 minutes, 0.1 M NaOH for 2 minutes, ddH_2O for 2 minutes and run buffer for 4 minutes. Run buffer comprised of 25 mM Sodium tetraborate pH 9.2. Run buffer was also prepared at this concentration with added surfactant Triton X-100. Borax run buffers was prepared with Triton at concentrations ranging form 0.0 to 0.5%. All wash and run buffers were prepared using Mili-Q deionized water and filtered using a 0.22 μm filter before use.

Results &Discussion

Prior to attempting separation assays, each miRNA was individually analyzed by CE-LIF. This allowed for a baseline to be established and a reference against which future runs may be compared. MiRNA-21 and miRNA-122 were individually hybridized with their respective fluorescently labeled probes. Conceptual structures of completely hybridized miRNAs are shown in Figure 1. Two unique temperature cycles were developed to optimize formation of either duplex (miRNA-122) or triplex (miRNA-21) hybrids. As miRNA-122 only hybridizes to its complementary DNA, the hybridization is fairly simple. Both miRNA and cDNA are prepared in incubation buffer, temperature of the sample is increased to 60 °C so as to melt away any unsanctioned self-annealing and gradually decreased 1 °C every 3 seconds to 20 °C. This gradual decrease in temperature ensures proper hybridization of complementary sequences.

Temperature sequence for the triplex miRNA-21 is slightly different. The sample is prepared in incubation buffer but is initially kept at 65 °C for 5 minutes to ensure all components are in their single-stranded state and no premature annealing remains. The sample temperature is gradually lowered to 45 °C and kept such for 60 minutes. This will ensure ample time for both annealing of the miRNA and cDNA as well as probe and cDNA. The temperature is then gradually lowered to 20 °C where hybridization is completed.

BODIPY 505/515 was used as internal standard for all CE runs. Use of fluorescein had initially been considered, however under analysis conditions used for this work, it was found to elute at a similar rate as certain analytes. Overlap of fluorescein and analyte signals rendered it unsuitable as an internal standard. BODIPY was added to each sample after hybridization. The standard was always added at a concentration roughly 2% that of the fluorescent probe used.

Once hybridized, samples were separated by CE-LIF, using a 25 mM borax run buffer at pH 9.2. Before each run the capillary was washed with 0.1 M HCl as cleaning agent, 0.1 M NaOH to re-ionize the interior capillary wall, ddH_2O and run buffer to prepare the capillary for the run. Between runs, samples were kept at 4 °C while the capillary was kept at a constant 15 °C.

Although we would like to assume the hybridization proceeds to completion, this is rarely the case. For this reason, samples are prepared with an excess of fluorescent probe. This will maximize the target analyte (miRNA) that is hybridized and detectable. Using an excess of probe will also generate a unique fingerprint of peaks when the sample is separated by CE-LIF. In Figure 2 the peak signature for hybridized miRNA-122 can be seen. The top electropherogram was recorded when cDNA-122 probe was run alone, the bottom electropherogram was recorded after hybridization of miRNA-122 and cDNA-122. The excess probe in the hybrid sample can be identified as the peak

with migration time equivalent to that observed in the probe reference run. In Figure 2, we notice this at 11.5 minutes; a peak with small shoulder peak. The excess probe peak is followed shortly after by a second peak; the hybrid. This combination is a fingerprint of miRNA-122 hybridization with fluorescently labeled cDNA.

The same analysis was performed on the miRNA-21 triplex; this can be seen in Figure 3. Starting from the top, the first electropherogram was recorded when cholesterol probe was run by itself and can be used to identify the probe in subsequent runs. The second electropherogram contains a duplex of cholesterol probe and cDNA-21 hybrid. The third electropherogram now contains the triplex hybrid of cholesterol probe, cDNA-21 and miRNA-21. This is apparent by the sharp three peaks present between 11 and 14 minutes. When all three electropherograms are aligned, each peak can be identified with certainty. The bottom-most electropherogram in Figure 3 is a control for impurities that may originate in the internal standard.

The cDNA probes were designed with the intent that they could be separated using MEKC facilitated CE-LIF. When micelles are present in the run buffer each analyte is in kinetic equilibrium; moving between the micelle and surrounding run buffer. It is thought that the addition of a cholesterol moiety on one probe and not the other will create a sufficient difference in the comparative hydrophobic character that this equilibrium can be altered. It is thought that the probe with the

hydrophobic cholesterol moiety will have a higher affinity for the hydrophobic core of the micelle then its sterol-lacking counterpart. The difference in electrophoretic mobility between a probe alone in run buffer and a micelle is such that any association with the micelle will alter that probes overall migration rate.

After first having successfully catalogued fingerprints for each target analyte, a suitable micelle constituent needed to be found. Triton X-100 was chosen as it is a non-ionic surfactant. RNA and DNA are negatively charged due to their phosphorelated backbone. Many commonly used surfactants such as sodium dodecyl sulphate (SDS) are also anionic. Using SDS as a surfactant for example would produce a micelle with a negatively charged external surface. Such a species would have electrophoretic mobility too similar to that of the nucleic polymers we intend to separate. A cationic surfactant on the other hand would create a positively charged micelle and separation would occur too quickly. This author dares not imagine the fate of a zwitterionic surfactant species in electrophoretic conditions…

It was imperative that during separation the surfactant be present in run buffer above its critical micelle concentration (CMC). Any concentration below this would not be sufficiently concentrated to form the micelles required for separation. A theoretical CMC value of 0.22-0.24 mM (or ~0.01% v/v) was used as reported by Sigma-Aldrich.[29] A Triton concentration titration was devised around this value and can be seen in Figure 4. Triplex hybrid was run using

standard 25 mM borax run buffer into which Triton was added. Concentrations ranged form 0.00% to 0.5% v/v incrementally. At 0.005% (below the CMC) only peak broadening is noticeable with very minor change in elution time. At 0.01% a significant decrease in elution time can be observed until migration time seems to stabilize above 0.05% v/v. This observation confirmed the previously calculated CMC of ~0.01%. For future separations, it was decided that a triton concentration of 0.05% v/v would be used.

A Mixture of miRNA-122 and miRNA-21 was prepared for the separation assay. MiRNAs were added in equal ratios (50nM each). 100nM of respective cDNA and 200nM of cholesterol probe were added. MiRNA hybrids were also prepared separately as references. The sample and all controls were hybridized according to protocol previously mentioned. After hybridization, each sample was analyzed in bothe regular 25 mM borax run buffer and Triton 0.05% run buffer. The results are presented in Figure 5.

It should be noted in Figure 5 that the top and middle pair of electropherograms are triplex and duplex only samples respectively, and run as a reference to the bottom electropherogram, which contains a mix of both. When comparing the triplex and the duplex hybrids as they are individually run in triton containing run buffer, only the triplex (containing cholesterol moiety) has an alteredelectrophoretic mobility and migration time. This confirms our prediction that the hydrophobicity of a probe will mediate its interaction with micelles in the

run buffer. Even when both miRNA are hybridized in the same sample (bottom pair of electropherograms) only the hybrid with a distinctly hydrophobic group will interact with the micelles. This distinction is enough to efficiently resolve the two hybrids when in a mixture.

Conclusion

We have successfully demonstrated that two miRNAs can be selectively isolated and detected using CE-LIF. We have further demonstrated as an initial proof of concept that the two distinct miRNAs can then be successfully resolved with the use of appropriately designed cDNA probes. This should only be the first step in a very promising direction of miRNA based biomarker detection. Future work in this area should include the establishment of a limit of detection (LOD) for single and multi-miRNA detection assays. Detection assays should be undertaken in more complex solutions then just the incubation buffer used for this project. Identification and resolution assays should be undertaken in the presence of foreign or random sequence DNA/RNA libraries and possibly cell lysate or blood serum samples.

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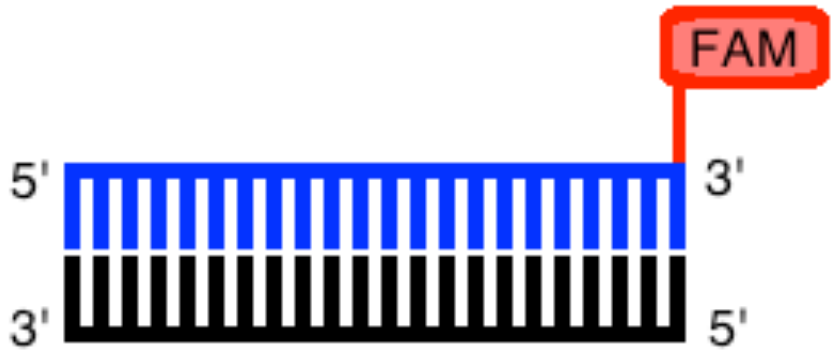
Appendix

A)



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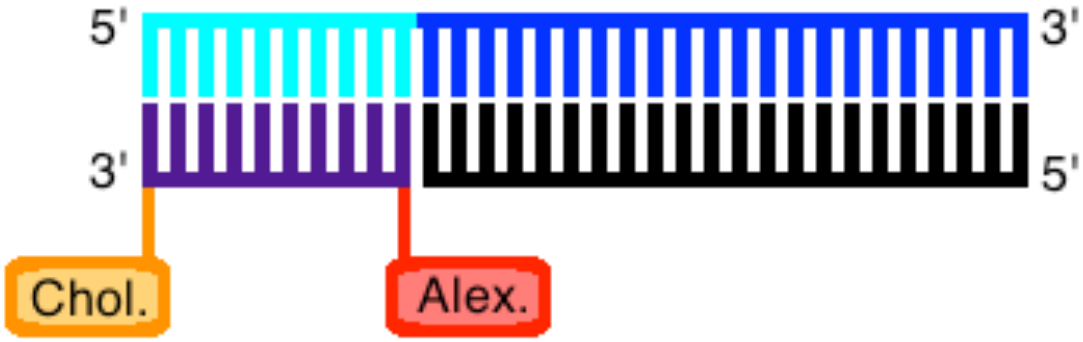

Figure 1. Artistic rendition of A) Hybridized miRNA-122 and cDNA-122 probe, B) Hybridized miRNA-21, cDNA-21 and Cholesterol probe. MiRNAs are identified in black, complementary DNA in dark blue, poly-A tail in light blue, poly-T sequence in purple, fluorescent probes in red and cholesterol moiety in yellow.

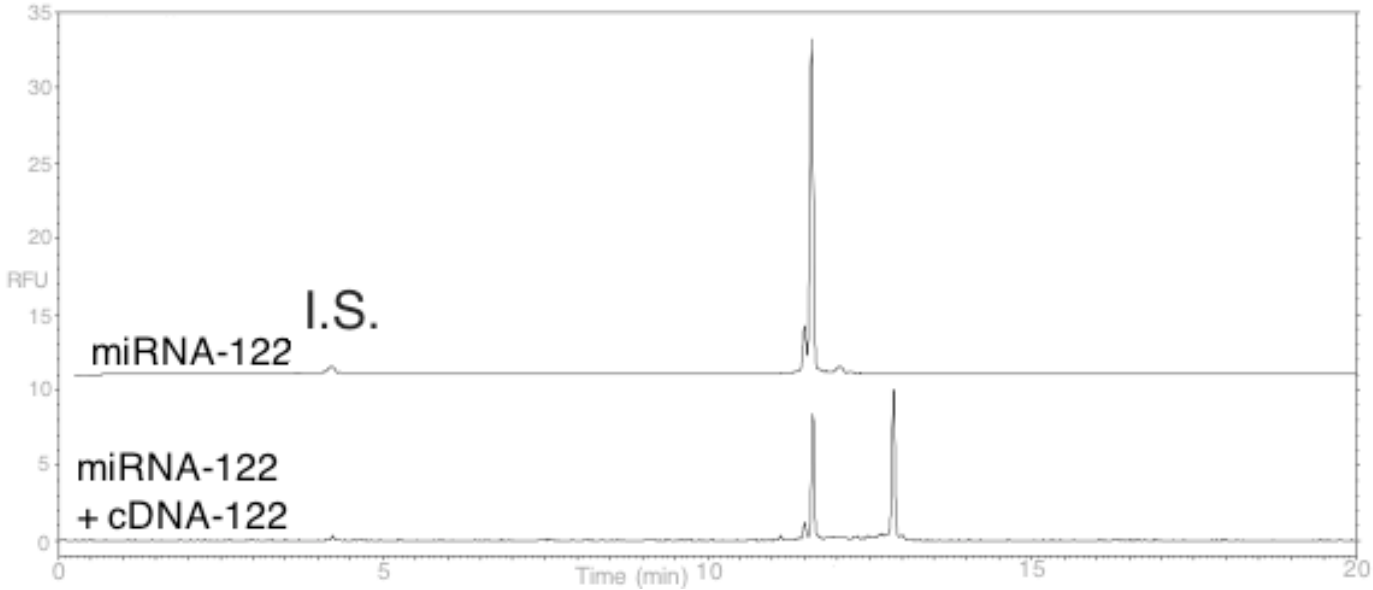

Figure 2. Initial analysis of miRNA-122 by CE-LIF using 25mM borax pH 9.2 run buffer and BODIPY as internal standard (I.S.). Top, electropherogram of cDNA-122 probe alone. Bottom, electropherogram of miRNA-122 and probe (duplex) hybrid.

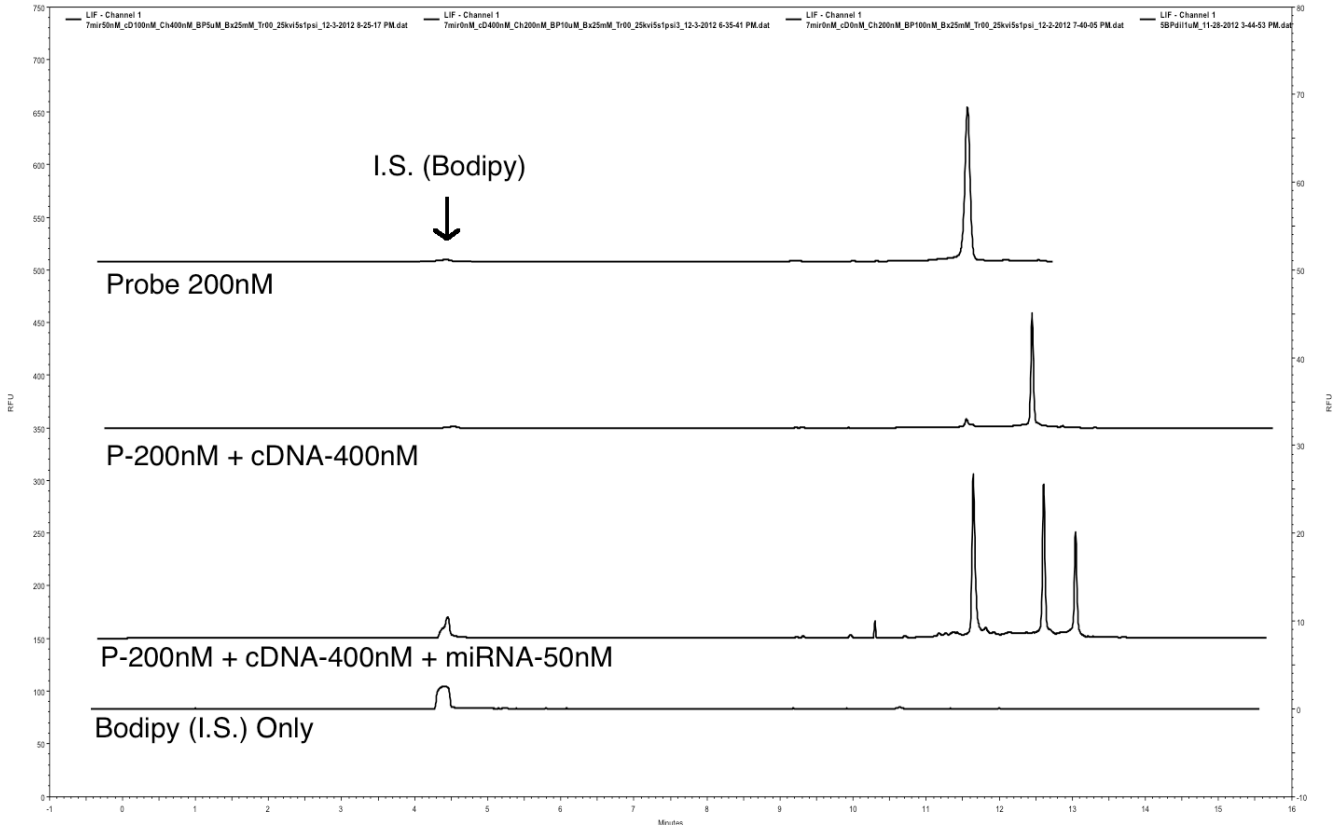

Figure 3. Initial analysis of miRNA-21 by CE-LIF using 25mM borax pH 9.2 run buffer and BODIPY internal standard (I.S.). Electropherograms from top to bottom: Cholesterol probe run alone. Cholesterol probe and cDNA-21 hybrid. Triplex hybrid. BODIPY run alone.

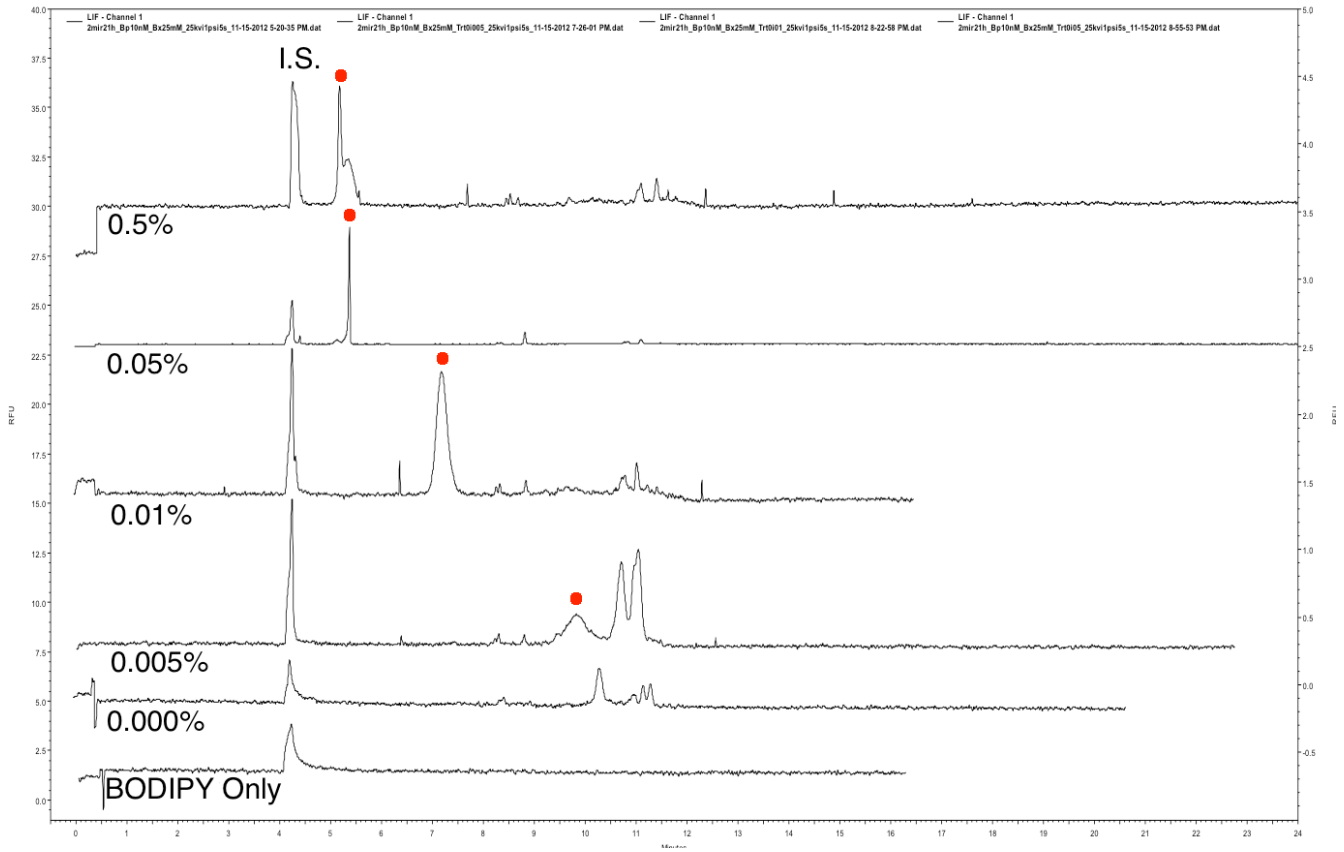

Figure 4. Titration of the non-ionic surfactant Triton X-100 in 25mM borax run buffer from 0.00% to 0.5% v/v. Triplex hybrid is run in each titration increment to determine resolution potential of micelles by MEKC at varying concentrations. Red mark indicated position of cholesterol probe. BODIPY is used as internal standard (I.S.).

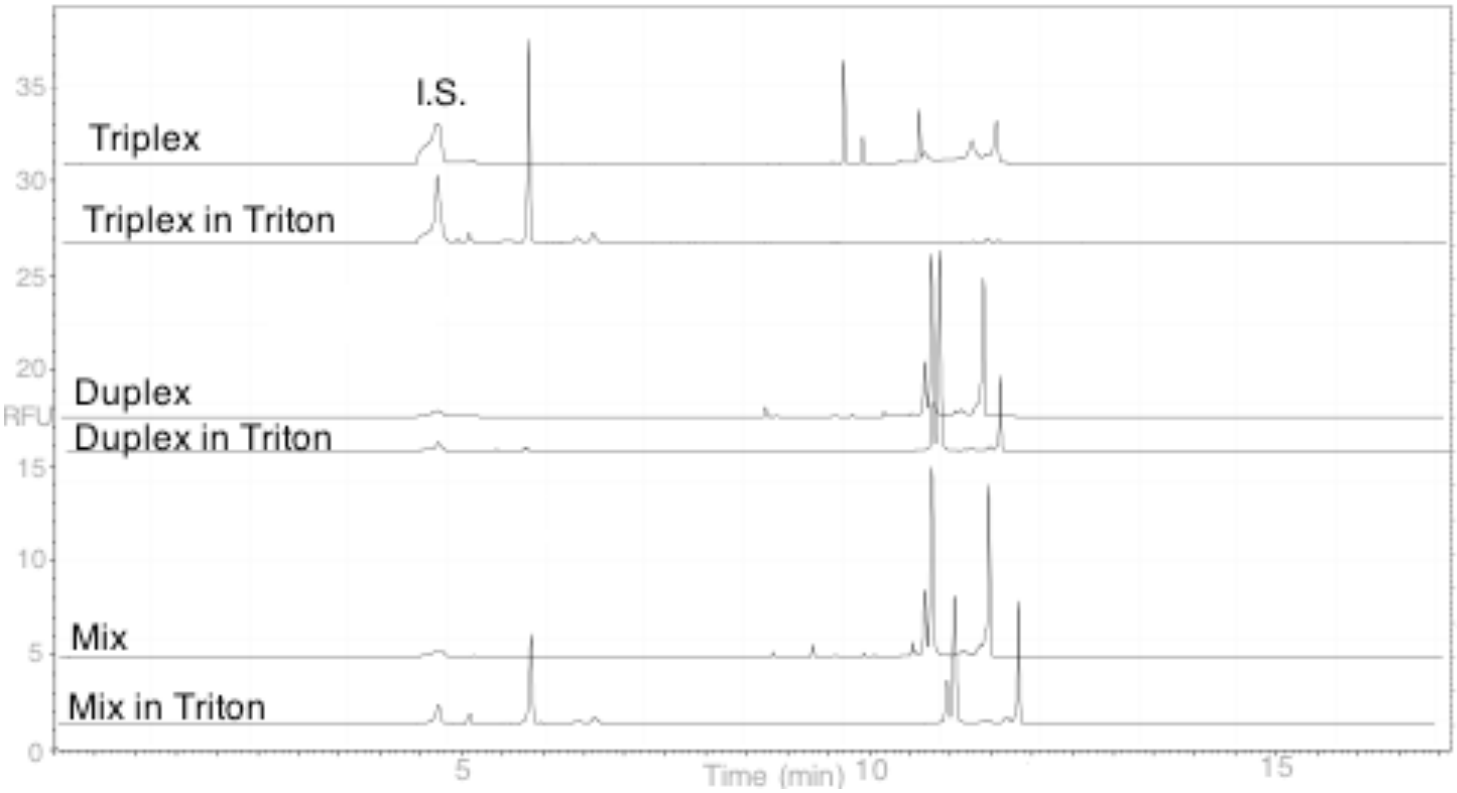

Figure 5. Separation of miRNA-122 (duplex) and miRNA-21 (triplex) hybrids by MEKC modified CE-LIF using 25 mM borax run buffer containing 0.05% Triton X-100. BODIPY used as internal standard (I.S.). Mixed hybrid sample (bottom pair) run, reference sample containing only duplex (middle pair), and reference sample containing only triplex (top pair). Each sample is shown after analysis using run buffer containing Triton (lower of each pair) and in run buffer containing no Triton for comparison (upper of each pair).