DNA in Ionic Liquids and Polyelectrolytes

by

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Author's Declaration

I hereby declare that I am the sole author of this thesis. This is a true copy of my thesis, including

any required final revisions, as accepted by my examiners.

I understand that my thesis may be made electronically available to the public.

Abstract

DNA has been widely studied in a variety of solvents. The majority of these solvents consist of either aqueous or organic components. The presence of ions or salts in these solvents can further alter DNA properties by changing the melting point or helical structure. The size, charge, and concentration of these additional components can all affect the behaviour of DNA. A new class of solvents, known as ionic liquids have recently gained popularity. Ionic liquids are comprised of entirely of ions and can be liquid at room temperature. Due to their low volatility and ability to dissolve both polar and non-polar substances, they are generating high levels of interest as 'green solvents'. Although the interaction between DNA and ionic liquids has been characterized, the potential of this interaction is still being studied. It was discovered that when DNA mixed with DNA intercalating dyes was added to ionic liquids, there was a large reduction in fluorescence. Although this fluorescence drop was believed to occur to removal of the dye molecule from the helix, the strength of this interaction has not been researched.

In this thesis, the interaction between different intercalating dyes and different ionic liquids was evaluated. We reasoned that perhaps the difference in interaction could be used as a method of separating the DNA-dye complex, which has previously never been accomplished. For example, it has been established that both DNA and cationic dyes have an affinity for ionic liquids. The relative strength of this affinity is undetermined, as well as the comparison to normal aqueous mediums. Although ionic liquids can drastically alter the stability of the DNA duplex by either raising or decreasing the melting point depending on the ionic liquid chosen,

we found that the DNA actually has a higher affinity for the aqueous phase. Conversely, intercalating dyes prefer to partition into the ionic phase. The relative affinities of the two components are strong enough for their respective phases that the complex can be split apart and each component can be extracted, allowing for separation of the two.

Acknowledgements

Dedication

I would like to dedicate this thesis to my friends and family for their encouragement and support.

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List of Abbreviations

A   adenosine

AuNPs   gold nanoparticles

C   cytosine

cDNA   complimentary DNA

dmso   dimethyl sulfoxide

dsDNA   double stranded DNA

EB   ethidium bromide

FACS   fluorescence activated cell sorting

G   guanosine

IL   ionic liquid

MgCl2   magnesium chloride

PAA   polyacrylate

PEG   polyethylene glycol

PN   propylammonium nitrate

SG   SYBR Green I

ssDNA   single stranded DNA

T   thymine

TO   thiazole orange

Chapter 1 Introduction

1.1 DNA Structure and Properties

DNA is a biomolecule that can be found in all living organisms. DNA stores the genetic information that allows cells to replicate and organisms to grow and is capable of storing vast amounts of information (the average human has three billion base pairs which allow for approximately 100 000 proteins to be developed).[1] However, due to the strong recognition capabilities of DNA, the biomolecule can also be used in the fabrication of biosensors. Many of the biosensors take advantage of the ability of DNA to bind selectively to the corresponding strand via hybridization between the base pairs.[2] Upon hybridization with a complimentary strand, a double helix is formed; that is, the double stranded DNA (dsDNA) winds around creating both minor and major groves as well as secondary structures.[3,4]

In order to understand how secondary structures are formed, one must first understand the individual components of DNA. There are four main nucleosides which make up individual strands. Adenosine (A) and guanosine (G) are purines, which means that the nucleoside consists of two aromatic rings. Thymine (T) and cytosine (C) are pyrimidines, and are composed of only one aromatic ring.[3] Upon hybridization, A binds to T and C binds to G through hydrogen bonds. The A-T bond consists of 2 hydrogen bonds, while the C-G pair consists of three hydrogen bonds.[1] Individual nucleoside that make up DNA strands are linked by phosphodiester bonds.[3] One important structural marker as a result of the phosphodiester bond is that all of the corresponding sugars in the nucleotides are planar stacked; that is, all of the sugars have the same orientation which allows for closer packing of the nucleosides. This also leads to strong

electrostatic interaction between neighboring bases, which can impact the structure based on the sequence.[5]

Another consequence of the hydrogen bonding between complementary bases is the stability of the duplex. As C-G pairs have one more hydrogen bond, one would expect that this pair is more stable than the A-T combination. Indeed, this extra hydrogen bond is often utilized by creating C rich or G rich sequences to assist in the duplication of DNA through polymerase chain reaction. In this method, the duplex is first heated to a point beyond denaturation such that the duplex splits into individual strands. Incubation with the corresponding primers allows the strands to be replicated, exponentially enriching the DNA.

If a mismatch does occur, the resulting duplex is much less stable. A T-C mismatch has been found to destabilize the duplex by up to 5.8kcal/mol. If one considers that the hydrogen bonds add up to 1.5kcal/mol, it can be seen that the destabilization effect is much larger. This suggests that a mismatch between bases causes more damage than just the missing hydrogen bonds, possibly even resulting in structural distortion of the helix.[1]

Due to the large effect of electrostatic interactions, DNA duplex stability can be influenced by surrounding environments.[6] Different buffer conditions, small molecule concentrations, higher salt concentrations, or even different solvents can all affect the stability of DNA.[7,8] Binding of the DNA to a substrate can affect the secondary structure, which also changes the stability.[9] This is a key component in the design of biosensors or the use of DNA for targeted applications, and will be discussed in more detail. If the DNA is being used for drug delivery, the drug molecules themselves can result in perturbation of the structure.[10]

1.2   DNA Melting

DNA melting has been used as a measure of DNA stability since melting analysis was first combined with PCR.[11] The theory behind DNA melting is the hybridization of dual stranded DNA. Depending on the number of mismatches in the complimentary sequences, or the strength of the buffer solution, the ability of the DNA to remain in its double helix form as the temperature increases changes. Measuring these changes can be done through the use of fluorescent dyes, which exhibit significantly weaker fluorescence when bound to single stranded DNA. The melting temperature, Tm, indicates when the hybridization begins to fall apart and the duplex is split.[12,13]

This method has been shown to detect single nucleotide replacements in both homoduplex and heteroduplex fragments of up to 167 base pairs. This indicates a high level of accuracy in the technique, as the two samples are almost identical.[14] This technique can also be used to determine the effect of solution conditions, which have been found to influence melting temperature. Solution conditions include, but are not limited to, buffers, pH, hydrophobicity, solutes, analytes, or even surfactants. High salt concentrations have been found to stabilize duplex DNA, resulting in higher melting points.[13] The same effect is found with the use of PEG, where higher molecular weight or concentrations can stabilize the DNA up to a certain temperature.[15] Cationic surfactants can allow for denaturation at much lower temperatures by stabilizing the individual strands. In fact, cationic surfactants have been found to be much more efficient at manipulating melting temperature as compared to other salts.[16]

Excluded volume is another factor that can influence the stability of hybridized DNA. Excluded volume refers to the area in a solvent that is occupied by another molecule. Upon denaturation of duplex DNA, two individual DNA strands are obtained. These strands take up a larger effective volume then the duplex version. Hence, as the environment becomes more crowded, denaturation of the duplex form becomes harder to achieve as it becomes less thermodynamically possible from a crowding perspective.[15]

1.3   DNA Binding Dyes and Interaction

DNA dyes and other small molecules generally attach onto DNA through two different methods. Of the two, intercalation is the most common for the dyes used in our experiments. In this process, the planar structure of the dye or small molecule forces its way into the grooves created by the helical structure. It is important to note that this is an enthalpically driven process, which means that it is generally spontaneous at room temperature. It should also be noted that insertion causes the helix to unwind slightly as well as lengthen to accommodate the new molecule.[4,17]

The second method of binding occurs though groove-binding. In this process, the molecule is small enough to fit into the minor grooves of the helix. This causes very little disturbance, and does not require any unwinding of the helix. For this reason, this process is mainly entropically driven. It is also worth mentioning that the dominant forces involved are electrostatic intercalation. Some components exhibit both binding mechanisms, while others are specific to either groove-binding or intercalation.[4]

1.3.1 Types of Dyes

There are many different types of DNA labelling dyes. Most of these dyes interact chemically with DNA, resulting in significantly increased fluorescence. Dyes can be either cationic or anionic, and have different excitation and emission wavelengths depending on the chemical structure. All of the dyes used can be seen in Figure 1.1.
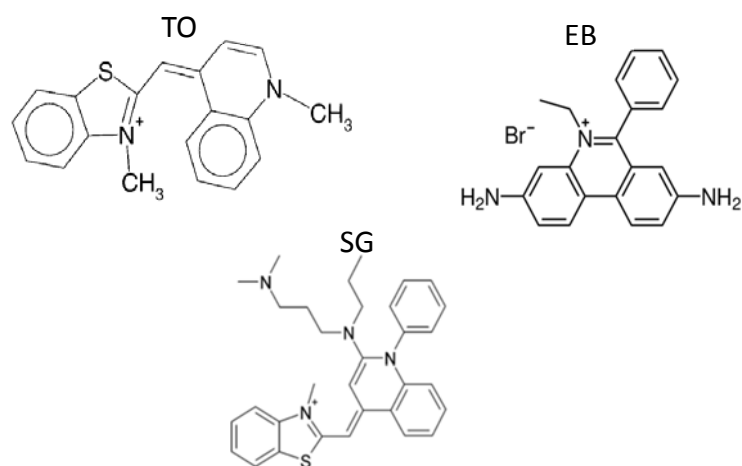
Figure 1.1: DNA labelling dyes used in experiments. Modified and reproduced with permission.[36]

1.3.1.1 Ethidium Bromide

Ethidium bromide is a commonly used dyes to probe DNA location and interaction.[18–22] EB is useful for several reasons. First, the background is low, leading to high signal and sensitivity upon conjugation with DNA. Binding of the dye to the DNA also increases the fluorescence lifetime. Due to the high affinity of the dye for double stranded DNA, EB can also inhibit many different biological purposes such as DNA synthesis or translation.[23] Unfortunately, this also makes EB a strong mutagen that must be handled and disposed of carefully.

EB binds with DNA through the intercalation method, where the planar structure of the dye squeezes in between adjacent base pairs.[24] Intercalation causes the DNA to lengthen as the bases are pushed apart.[19] This means that EB binds much more strongly to dsDNA as compared to ssDNA. However, EB can still bind to ssDNA, although the fluorescence signal will be much weaker.[23] In fact, binding of EB to triplex is even stronger then binding to a duplex.[25]

Small molecules in the solution can also affect the ability of EB to bind to DNA. For example, magnesium chloride ($MgCl_2$) has been found to decrease the strength of the interaction, even though the number of binding sites remains unchanged.[26] Higher salt concentrations can result in enhanced fluorescence, even changing the method of binding. At low salt concentrations, binding can also occur via electrostatic interaction to phosphate groups as well as intercalation, although this method shows reduced fluorescence.[27]

1.3.1.2   SYBR Green

SYBR Green I (SG) is a commonly used dye for PCR amplification of DNA.[28–32] SG is different from EB in that it binds to the minor grooves of dsDNA. Subsequent to binding, the fluorescence increases by a magnitude of 100 times, thereby presenting a strong signal as compared to the baseline.[30] SG can also bind to ssDNA, but this is at least 11 times weaker then compared to dsDNA. The exact efficiency changes with respect to the four bases, as SG is sequence specific.[33] SG is preferred over other staining dyes during PCR because it is reliable, easy, and relatively cheap.[32]

One of the problems associated with using dyes for PCR is the idea of preferential binding. This is not so much an issue when amplifying DNA, but rather when conducting melting point or stability experiments. It has been found that SG binds preferentially to C-G rich areas when the SG is in excess. This could cause slight errors by showing a melting point that is higher than it should be, as detachment of SG from other areas frees up excess dye.[31] Another problem associated with SG is that it can inhibit PCR. However, this can be overcome by optimizing the reaction mixture or by adding the concentration of $MgCl_2$.

SG has been previously used to detect *Salmonella enterica* using PCR. Although some problems exist, SG was overall easy to perform and reproducible.[28] SG can also be coupled with flow cytometry to measure cell concentrations or distinguish between bacteria and cells. [34] If coupled with capillary electrophoresis, SG is able to distinguish between different sized DNA fragments.[35] Although several problems have been identified, SG is highly viable as the problems do not cause a large enough deviation to throw off results in the majority of cases.

1.3.1.3 Thiazole Orange

Thiazole orange (TO) is another intercalating dye used for staining nucleic acids.[36–40] Upon binding with DNA, rotation around the bond connecting two aromatic rings is hampered, which is believed to be the source of increased fluorescence. TO fluorescence is about 10 times stronger when bound to dsDNA as compared to ssDNA. If the ssDNA is composed mainly of pyrimidines, this binding strength drops to up to 100 times weaker. TO has two main forms of binding, either as a monomer or dimer. The form of binding depends on the nucleoside in

question. For dsDNA or poly A DNA, TO generally binds as monomer. In the case of poly C or poly T, TO prefers the dimer configuration. TO can bind as either a monomer or a dimer to poly G.[36]

As with ethidium bromide, TO has negligible fluorescence when unbound to DNA. One of the benefits of TO for biological applications is it is cell membrane permeable, and can absorb at 509nm, which allows for excitation from an argon source. Compared to the unbound form, the fluorescence is 1000 times stronger.[41] This is much higher than even the fluorescence enhancement for EB.[39] It should also be noted that TO binds differently to DNA as compared to RNA; in DNA, saturation is reached when there is one molecule for every two base pairs, while this drops down to one molecule for every 10 base pairs in RNA.[42]

TO is a cationic dye that can be modified to enhance binding with the negatively charged DNA. Conjugation with a positive side chain can drastically increase the affinity of the dye. Using side chains or other modifications, affinity for all nucleosides equally can be obtained.[43] TO has also successfully been used to stain blood samples for counting or sorting by fluorescence activated cell sorting (FACS) or for sorting PCR products with capillary electrophoresis.[42,44]

TO has also been successfully used as a biosensor indicator. As TO binds to ssDNA, this can be used as a probe for complimentary strands. Upon hybridization, the fluorescence drastically increases which represents the detection of that particular strand. One of the problems associated with this technique is that the DNA can loop back on itself, simulating dsDNA locally and causing an increased fluorescence.[45]

1.4 Solvents for DNA Chemistry

1.4.1 Salts in Water

A solution can be modeled by a large group of molecules that are all held together through some sort of non-covalent interaction.[46] Size, shape, orientation, hydrophobicity, and change of these molecules plays a large role in determining how other molecules such as DNA will react in the solution.[47–50] We have already found that salts in the solution can change the ability of dyes to intercalate into the DNA.[51] Whether by some sort of charge screening or stabilization, small molecules can also change the speed at which binding will occur.[52] Charged ions in the solution can change the electrostatic interactions, resulting in different structures or packing.[53]

Ion size and charge plays a large role in the stabilization of a DNA duplex. Although both Na^+ and Mg^{2+} can stabilize the duplex and increase the melting temperature, Mg^{2+} is much more effective.[47] It should be noted that DNA itself is anionic.[52] Longer DNA is much more stable, due to the stronger attractive field for bound ions. Similarly, because dsDNA is more negative than ssDNA, the effect in the presence of ions is much more pronounced.[47]

Aside from immediate effects, secondary effects as a result of molecular interactions must be considered. For example, intercalative molecules force the helix to expand and slightly unwind. This then results in a locally straighter chain that is also stiffer. This structural change affects the overall parameters of the solution, influencing viscosity or aggregation. Contrary to what is expected, the intercalation of some molecules causes enhanced viscosity, even though the coil becomes less compact.[54]

If the ions in solution are not intercalative, condensation can occur. As DNA is negatively charged, close packing should generally be accompanied by high repulsion. The presence of cations, even at low concentrations, can help to facilitate DNA collapse by stabilizing the condensed state.[55,56]

Although small molecules play a large role, the solvent itself is important to consider in DNA interaction. Water molecules themselves can bind at the interface and cause a change in heat capacity. In a study performed on the interaction between DNA and proteins, water molecules were found to occupy the space between the major groove and the protein, effectively bridging the two.[57] These molecules were then able to form hydrogen bonds with both the protein and the DNA, suggesting that the choice of solvent and solutes is extremely important to facilitate interaction.

1.4.2   Small Molecular Solvents

DNA has been found to react differently in small molecule solvents. Organic solvents especially can cause DNA to denature, resulting in a lower melting point. DNA has also been found to precipitate in ethanol, making it useful for DNA extraction.[58] Surprisingly, the rate of hybridization is actually increased in organic solvents. It is believed that this occurs due to the reduced activation energy barrier.[59] The melting temperatures of DNA in methanol, ethanol, and isopropanol are shown in Figure 1.2.
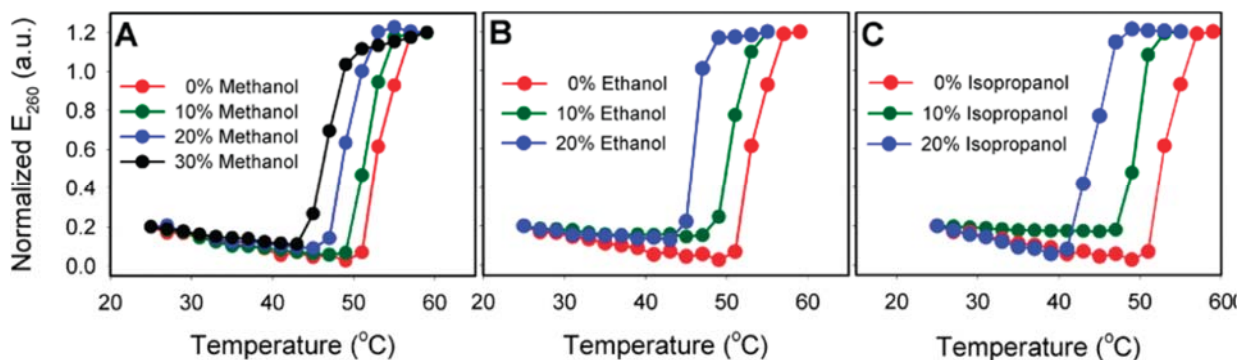
Figure 1.2: DNA melting point in methanol, ethanol, and isopropanol. Reprinted with permission.[58]

In aqueous mediums, DNA is often considered as stable. However, without refrigeration, DNA has a shelf life of less than one month. Although the duplex structure may be maintained, it has been found that the DNA helical structure is lost.[60] This is important for applications that require a specific structure for recognition or binding, as it can render the DNA functionless unless the original structure can be reacquired.

Dimethyl sulfoxide (DMSO) has also been found to enhance transcription and amplification. Normally, prevention of hybridization would be unwanted for sensors, as signal would not be generated. However, in the case of amplification, annealing of the templates can sometimes retard amplification. Even 5% of DMSO has been found to increase amplification by preventing renaturing of the templates.[61]

1.4.3 Ionic Liquids

Ionic liquids (IL), also known as organic salts, are liquids that are comprised entirely of ions. Unlike molecular solvents, every particle in an ionic liquid has an ionic charge. Even more interesting is the fact that many of these are liquids at room temperature. The presence of the

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ionic species results in vastly different thermodynamically properties, making them highly interesting. Additionally, ionic liquids have no identifiable vapour pressure, resulting in no detectible emission of volatile organic compounds, allowing ionic solvents to be perfect for 'green' methods.[62–68] A large source of environmental pollution is the volatile organic compounds that are the by-product of traditional industrial solvents. Using ionic liquids would mitigate this emission.[63]

Another interesting consequence of ionic solvents is that they are able to dissolve both polar and non-polar substances. As well, they remain in a liquid state for a much larger range of temperatures as compared to molecular solvents.[66] Many ionic solvents are immiscible in other molecular solvents, allowing for phase separation as a means of extraction or the fabrication of multiple phase systems.[69]

Depending on the eventual goal and use, ionic liquids can be tailored to fit the desired objective. For example, ionic liquids can be prepared to be either hydrophobic or hydrophilic simply by changing the anion. Hydrophilic anions, such as iodide and chloride, result in ionic liquids that are miscible in water. If hydrophilic anions such as PF_6^- are used, the resulting ionic liquid is hydrophobic and immiscible in aqueous mediums. Increasing the chain length of the cation has also been found to increase the hydrophobicity.[70]

1.4.3.1 Applications in DNA Chemistry

Although interest in ionic liquids has surged recently, there are several cases in which ionic liquids have been utilized in conjunction with DNA. Many of these examples deal with

electrochemistry, where the DNA is stabilized within an ionic liquid network. The ionic liquids present a good environment for electrochemical applications due to their high conductivity and good solubility.[71–75] ILs have also been used to create DNA films with high conductivity.[76,77]

One application of electrochemistry is the ability to detect hybridization of dsDNA without the use of a label or dye. Upon hybridization, the impedance changes. If the probe DNA strand is first immobilized onto an electrode, the hybridization can be detected through the increase in electron transfer resistance. Although this is possible without the use of ILs, their presence could largely enhance the sensitivity.[78]

ILs can also be used to enhance the detection of specific bases. Both adenine and guanine show characteristic oxidation peaks. This allows for a comparison of the ratio between the peaks, such that a single strand of DNA can be measured for both A or G content.[79] It was found that the presence of IL in these sensors can greatly enhance the peak, essentially increasing the sensitivity of the probe.[75]

Although ILs are said to be inert, this does not infer that there is no interaction between DNA and the ionic liquid. A detailed study was carried out to determine the interaction characteristics between DNA and 1-butyl-3-methylimidazolium tetrafluroborate. It was found that the IL could interact with DNA through electrostatic interactions, even going so far as to be able to replace some molecules that had already bound to the DNA.[80] A schematic of this interaction is shown in Figure 1.3.[81] ILs can also interact with ENA through hydrophobic interactions with the hydrophobic bases of DNA, which results in compacting of the DNA coil and a lower hydrodynamic radius.
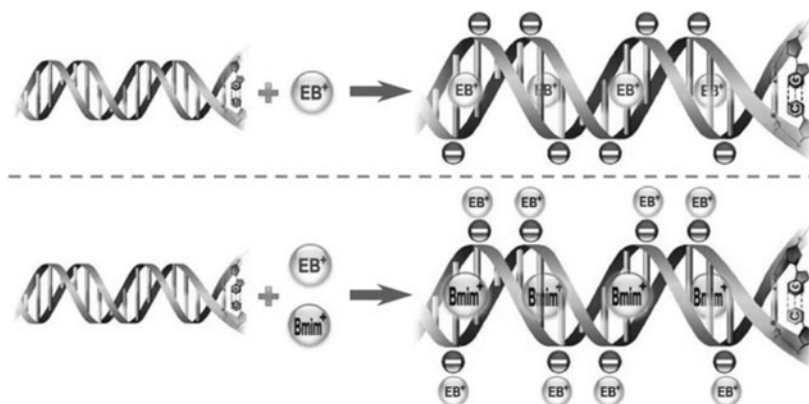
Another experiment showed that the addition of IL with the DNA dye conjugate could cause a decrease in fluorescence. It was hypothesized that this IL bound competitively with the DNA, eventually being able to completely displace the EB completely.[82] Our experiments will show that perhaps a stronger explanation of this phenomenon is the interaction between the dye and the IL, rather than the DNA and IL.

With respect to the DNA itself, some ILs such as choline dihydrogenphosphate have been found to significantly increase DNA stability. Generally, DNA is stored under refrigeration, as the double helix structure is disrupted in just a month of storage at RT. However, if stored in ILs, this structure remains uncompromised, even if left at room temperature for up to six months. Additionally, it has been reported that ILs can also improve temperature stability of the duplex by preventing denaturation at higher temperatures as compared to an aqueous medium. In some hydrated ionic liquids, DNA can be stabilized up to a temperature of 100 $^{\circ}$C, which is significantly higher than in a fully aqueous medium.[60]

Other ILs, such as the hydrophobic propylammonium nitrate (PN) has been found to destabilize the DNA duplex by lowering the melting point. As low as 1% was enough to change the melting point by 8 °C. Higher concentrations can bring this melting point to below room temperature, as shown in Figure 1.4.[83]
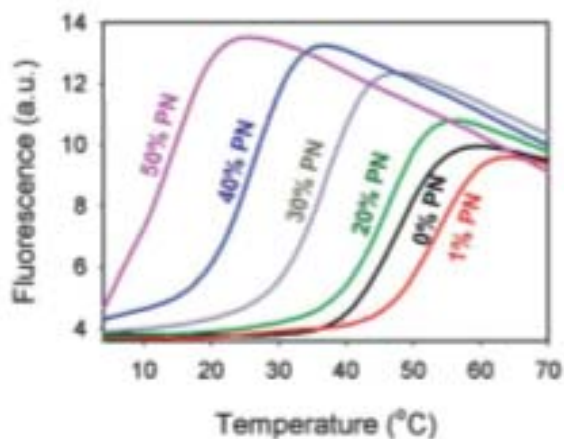
Figure 1.4: Melting temperature of DNA in PN. Reprinted with permission.[83]

1.4.3.2 Applications for Extraction

Liquid-liquid interfaces are often used for separation of different molecules or biomaterials, but they suffer from high toxicity and are generally expensive. Disposal of these diluents is also a problem. Previously, these interfaces were formed though a mix of an organic solvent and aqueous phase, as this would create the phase separation necessary. However, the focus is now being shifted to ionic liquids, as many of these are also able to create the phase separation and show high solubility for organic components.[84,85]

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It has been reported that specific ILs are able to completely remove DNA from the aqueous phase into the ionic phase.[86] This is contrary to our experiments, which show that it is the dye only that extracts into the ionic phase, rather than the DNA. This will be discussed in more depth in chapter 2.

Ionic liquids have also shown the ability to remove heavy metal ions from aqueous solutions. In order to facilitate this extraction, pH must be controlled as the distribution ratio is highly dependent. Using dithizone as a chelator, a neutral metal-dithizone complex is formed. This complex can then be extracted into the ionic phase, thereby removing the metal contamination from the aqueous phase. The extraction efficiency of IL was higher than that of with chloroform, and over 90% could be removed as long as the pH was higher than 4.[87] Strontium, another toxic heavy metal, has also been successfully extracted by IL. Again, the extraction efficiency was higher in ILs than organic solvents.[88]

1.4.3.3   Applications for nanoparticles

ILs have also received much attention for being able to stabilize the synthesis of very small nanoparticles. There are several parameters of ILs that contribute to this stability. ILs have very low interface tension, allowing very small particles to be generated. Due to their high polarity, they can actually form hydrogen bonds allowing for supramolecular organization, which aids in ordering for nanoscale structures.[89]

Gold nanoparticles have been successfully synthesized using ILs. ILs can stabilize the nanoparticles, preventing aggregation until a target is introduced.[90] Phase separation of ionic

liquids can also be used to stabilize interfacial polymerization by removing the need for catalysts or phase transfer agents. The high solubility of polymeric compounds in ILs allows for even the poorly soluble compounds to be fabricated much easier.[91] This can lead to smaller particles sizes and reduced toxicity to the environment by limiting the use of organic solvents.

1.4.3.4 Applications as catalysts

Ionic liquids can also be used as catalysts by stabilizing intermediary complexes. As mentioned, one advantage of ionic liquids is their high solubility for ionic species. Many acid mediated processes require cationic intermediary complexes, which can be hard to stabilize without significant manipulation. One example of an acid catalyzed reaction is the conversion of carbon dioxide into epoxides. This reaction has been catalyzed by ILs and found to have 100% yield after only 6 hours.[92]

ILs have also proven to be useful in stabilizing many enzymatic reactions. For example, Z-aspartame was successfully synthesized when catalyzed by thermolysin in an IL. The activity was equal to that observed in ethyl acetate and water. Similarly, some lipases have also been investigated in the presence of ILs. Enzymatic activity was equal to that observed in normal organic media, and in some cases, the activity was even improved.[92]

1.4.4 Non-ionic Polymers

Non-ionic polymers such as polyethylene glycol (PEG), polyethylene oxide, or polyoxypropylene are widely used in the field of DNA chemistry. These polymers can be used to stabilize

nanoparticles or DNA, aiding in the delivery of anti-cancer drugs, proteins, or even DNA into cell.[93–95] One of the main advantages is their ability to morph into supramolecular structures depending on their configuration and copolymer chains.[96] This adaptability and customizability makes them useful carriers for biomolecules.

Polyethylenimine has a high cationic potential, which makes it a useful carrier of DNA. Unfortunately, as the molecular weight is increased, the toxicity is also increased. By creating a copolymer with PEG, the molecular weight can be increased with mitigated toxicity. Addition of the block copolymer can also prevent degradation. Using this compound to deliver DNA increased the efficacy three fold as compared to a lower molecular weight version.[94]

These polymers have also been shown to be highly useful for the stabilization of nanoparticles. Due to the small size, aggregation is often a large problem. Nanoparticle stability can be significantly enhanced by adding a PEG monolayer. Longer PEG chains resulted in higher stability.[97] Interestingly, PEG can enhance hybridization onto DNA functionalized gold nanoparticles (AuNPs), but at the same time prevent DNA adsorption by competing for binding sites.[98] This suggests that the order in which PEG or other non-ionic polymers is highly important for successful application.

1.4.5 Polyelectrolytes

Polyelectrolytes have many uses in the field of DNA chemistry. One of the primary uses of polyelectrolytes is to influence DNA stability. This can refer to either the stability of the DNA duplex itself in different solutions, or to stabilize other structures or molecules such that they

can interact with the DNA.[15,99,100] Polyelectrolyte films have also been used successfully in the past to promote delivery of DNA into cells.[101] Similar films can also aid in the immobilization of DNA or other biomolecules by presenting a charged surface for adsorbtion.[102]

Polyanions have been found to stabilize the DNA duplex much more than non-ionic polymers. As DNA itself is negatively charged, it is reasonable that repulsion caused by the polymer stabilizes the duplex form over single strands. However, effect of the cation used to prepare the anionic polymer must also be considered, as these cations can also influence the stability of the duplex. Khimji et al. found that sodium polyacrylate was able to stabilize the DNA duplex (as measured by melting temperature) much more than polyethylene glycol, even when the salt concentration was manually adjusted. A schematic of this interaction is provided in Figure 1.5[15]
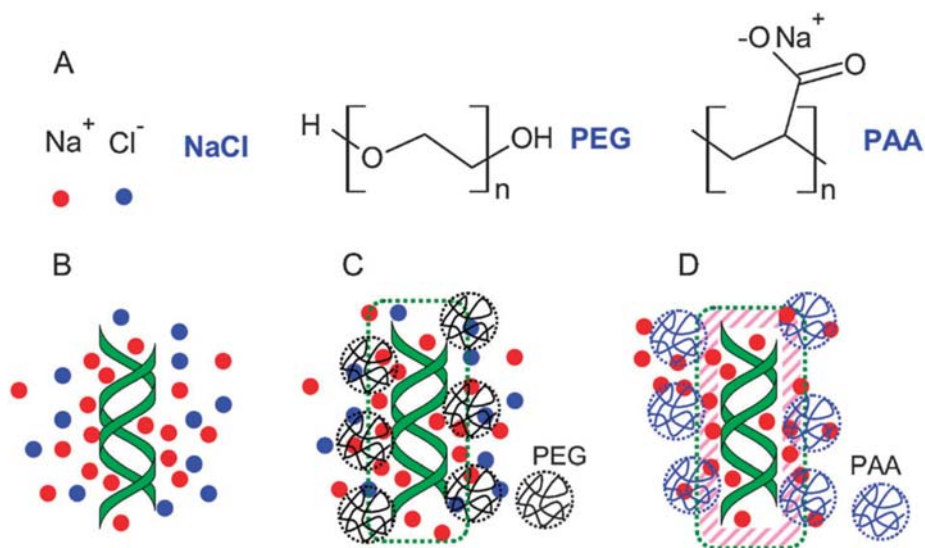

Figure 1.5: DNA interaction with NaCl, PEG, and PAA. Structure of each component (A), and schematic of the interaction between the compounds (B-D). Reprinted with permission.[15]

Depending on the strength of the counterion, polyelectrolytes have also been found to induce DNA condensation. This suggests that caution must be taken to ensure the correctly polyelectrolyte is used. Electrostatic interaction is the main force that drives either stabilization or condensation of DNA.[103] Although EB binds readily to DNA, use of the wrong polyelectrolyte can result in anticooperativity, resulting in less binding of EB to DNA.[99]

1.5   Thesis Objective

As discussed, ILs have many unique properties. The full extent of these properties, as well as how they interact with different molecules has yet to be discovered. Although many different ionic liquids have been discovered, their full use for biomolecules is still unknown. Some studies have shown that ILs do interact with DNA and other biomolecules, although alternating conditions could change the avenues of this interaction.

Although both DNA and dyes have been exposed to ILs, concerns still remain about the competitive interaction when the complex is mixed with both an ionic phase and aqueous layer. The majority of the focus has dealt with individual components and their interaction with IL, rather than both together. The goal of this thesis is to further explore the interaction between DNA, dye, IL, and water to generate a model of preference. The liquid-liquid system will be investigated for separation capabilities and characterized as a function of concentration. My findings, which show that the phase separated system is a suitable mechanism for separating the DNA-dye complex, as well as for the separation of pure dyes will be subsequently discussed.

Chapter 2 DNA in Ionic Liquids

2.1 Introduction

The main goal of this project was to study the behaviour of DNA and DNA staining dyes in ionic liquids. DNA can adopt different secondary structures in different solvents, changing its stability or conformation. Our specific objective was to examine which medium DNA has a stronger preference for to determine if a viable separation method with other components can be accomplished. More specifically, can the ability of ionic liquids that phase separate with aqueous mediums be used to separate DNA from dye molecules that have been intercalated into the structure.

2.2 Results and Discussion

Although many ionic liquids exist, only two were used for the experiments. These ionic liquids were chosen due to their ability to phase separate with water. In order to create an adequate liquid-liquid interface for extraction, we needed to ensure that the solubility of the IL was sufficiently low. High solubility would result in the lack of phase separation, making it that much harder to determine which component had higher affinity for the molecules being examined. The structure of each ionic liquid is shown Figure 2.1. As can be seen, both ILs have an immiscible anion, which translates to their ability to phase separate with aqueous mediums.
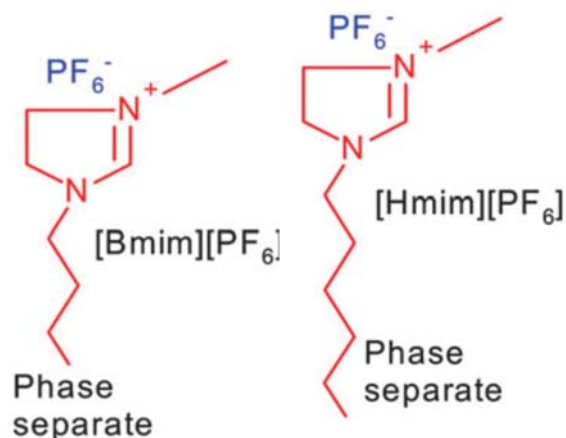
2.2.1   Dye Partitioning

Our first objective was to determine the interaction, if any, between free dye and the ionic liquid. The goal was to examine if the dye had a preference on remaining in the aqueous phase or extracting into the ionic portion. To test this, we used different cationic dyes (SYBR, EB, and TO) and also tested two different ionic liquids.

2.2.1.1   Effect of Dye

We first tested the effect of two different intercalating dyes free in solution. EB and TO were selected as they are readily available and very commonly used as DNA markers during amplification or gel electrophoresis. All three of these dyes have a greater affinity for dsDNA as compared to ssDNA, and none of them have been previously separated after having been bound to DNA. Fluorescence is significantly enhanced upon intercalation with DNA, making it easy to determine the combined presence. If the concentration is sufficiently high, the colour of these dyes in the solution can be seen without the need for UV excitation.

In order to examine the effect of ionic liquid on these dyes, we first added the dye to an aqueous medium. Ionic liquid was then added which promptly sunk to the bottom due to the heavier weight. Colour could be seen in the aqueous portion following addition of the dye. The sample was then sonicated to fully mix the two phases. Centrifugation was used to speed up the phase separation.

As shown in Figure 2.2, all of the dye moved down to the ionic phase. No dye could be found in the aqueous phase. Interestingly, it seemed as if there was some interaction with the ionic liquid that is similar to the interaction with DNA, as the fluorescence increased after it moved down to the ionic liquid. [Hmim][PF6] was used to determine the effect of different dyes.
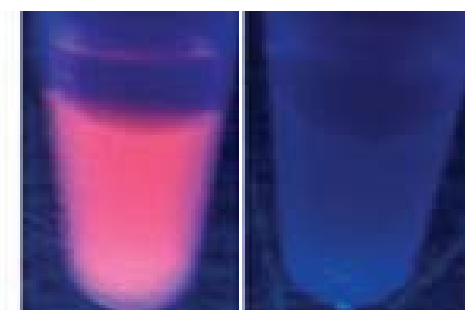
Figure 2.2: Free dye partitioning into the ionic phase. EB (left) can clearly be seen partitioning into the bottom ionic phase. TO (right) fluorescence without DNA is too weak to be observed. Modified and reproduced with permission.[104]

2.2.1.2 Effect of Ionic Liquid

We next tested these two dyes in two different ionic liquids that were both found to phase separate with an aqueous medium. Our goal was to determine if this partitioning effect was universal or specific to that one type. As before, dye was added to the aqueous phase and then the ionic liquid was added. As a comparison, fluorescence in the aqueous phase is also provided in Figure 2.3.

We found that regardless of which ionic liquid was used, the dye would always partition into the ionic phase. Fluorescence in the ionic phase was also stronger in both cases, suggesting there is some interaction between the dye and the ionic liquid. Careful examination also shows that the fluorescence is slightly different between the ionic liquids, suggesting that the interaction between them is slightly different. Dye and IL concentration was the same for all cases.
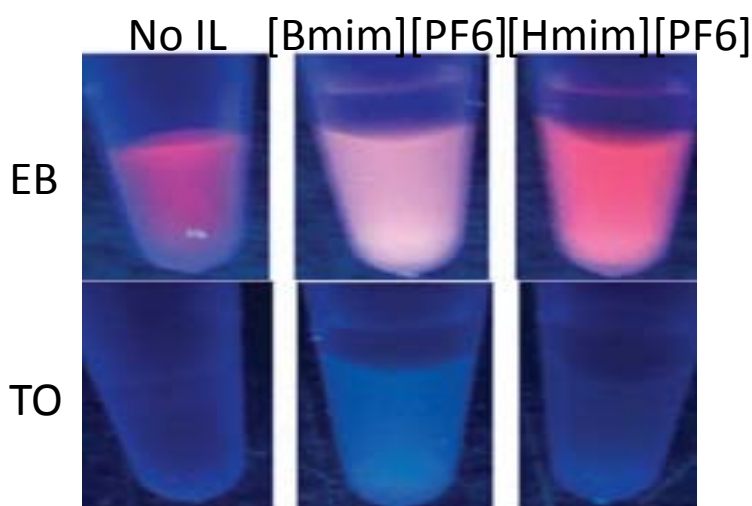
Figure 2.3: Dye partitioning in different ILs. Modified and reproduced with permission.[104]

Another interesting point to notice is that all of the ionic liquids tested were cationic. This is the case for most DNA staining dyes. However, a few anionic staining dyes such as deoxyadenosine do exist. We found that this could not be extracted by either ionic liquid, suggesting that the charge is vital for extraction into ionic liquids. Although this would normally suggest that the interaction between the dye and the ionic liquid is electrostatically based, several other anionic dyes have been successfully extracted from water into IL. It was found that increasing alkyl chain length of the cation increased the extraction efficiency, as did increasing temperature.[105] This means that it is too early to claim that deoxyadenosine cannot be removed; further

investigation is required to examine other ionic liquids with different cationic chain lengths and different temperatures.

2.2.2   DNA Partitioning

2.2.2.1   Ionic Liquid Type

As we observed that the dye itself preferred to partition into the ionic phase, we next wanted to see how this would change if the dye was intercalated into the DNA. If the DNA-Dye attachment was stronger than the interaction between the ionic liquid and the dye, we would expect that either the DNA-dye conjugate would both be removed from the aqueous phase. If the interaction between the DNA and dye was not as strong, then perhaps only the dye would become separated. The third option is that both would remain in the aqueous phase.

To test this, we utilized 24mer dsDNA. The interaction between intercalating dyes and double stranded DNA is much stronger, which would make it harder for separation to occur. Dye was added to a DNA solution and allowed to interact. After a short period (5 minutes) the ionic liquid was added. The sample was sonicated until it turned milky and then centrifuged to promote phase separation. A control sample with no ionic liquid was also prepared. Following phase separation, the aqueous phase was collected and the fluorescence was measured. The value obtained was then normalized.
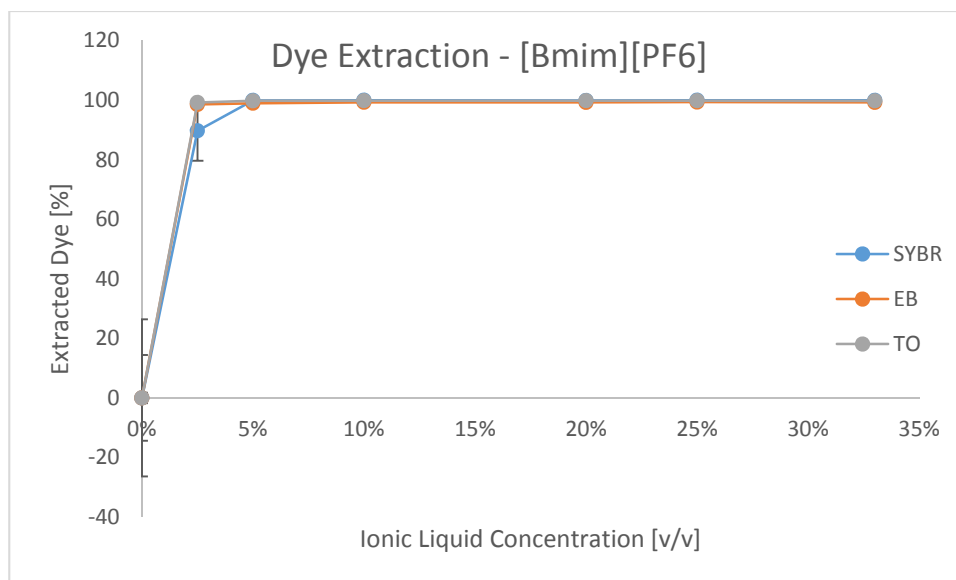
Figure 2.4: Dye extraction from dsDNA in [Bmim][PF6]

As can be seen from Figure 2.4, even 2.5% of ionic liquid by volume was able to extract close to 95% of the dye. From our free dye experiments, we know that interaction with ionic liquid does not quench the fluorescence; it actually enhances it. However, in this case, 95% of the fluorescence was removed from the aqueous phase, indicating that either the dye was not present or the DNA-dye conjugate was not present. Essentially, either just the dye or both components partitioned into the ionic phase. However, when fresh dye was added to the aqueous phase following separation, bright fluorescence was observed. This suggests that the DNA remained in the aqueous phase, while only the dye partitioned into the ionic phase. When the ionic liquid concentration was increased to 5% by volume, 100% of the dye was extracted. This was observed repeatedly and with low error. Figure 2.5 shows extraction from the second ionic liquid, which was also able to remove the dye from the aqueous phase.

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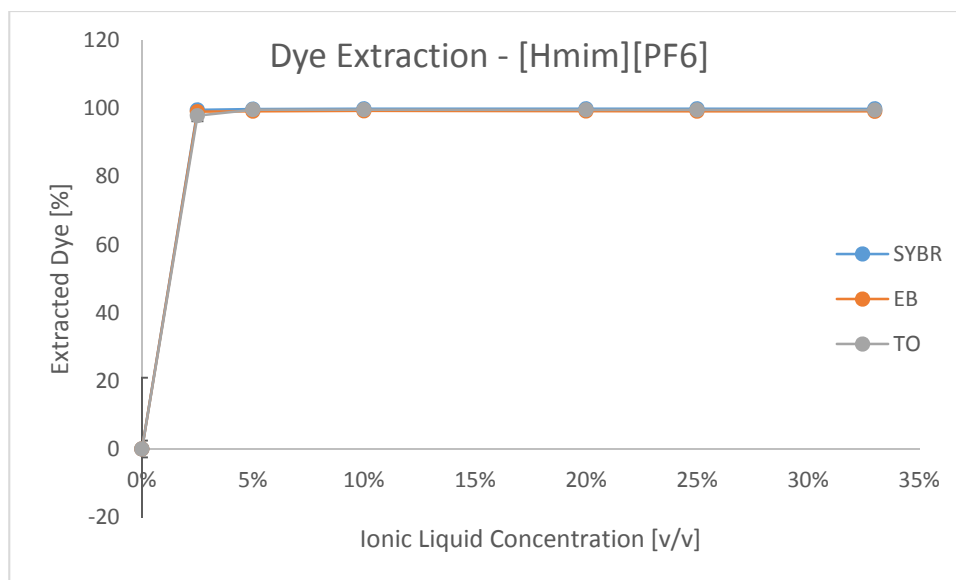
Figure 2.5: Dye extraction from dsDNA in [Hmim][PF6]

Dye concentrations of less than 2.5% were not tested due to the slight solubility of the ILs in the aqueous medium. Approximately 2% was found to be soluble, and thus no phase separation could be achieved. The high values obtained at 2.5% then suggest that even at such a low concentration, the extraction capabilities were not hindered. This indicates that the system is highly effective for the separation of these substances. Further research would be required to determine if other cationic substances could also be separated with the same efficacy.

Extraction of metal ions has already been proven with different ILs. A possible next step would be to determine if these ILs also have the capability to separate the same metal ions if they have already bound to DNA. This would present a possible method of extracting and regenerating DNA sensors or extraction systems. This could also provide a novel way to clean membranes used to filter out heavy metals from water.

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Further research is also required to determine if the method of binding to DNA is important for the separation. Currently, all separated molecules are intercalated into the DNA. However, this is only one method of binding. Small molecules can also attach to either major or minor grooves in the double helix. Further investigation is required to determine the exact requirements for extraction. This would also provide further insight as to exactly why the cationic dyes prefer the IL; is it solely electrostatic based, and if so, is there a charge threshold for extraction.

2.2.2.2   Ionic Liquid Concentration

Having found that the dye preferred the aqueous phase, our next goal was to confirm the location of the DNA. A paper published by Wang et al. indicated that [Bmim][PF6] was able to completely remove DNA from the aqueous phase.[86] The return of fluorescence suggested that this was not the case in our experiment, so further tests were run to fully confirm the location of the DNA. Tracking the location of the DNA would also allow us to infer the interaction strength between the dye and ionic liquid.

To test this, we utilized fluorophore functionalized DNA. These DNA strands were 15 nucleotides long, and composed of A, C, G, and T rich sequences. As before, a solution containing the DNA was prepared. Ionic liquid was added and the sample was sonicated until a milky composition developed. Centrifugation was used to speed up the phase separation. The aqueous layer was collected and the fluorescence was measured.

Figure 2.6 shows the fluorescence in the aqueous phase. As indicated, regardless of the ionic liquid concentration, there is no significant decrease in fluorescence following the

centrifugation. In some cases, the fluorescence is actually higher. This suggests that 100% of the DNA is located in the aqueous phase. If any DNA partitioned in the ionic phase, we should see some decrease in fluorescence.
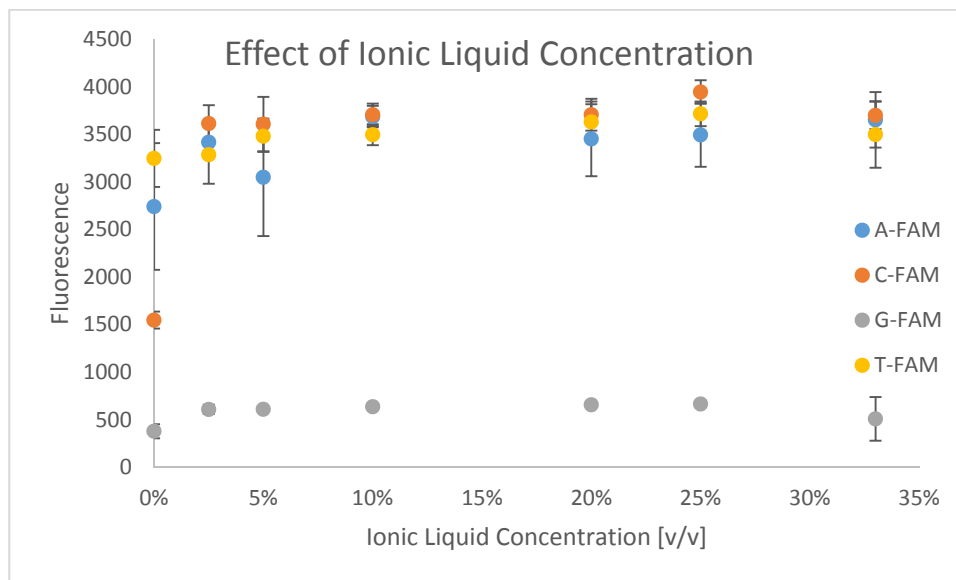
Figure 2.6: Aqueous phase fluorescence as a function of IL concentration

This confirms that only the dye is moving into the ionic phase, as the DNA on its own prefers the aqueous phase. Essentially, this means that the ionic liquid was able to remove the dye from the aqueous phase, but also separate the DNA-dye complex. Hence, the interaction between dye and the ionic liquid was stronger than that of the DNA and dye. Unless the fluorophore was becoming detached from the DNA, which is unlikely, this result also confirms that the DNA does not actually partition into the ionic phase as previously reported.[86]

Another conclusion that can be drawn from this data is regarding the interaction strength of both the aqueous phase and ionic phase. It was found that when DNA intercalated with EB was

placed in an IL, the fluorescence disappeared. Previously, this was believed to have occurred

due to the much stronger interaction between the DNA and the IL, to a point where the IL was

able to actually remove and replace the EB within the DNA.[82] However, this data seems to

indicate that the interaction of DNA and water is actually much stronger than the interaction

with the IL. Only the dye prefers interaction with the IL, suggesting that the true reason for

decreasing fluorescence is the stronger interaction between EB and the IL, not the DNA.

Previously, there have been no reports which in which DNA-dye separation is achieved.

Methods such as capillary electrophoresis are able to separate DNA based on different lengths,

but they are unable to separate the DNA-dye complex. This presents a novel way to regenerate

or retrieve DNA that has already been used. As the dye's themselves could interfere with the

sensitivity of a biosensor, or create a higher baseline and decrease sensitivity, it is important

for the reusability of a sensor that the dye can be removed.

2.2.2.3 DNA Concentration

We next attempted to run a concentration study. Although unlikely, a chance existed that at a

certain concentration, the DNA was more likely to partition in the ionic phase beyond the

threshold. To examine this, we used both our dsDNA from earlier as we as high molecular

weight DNA obtained from salmon testes. The ionic liquid concentration was fixed at 33% by

volume and only the DNA concentration was varied.

As previous, the samples were mixed and after the DNA concentration was reached and

allowed to equilibrate, ionic liquid was added. The key difference in this experiment was that

no dye was added prior to mixing of the ionic liquid. In order to generate signal, dye was added

after the aqueous phase had been removed and collected. As per the results by Wang et al. EB

was added at a concentration of 5ng/mL at a 1:1 ratio.[86] Figure 2.7 shows the effect of long

DNA at the higher concentrations, while Figure 2.8 shows the duplex DNA at very low

concentrations.
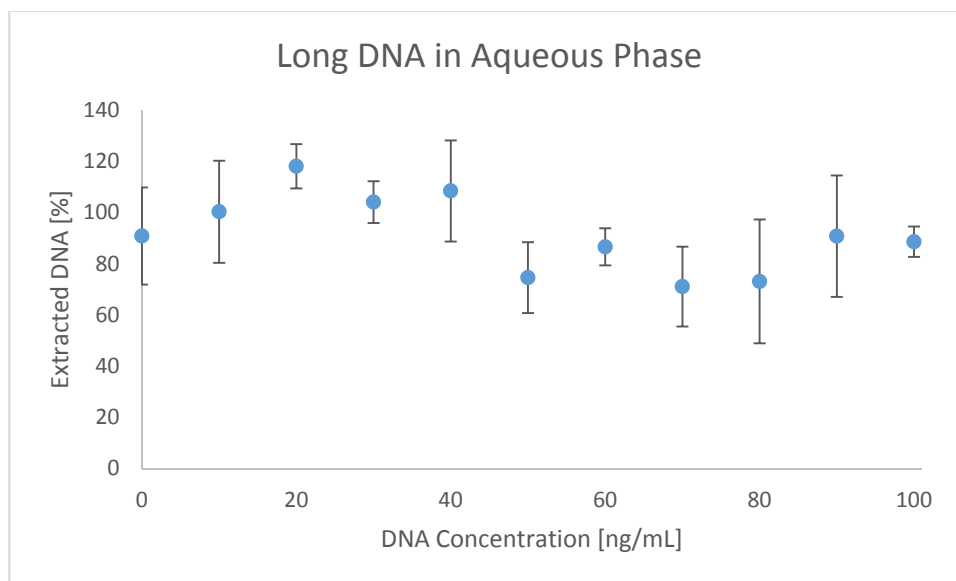
Figure 2.7: Fraction of the DNA in the aqueous phase

As can be seen, although there was wide variation in the results, there was no significant

difference regardless of the DNA concentration. In all cases, the DNA in the aqueous phase was

close to 100%. At a concentration of 0ng/mL, the fluorescence is caused by the ethidium

bromide itself. These results suggest that at no concentration in the range tested does the DNA

partition into the ionic phase. Even at higher DNA concentrations, there is no saturation of the

aqueous phase that causes DNA to diffuse or partition into the ionic phase.
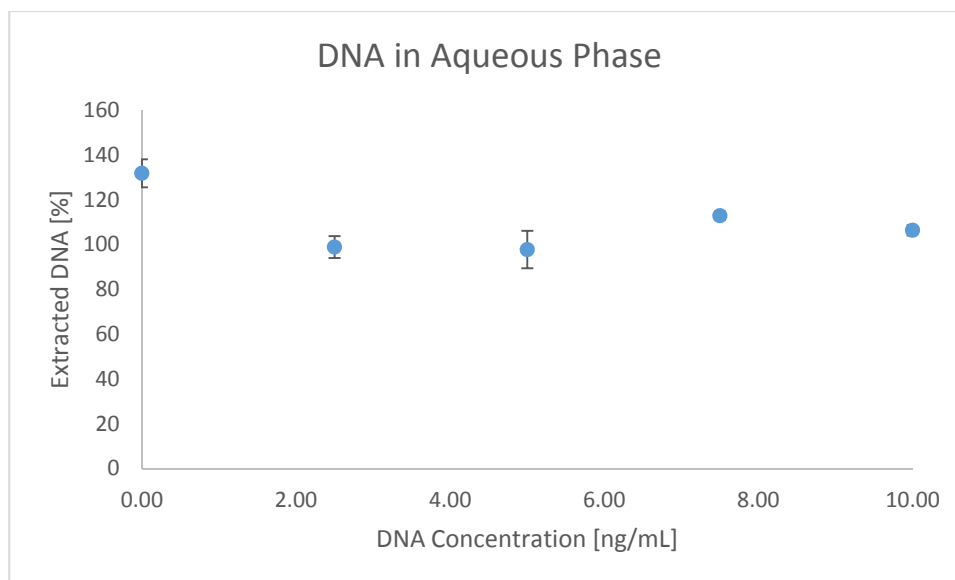
Figure 2.8: Fraction of the DNA in the aqueous phase at low concentrations

These results also indicate that the length of the DNA was not a factor in partitioning. In either case, close to 100% of the DNA remained in the aqueous phase. Since both long DNA with a high molecular weight and 24mer DNA was used, and both showed the same trend, it can also be assumed that regardless of how long or short the DNA is, it will still prefer to remain in the aqueous phase.

2.2.2.4 DNA Composition

As a point of interest, we next tried to see if the DNA composition affected the partitioning of DNA. This would indicate whether or not the interaction with ionic liquid was stronger depending on the nucleotide sequence. We utilized our 15mer homopolymer DNA to examine the difference in partitioning. The same method was employed to mix the ionic liquid and DNA solution. No dye was required as the DNA was already fluorescently tagged.

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Figure 2.9 compares the fluorescence of the control, which had the same DNA concentration but no ionic liquid to the aqueous phase, which had been mixed with ionic liquid and collected after centrifugation. Again, although there was variation, A and T bases exhibited no significant difference between the control and collected aqueous phase, suggesting that there is absolutely no partitioning into the ionic phase. For C rich DNA, there was a lower fluorescence which could suggest a small amount either partitions into the ionic liquid or prefers to remain at the interface between the separated phases. This portion was extremely hard to extract without also removing some ionic liquid, and so care was taken to avoid this. In the case of G rich DNA, the fluorescence actually increased slightly. This could be due to the destabilization of the G-quadruplex secondary structure, which could have been quenching the fluorescence initially.[106] In fact, this IL has been found to cause to cause structural changes when mixed with DNA,[82] giving evidence to the disruption of the G-quadruplex.
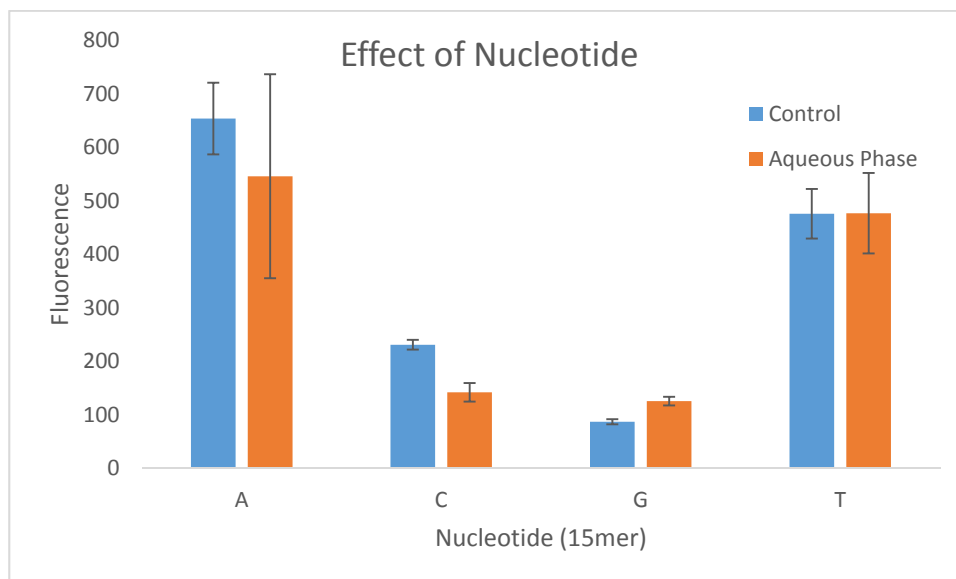
Figure 2.9: Effect of the nucleotide on extraction into the ionic liquid

2.2.3 Back Extraction

As a further validation, we attempted to do a back extraction of the DNA from the ionic liquid. Using a pH 4 phosphate citrate buffer that was reported to remove DNA that had partitioned into the ionic phase,[86] any signal generated here would suggest that some DNA had gone into the ionic phase. As specified, the extraction buffer was supposed to be able to remove as much as 30% of the DNA extracted into the ionic phase. As previous, ionic liquid was added to the DNA solution. However, instead of collecting the aqueous phase, the ionic phase was also collected. Extra care was taken to remove only the ionic phase and thus avoid also collecting any DNA from the aqueous phase. This ionic portion was then added to the back extraction buffer and sonicated until milky, after which centrifugation was used. The aqueous portion of this second mixing was collected and measured for fluorescence.

Figure 2.10 shows the back extraction of the high molecular weight DNA compared to the control. A fresh sample containing the same concentration of DNA was used as the control sample. As indicated, there is no significant difference from the baseline. This indicates that the back extraction buffer was unable to pull any DNA out of the ionic liquid. Although a couple of data points suggested back extraction was successful, it is important to note the high standard deviation of these samples. Experimental error could explain this, especially if some of the aqueous layer was accidentally also collected when isolating the ionic phase.
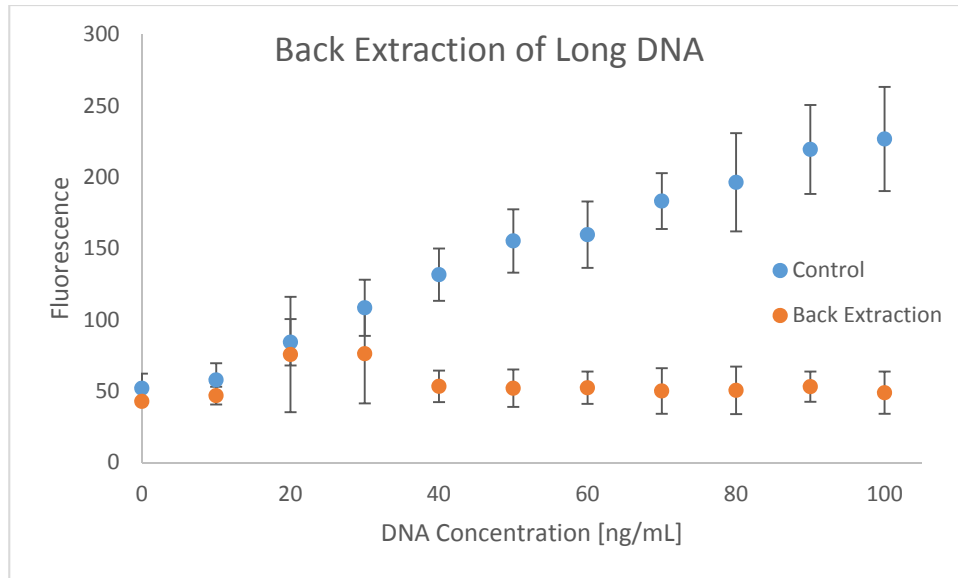
Figure 2.10: Back extraction of high molecular weight DNA from salmon testes

We also performed the back extraction on the A, C, G, and T rich DNA sequences, shown in Figure 2.11. As an added control, we also back extracted into water. As with the high molecular weight DNA, almost no DNA was retrieved following the back extraction. Instead of comparing these values to the control, we compared them to the aqueous phase that we had collected. All of the values have been normalized over the control to indicate the percentage of DNA retrieved in each phase instead of the relative fluorescence.
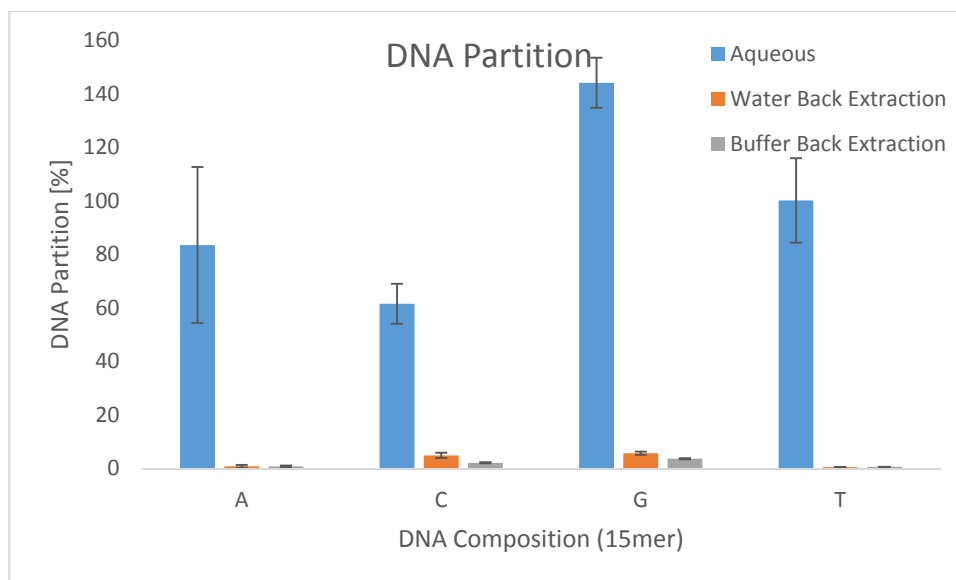
Figure 2.11: Homopolymer DNA partitioning in the aqueous and back extracted phases.

Again, both A and T suggested that no DNA was partitioning into the ionic phase. C and G rich DNA showed some signal following the back extraction, but this represents only 3-5% of the total DNA. If we consider that the mixing of ionic liquid causes destabilization of the fluorescence quenching G quadruplex (indicated by the extremely high aqueous percentage), then even less was collected in the back extraction. Again, the value for C was lower than the expected 100%, which may also indicate that out of all the bases, C rich DNA is the most comfortable in the ionic phase.

2.2.4 PCR Amplification

The higher signal observed for G-rich sequences suggested that there may be some structural changes involved during the separation process. In order for this technique to be a viable

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method for the regeneration of DNA-dye based biosensors, we also need to ensure that there is no damage to the DNA. Removing the dye would be useless if the DNA is no longer functional. To experiment the effect of the ionic liquid, we attempted to run PCR on the extracted sample. The aqueous phase was collected and run through PCR alongside a control sample where the concentration was known.

We tested three different concentrations of DNA. In both the 10nM and 1nM cases, both the control sample and the extracted sample showed almost the exact same amplification curve. This suggests that the DNA was not structurally damaged in a way that would impede the amplification. Also, the fact that both curves in Figure 2.12 are almost super imposed on each other also indicates that both samples contained the exact same concentration, further corroborating our earlier results.
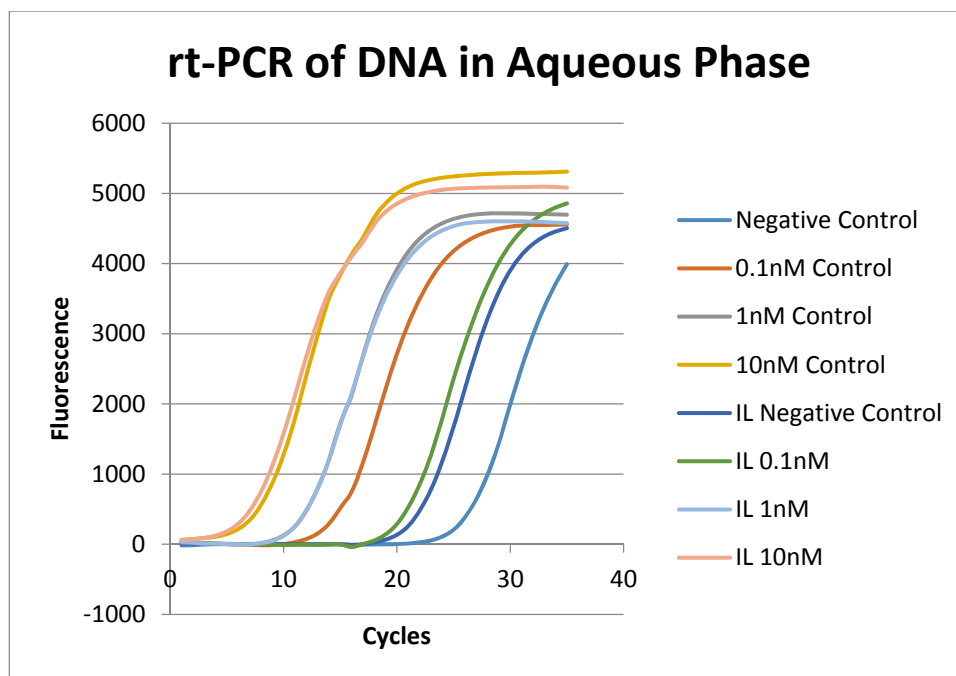
Figure 2.12: rt-PCR of dye remaining in aqueous phase after being mixed with [Bmim][PF6]

At a concentration of 1nM, a difference can be seen if the control is compared to the IL sample. A possible reason for this is that at such a low concentration, it is possible that some DNA was lost when the aqueous layer was collected. Although the DNA remains in the aqueous phase, it may remain close to the boundary layer. As avoiding taking in any of the IL was a major concern, care was taken to stay away from this area. As a result, some DNA may have been lost, which would account for the difference in amplification.

Finally, we ran the amplified DNA through gel electrophoresis. The same vertical displacement on the gel shows that the strands were of the same length, further indicating that the DNA was not fundamentally damaged during the separation process, as can be seen in Figure 2.13. The intensities of the bands are all similar as these lanes hold the amplified product and not the initial extraction. The lack of smearing around the bands suggests that the product was all uniform, meaning that the DNA was the expected length.
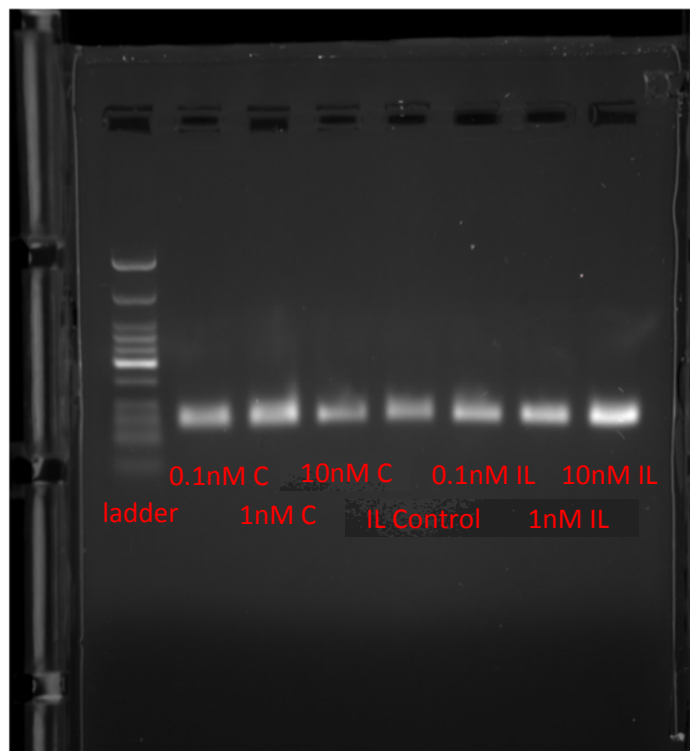
Figure 2.13: Agarose gel electrophoresis of amplified DNA after mixing with [Bmim][PF6].

2.3 Methods

2.3.1 Chemicals

All of the ionic liquids were obtained from Ionic Liquids Technologies (Tuscaloosa, AL). DNA staining dyes (EB, TO, SG) were purchased from Sigma-Aldrich. EVA-Green PCR reagent (supermix) was obtained from Bio-Rad. Buffers were prepared in the lab and the salts used were purchased from Mandel Scientific (Guelph, Canada). Milli-Q water was used to dilute the buffers and for all other experiments.

2.3.1.1   DNAs Used

Several different sequences were used in this work. All of the oligonucleotides were obtained

from Integrated DNA Technologies (Coralville, IA). The sequences are summarised in Table 2.1.

High molecular weight DNA from salmon testes was purchased from Sigma-Aldrich

Table 2.1: DNA Sequences used in Experiments

DNA Name	Sequence (5' to 3')
24 mer	ACGCATCTGTGAAGAGAACCTGGG
cDNA	CCCAGGTTCTCTTCACAGATGCGT
FAM-A15	FAM-AAAAAAAAAAAAAAA
FAM-C15	FAM-CCCCCCCCCCCCCCC
FAM-G15	FAM-GGGGGGGGGGGGGGG
FAM-T15	FAM-TTTTTTTTTTTTTTT
Primer 1	CGTTAAGACCTCTATGAAATGAATGTA
Primer 2	GAAAGGTAAGTACAGGGAAAGG
PCR Template	GAAAGGTAAGTACAGGGAAAGGACCTTCCTCCGCAATACTCCCCCAGGTTCTCT TACATTCATTCATAGAGGTCTTAACG

2.3.2   Experimental Procedures

In order to test the separation efficiency of individual dyes, an aqueous buffer solution was first

prepared. This buffer contained 50mM NaCl and 20mM HEPES. 200µM of ssDNA and the

complimentary strand was added and allowed to hybridize in solution. The dye being tested

was then added at a concentration of 5µM and allowed to intercalate with the DNA, generating

strong fluorescence. Ionic liquid was then added at the desired concentration by pipette. The ionic liquid immediately sunk to the bottom, so the sample was placed in a sonicator for 30 minutes. The sample was manually agitated at several intervals to ensure complete mixing. Once the sample became milky, and phase separation could not be seen, the sample was removed and centrifuged for 6 minutes at a speed of 6800 rpm. This caused the phase separation to occur, with the aqueous phase on top and the ionic phase at the bottom. The aqueous layer was the collected and placed in a plate reader for fluorescence measurement.

To trace the effect of ionic liquid concentration, DNA with a fluorophore attached was used. This removed the need for adding dye. The DNA concentration was fixed at 0.5µM, as this concentration could be easily detected yet did not saturate the detector. As previously, the DNA solution was prepared prior to addition of the ionic liquid. This method was also used to determine the effect of individual nucleosides on the ability of the ionic liquid to remove DNA from the aqueous phase.

In order to carry out the concentration study, different concentrations of DNA were prepared in buffer. Although this DNA did not contain a fluorophore, no dye was added prior to addition of the ionic liquid. This would be removed during the mixing, and would not be able to generate signal to track the DNA. As before, the ionic liquid was added and the sample was centrifuged. Ionic liquid was added to make a 33% v/v solution. After extracting the aqueous phase, the dye was then added to determine the quantity of DNA remaining in the aqueous phase. Only 5ng/mL of EB was added at a 1:1 ratio with the aqueous volume, as suggested by Wang et al.[86]

To perform the back extraction, we first performed an initial extraction as stated above. Instead of collecting the aqueous phase, we instead removed the ionic phase. This ionic phase was then added at a 1:1 ratio to either a stripper buffer composed of pH 4.0 phosphate citrate buffer or back into the initial buffer or water. This mixture was the re-sonicated and centrifuged until milky. After this second mixing, the aqueous phase was collected and the signal was measured.

Viability of the DNA was confirmed by PCR. As before DNA solution was prepared and mixed with the ionic liquid. After the extraction, instead of adding dye, 1μL of the aqueous phase was removed and added to the PCR mix. The mix contained 50% EVA Green Supermix, 1μL of the forward and reverse primers for a concentration of 400nM, and topped up to a total volume of 20μL with Milli-Q water. These samples were then placed into the rt-PCR, which was allowed to run until the control sample also began to show amplification. Recommended settings were used. Enzyme activation occurred at 95 ^{O}C for 30s, followed by denaturation at 95 ^{O}C for 35s. Annealing and extension occurred for 5s at 55 ^{O}C. All of the ionic liquid separated samples were compared to their own controls to fully investigate the effect of the ionic liquid.

2.3.3 Measurement Techniques/Instruments Used

A UV plate reader was used for all fluorescence measurements. An excitation wavelength of 490, 480, 480, and 535 was used for FAM, SG, TO, and EB respectively. For FAM, SG, and TO, the emission wavelength was set to 525. In the case of EB, an emission wavelength of 600 was used. These are summarized in Table 2.2.

Tag/Marker	Excitation (nm)	Emission (nm)
FAM	490	525
SG	480	525
TO	480	525
EB	525	600

A Bio-Rad CFX96 thermo cycler was used for real time monitor of PCR amplification. Recommended settings were used to track the fluorescence increase. A sonicator and centrifuge were used as needed in accordance with the procedures listed above.

2.4 Conclusion

We have demonstrated that both ionic liquids have the capability to extract hydrophobic dyes from an aqueous medium. This is true for both free dye and dye that has been bound to DNA. We have also demonstrated that the DNA prefers to remain in the aqueous phase. None of the DNA partitions into the ionic phase. Prior to this research, studies indicated that the DNA preferred to partition into the ionic phase. In fact, it was previously determined by Wang et al.[86] that all of the DNA actually is removed from the aqueous phase, and the use of a stripper buffer is only minimally effective in recovering the DNA. In all of our experiments, we found that this was not the case. The DNA actually remains in the aqueous phase. Care was taken to ensure that experimental conditions were as similar to previous experiments as possible. To

confirm, we tested several different DNA strands (dsDNA, homogeneous DNA, long and short DNA) and all of them seemed to prefer the aqueous phase. Tracking the location of the DNA was carried out through fluorescence readings (addition of dye into the aqueous phase or FAM labelled DNA) and by PCR. PCR amplification showed near simultaneous enhancement for the extracted sample and the control, which contained no ionic liquid. This indicates that the same concentration of DNA was present in both cases, which means that none of the DNA was partitioning into the ionic phase.

The other conclusion was the ability to separate the DNA dye complex. This has previously never been accomplished, as even DNA separation methods such as gel electrophoreses are unable to separate the two. In electrophoreses, the dye moves through the gel with the DNA. This presents a way of potentially re-using DNA that has already been bound to dye to generate signal. Again, several dyes were tested to ensure this result is consistent. It was found that only the cationic dyes could be separated consistently. A few anionic dyes were also found to extract into the ionic liquid, although deoxyadenosine could not be separated. It is probable that factors such as charge or hydrophobicity play a large role in determining whether or not separation will occur. We also found that there is some interaction between dye and ionic liquid that causes enhanced fluorescence. Although not as bright as when affixed to DNA, there is definitely fluorescence increase between dye in the aqueous phase and dye in the ionic phase.

Having proven that cationic dyes could be separated, it is possible that other substances can also react in a similar manner. For example, other small molecules that intercalate into the DNA may also detach and separate by partitioning into the ionic liquid. This would allow for DNA to become a carrier or vessel to transport small molecules in aqueous mediums without releasing

them. When the molecules need to be removed or collected, the phase separation with ionic liquids can be used. More research is required to determine exactly what interaction exists between the dye and ionic liquid. As well, further research would allow the generality of the observations to be further investigated. A simple experiment would be to test other cationic molecules to determine where they partition if they can be separated from the DNA. This may also allow for separation strength to be determined.

In order to expand upon this technique, small molecules with different binding pathways should also be tested. Molecules that bind into the grooves of duplex DNA should be examined to determine if the binding pathway is a factor in determining separation. This should also give an idea of the efficacy of the technique; how strong is the interaction and what binding strength can be displaced. Finally, although we know some interaction exists between cationic dye molecules and the ionic liquid, the exact method of interaction can be probed and examined.

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