Study of dynamics and nanosegregation in ionic liquids and deep eutectic solvents by fluorescence correlation spectroscopy

by

Deyny Leticia Mendivelso-Pérez

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Program of Study Committee:
Emily A. Smith, Major Professor
Jared L. Anderson
Young-Jin Lee
Jacob W. Petrich
Igor I. Slowing

The student author, whose presentation of the scholarship herein was approved by the program of study committee, is solely responsible for the content of this dissertation. The Graduate College will ensure this dissertation is globally accessible and will not permit alterations after a degree is conferred.

Iowa State University

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DEDICATION

This dissertation is dedicated to the loves of my life, my husband and my family

(grandmother, mother and siblings)
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Ionic liquids (ILs) and deep eutectics solvents (DESs) have emerged as a promising alternative to traditional organic solvents. This is due to their unique properties such as extremely low volatility, electroconductivity, unusual solvation and tuned miscibility, among other properties. Although, ILs and DESs are different types of solvents based on their molecular structures, they share many characteristics and properties that make them potentially attractive for a diversity of applications such as electrochemistry, synthesis, separation technologies, catalysis, materials science, and biochemistry. These unique properties have been interpreted as the result of their organization at the nanoscale level. Thus, the presence of nanosegregation in ILs and DESs is proposed to be important for many applications using these solvents, yet this nanoscale heterogeneity is poorly understood. In this dissertation, the translational diffusion dynamics of fluorophores in ILs and DESs films is reported as measured by fluorescence correlation spectroscopy.

Theoretical studies have predicted a high degree of nanosegregation in tetraalkylphosphonium-based ILs. However, experimental studies that confirm these findings are scarce. To this end, fluorescence correlation spectroscopy was used to study molecular diffusion in a series of tetraalkylphosphonium ILs films. The primary motivation for this study was to understand how the nanostructural organization affects the diffusion behavior of fluorophores of different polarities, polar (Atto 590), and nonpolar fluorophore (DiD), when the cation and anion in tetraalkylphosphonium ILs are altered. From the results, it was concluded that spatial heterogeneity is present in these classes of ILs, given that the diffusion of the fluorescent probes deviates from the Brownian diffusion behavior. These deviations are attributed to the presence of structural heterogeneities in the tetraalkylphosphonium ILs.
DESs have demonstrated increased potential for a diversity of applications, especially in separation technologies. Similar to ILs, nanostructural heterogeneity has been observed in DESs by theoretical and experimental studies. However, the fundamental understanding of DESs structure at the molecular level remains in a relatively early stage. To gain further insight into the presence of nanostructural heterogeneity in carboxylic-based DESs, fluorescence correlation spectroscopy experiments were performed for studying the translational diffusion properties of a hydrophilic (Atto 590) and a hydrophobic (DiI) fluorophore. Anomalous diffusion behavior was observed for the fluorescent molecules in all studied DESs. This anomalous diffusion behavior is characteristic of heterogeneous systems.
CHAPTER 1. GENERAL INTRODUCTION

Introduction to Ionic Liquids and Deep Eutectic Solvents

Basics of Ionic Liquids (ILs)

In 1914, Paul Walden reported the synthesis of an ionic salt, ethylammonium nitrate, [EtNH$_3$][NO$_3$] (melting point of 12.5 °C) and that was synthesized by the neutralization of ethylamine with concentrated nitric acid.$^{1,2}$ Although there is evidence of the early synthesis of these ionic compounds, it is generally accepted that the birth of ionic liquids (ILs) took place with Walden’s report. According to his definition, ionic liquids are materials composed of cations and anions that melt at or below 100 °C (which is an arbitrary temperature that he chose), which sets them apart from molten salts that exhibit higher melting points. Many ILs have melting points lower than or equal to room temperature, and these ILs are called room temperature ionic liquids (RTILs).$^3$ The low melting point of ILs is due to the chemical structure of the ions, and the interactions between them, where the cation has a low degree of symmetry and the anion has considerable delocalization of the electron cloud. These aspects tend to frustrate packing and to reduce the lattice energy of the crystalline form of the salt and explain the lower the melting point.$^4$

A general approach for the synthesis of ILs is by combining bulky cations (imidazolium, pyridinium, sulfonium, phosphonium, etc.) with a range of organic or inorganic anions. Figure 1 shows the most common cations and anions that form part of the structure of ILs. The chemical structure of the cation and anion gives ILs unique physical and chemical properties (i.e., extremely low volatility, unusual solvation and tuned miscibility, electroconductivity, among other properties), which make them potentially attractive compounds as an alternative to traditional organic solvents. Thus, the macroscopic properties observed in ILs can be fine-tuned
by careful selection of the ions for a giving application. For this reason, ILs are considered to be “designer solvents”, giving the possibility of designing an IL that matches the characteristics required by a specific application. The structural diversity and properties characteristic of ILs make these materials valuable for a diversity of applications such as electrochemistry, synthesis, separation technologies, catalysis, materials science, and biochemistry.

It is estimated there are around $10^{18}$ possible anion and cation combinations for the synthesis of ILs. As mentioned before, the structure-property relationship that is characteristic of ILs allows compounds with specific properties such as melting point, viscosity, polarity, water miscibility, density, and hydrophobicity to be conceived. For instance, glass transition temperatures, melting temperatures, and viscosities of branched ILs are higher than those of their linear analogues. In imidazolium-based ILs with a common anion, as may be expected, increasing the alkyl chain length in the imidazolium cation leads to a considerable increase of viscosity. Nevertheless, the increase in viscosity is also dependent on the nature of the anion with which the imidazolium cations are paired. To this end, the combination of cation-anion should be carefully tailored for obtaining an IL with desired properties.
In the last two decades, deep eutectic solvents (DESs) have emerged as a new type of solvent with interesting and similar properties to ILs. In 2003, Abbot et al.\textsuperscript{10} prepared eutectic mixtures composed of a range of quaternary ammonium salts with hydrogen bond donors (HBD) that were liquid at room temperature and had interesting solvent properties. These mixtures exhibited a eutectic point much lower than the melting point of any of the individual components. Abbot at al.\textsuperscript{10} adopted the term deep eutectic solvents to differentiate them from ionic liquids. In 2019, Martins et al.\textsuperscript{11} proposed a more complete definition for DES systems. They suggested that “DES should be defined as a mixture of pure compounds for which the eutectic point temperature is below that of an ideal liquid mixture”. In this work, it is suggested that in order to differentiate a mixture as DES from other simple eutectic mixtures, the phase diagram should be known. DES systems exhibit strong negative deviations from ideality (\textit{i.e.}, the eutectic temperature is lower that of the ideal eutectic), as shown in Figure 2. Additionally, the phase diagram defines the range of compositions at which the mixture is liquid at operating temperature.
temperature. In 2019, Alhadid et al.\textsuperscript{12} suggested that “a simple eutectic mixture with a large depression in eutectic temperature relative to the melting temperatures of the pure constituents sufficient to form a liquid at operating temperature could be referred to as DES”, which requires knowledge and understanding of solid-liquid equilibria (SLE).

\textbf{Figure 2.} Representation of the comparison of the SLE of a simple ideal eutectic mixture (red) and a deep eutectic mixture (blue). The melting temperature of individual components are $T_{m,1}$ and $T_{m,2}$. Temperature depression corresponds to the difference ($\Delta T_2$) between the ideal ($\Delta T_{E,\text{ideal}}$) and the real ($\Delta T_E$) eutectic point and not as the difference ($\Delta T_1$) between the linear combination of the melting points of the pure components and the real eutectic point ($\Delta T_1$).

\textit{Figure adapted from Martins et al.}\textsuperscript{11}

Since 2003, several DES systems have been reported, and they have been classified into four types, as shown in Table 1. Cat\textsuperscript{+} usually corresponds to a quaternary ammonium, phosphonium, or sulfonium salt. The anionic moiety (X\textsuperscript{−}) is often involved in hydrogen bonding with protons from the hydrogen donor (RZ) group (R = alkyl group and Z = CONH\textsubscript{2}, COOH, or OH).\textsuperscript{13} Abbot et al.\textsuperscript{10,14} attributed the decrease in the freezing point at the charge delocalization
through the formation of hydrogen bonds between the halide anion and the HBD. Furthermore, it has been observed that the melting point of the mixture decreases as the symmetry of the cation decreases. Similar behavior is observed in ILs.\textsuperscript{10,14} Several experimental and computational studies have shown the presence of a more wide variety of hydrogen bonds in DESs; where this hydrogen bonding network leads to the strong decrease in the melting point and unique properties observed in DESs.\textsuperscript{15}

Table 1. General formula for the classification of deep eutectic solvents. Adapted from Smith et al.\textsuperscript{16}

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<th>Type</th>
<th>General Formula</th>
<th>Terms</th>
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<td>Type I</td>
<td>$\text{Cat}^+X^-\text{MCl}_x$</td>
<td>$M = \text{Zn, Sn, Fe, Al, Ga, In, Bi, Pb}$</td>
</tr>
<tr>
<td>Type II</td>
<td>$\text{Cat}^+X^-\text{MCl}_x \cdot y\text{H}_2\text{O}$</td>
<td>$M = \text{Cr, Co, Cu, Ni, Fe}$</td>
</tr>
<tr>
<td>Type III</td>
<td>$\text{Cat}^+X^-x\text{RZ}$</td>
<td>$Z = \text{CONH}_2, \text{COOH}, \text{OH}$</td>
</tr>
<tr>
<td>Type IV</td>
<td>$\text{MCl}<em>x + \text{RZ} = \text{MCl}</em>{x-1}^+ \cdot \text{RZ} + \text{MCl}_{x+1}^-$</td>
<td>$M = \text{Al, Zn}; Z = \text{CONH}_2, \text{OH}$</td>
</tr>
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The amount of research focused on fundamental studies or applications of DES has increased over the last two decades, and a wide variety of DESs have been reported. The most attractive feature of DES is the possibility to fine-tune the properties of the system by the appropriate selection of the individual components based on their molecular structure and chemical nature. This structure-property relationship observed in DESs allows them to be classified as a designer solvent, similar to ILs.\textsuperscript{17} However, despite ILs and DESs sharing similar physical properties, such as low vapor pressure, relatively wide-liquid range, low flammability, and good thermal stability, DESs exhibit several advantages over ILs.\textsuperscript{18} Particularly, DESs are usually less toxic and biodegradable. They are also cheaper and easier to prepare at moderate temperatures with no purification steps. These particular features, in combination with a
hydrogen bonding network (bulk nanostructure) and physical properties, make DESs attractive alternatives to traditional solvents (as well as some ILs) in a variety of applications.¹⁹⁻²³

**Nanostructural Organization in Ionic Liquids and Deep Eutectic Solvents**

The unique properties of ILs and DESs have been interpreted as the result of their organization at the molecular level.²⁴ For example, such an organization might be responsible for their ability to dissolve both polar and nonpolar compounds. This would be explained if the compounds distribute into different portions of the structurally heterogeneous system. It is believed that the size and the structure of the nanodomains would affect the transport parameters within ILs and DESs.²⁵ Thus, the study of the microstructure in ILs and DESs has received increasing attention, and many experimental and theoretical approaches have been used. In this context, the structural composition, water content, and temperature are factors that affect the extent of these structural heterogeneities in ILs and DEs.

**Effect of the Structural Composition in Nanosegregation**

Heterogeneity is one of the critical features in interpreting unexpected physical phenomena of ILs, such as heterogeneous self-diffusion, surface layering, and the formation of surfactant-like micelles in ILs-water mixtures.²⁶ The structural organization in ILs has been reported to be determined by the chemical structure of the cation.²⁷⁻³¹ Nanostructures form in imidazolium-, ammonium-,³¹ pyridinium-,³² piperidinium-,³³ and phosphonium-based ILs.³⁴,³⁵ IL nanostructures were first reported from MD simulations, where the charged anions and headgroups of the cations were shown to distribute homogeneously because of the strong electrostatic interactions, while the neutral tail groups tended to aggregate due to the collective short-range interactions. As a result, polar and nonpolar domains are observed in IL systems. For instance, several studies have reported nanosegregation in imidazolium-base ionic liquids with alkyl chain lengths longer than four carbon atoms, but no aggregation was reported for shorter
chain lengths.\textsuperscript{36} Thus, when the side chain of the cation is long enough, the domain formation of tail groups results in a liquid crystal-like structure. The structural heterogeneities occur over a spatial scale of a few nanometers (11-20 Å).\textsuperscript{29}

Experimentally, Tokuda et al.\textsuperscript{37} were the first to propose the existence of aggregates in bulk ILs based on viscosity and conductivity measurements on a family of alkylimidazolium bis(trifluoromethane)sulfonimide ([C\textsubscript{n}MIM][NTf\textsubscript{2}]) ILs. Furthermore, the size of the domains has been studied experimentally by many research groups. Domain sizes in the range of 10-100 nm were reported for imidazolium-based ILs, and the domain size increases with the alkyl chain length.\textsuperscript{38, 39} X-ray diffraction studies in imidazolium-based ILs have shown that ILs with an alkyl chain on their cation or anion segregate into polar and nonpolar domains over an intermediate length scale of typically 8-20 Å.\textsuperscript{40, 41} Thus, experimental and computational studies have provided strong insights into the presence of nanosegregation in a variety of IL systems.

Similar to ILs, heterogeneity has been observed in DESs.\textsuperscript{29, 30, 36, 40} However, the fundamental understanding of DES structure remains in a relatively early stage. Computational and experimental approaches have been used as a tool for investigating the microstructures present in the most common DES systems. It is believed that the hydrogen bonding network is not only essential for the DESs formation but also strongly influences the local structure and dynamics within DESs, where the extent of the hydrogen bonding network depends on the nature of the individual components of the mixture (i.e., HBD and HBA).\textsuperscript{42-50} Garcia et al.\textsuperscript{50} used DFT simulations to study hydrogen bonding in a set of 45 DESs to relate melting point with molecular structure. His studies demonstrated the presence of a cage-like structure, where the melting point depression is due to charge delocalization within the hydrogen bonding network. Ashworth et al.\textsuperscript{15} proposed that there is a substantial hydrogen bonding, an ‘alphabet soup’ of hydrogen
bonds, in DES systems that can vary in strength and number. Overall, they concluded that ChCl:U DES exhibits a diverse range of hydrogen bonding, which gives a highly disordered molecular structure to the ChCl:U DES system.

The amphiphilic behavior previously observed in ILs has been found in some types of DESs. Using MD simulations, McDonald et al. predicted, for the first time, amphiphilic nanosegregation in a series of alkylammonium bromide (alkyl: ethyl-, propyl-, and butyl-) and glycerol DES at a 1:2 molar ratio. In these systems, a polar and nonpolar domain were predicted, where the contact between bromide and the methyl group is reduced as the alkyl chain increases. The resulting nanostructures associate into a bilayer-like structure even for the shorter carbon chain (ethylammonium bromide), and that association and ordering increases with alkyl chain length. The same nanosegregation of polar and nonpolar domains was reported by Cui et al. and in nonionic DES consisting of N-methylacetamide:lauric acid (NMA:LA). Interestingly, Alizadeh et al. found strong heterogeneity in a series of alkylcholine chloride:ethylene glycol (1:2 molar ratio) DESs. Their MD simulations revealed that in all the DESs studied, the polar groups are always completely connected forming one domain, while the nonpolar groups tend to be highly dispersed within the polar parts for DES with alkyl side chains of four carbon units. However, DES with alkyl side chains of eight or more carbon units exhibit two distinctive polar and nonpolar domains. Thus, heterogeneity in these DESs increased as the alkyl side chain length increased, leading to an increase in the nonpolar domain size.

**Effect of Water Content**

The effect of water on the molecular structure of ILs and DESs has been studied by several research groups, and even residual water has an impact on these systems. ILs are hygroscopic and can absorb a significant amount of water from the atmosphere, and complete removal of water is extremely difficult. The presence of water affects physical properties in ILs
such as density, viscosity, ionic mobility, which are influenced even by small water concentrations.\textsuperscript{52,53} ATR-IR experiments and computational simulations have shown that water is present in two different states in a variety of ILs.\textsuperscript{54,55} At low water concentrations (\textit{i.e.}, $<0.2$ mole fraction), water molecules are in an isolated (non self-aggregated) state or exist in small independent water clusters in the polar cation-anion network in ILs. In these clusters, water molecules bound to the basic anion via hydrogen bonding. It has been found that the cations play a minor role in the water cluster formation, and the water molecule preferentially interacts via hydrogen bonding with two distinct anions by forming (Anion$\cdots$H–O–H$\cdots$Anion) associations.\textsuperscript{52,53,56,57} It has been observed that the water-anion interaction increases in the order $[\text{PF}_6]^\text{−} < [\text{SbF}_6]^\text{−} < [\text{BF}_4]^\text{−} < [\text{ClO}_4]^\text{−} < [\text{CF}_3\text{SO}_3]^\text{−} < [\text{NO}_3]^\text{−} < [\text{CF}_3\text{CO}_2]^\text{−}$.\textsuperscript{54}

Additionally, at high water concentrations (\textit{i.e.}, 0.2 to 0.8 molar fraction), water molecules self-associate, forming continuous water clusters. These clusters increase in size as the mole fraction of water increases, thereby slightly disturbing the polar IL network. These water clusters eventually percolate in the ionic network of the IL, and strongly change the properties of the system.\textsuperscript{58,59} However, when the water molar fraction is considerably high (\textit{>} 0.8 molar fraction), IL systems have shown a moderate degree of aggregation before the IL is fully dissolved in water just like any conventional electrolyte.\textsuperscript{60} Canongia-Lopez et al.\textsuperscript{61} demonstrated the existence of microphase segregation between polar and nonpolar domains, where hydrogen bonding dominates the interaction between water and anion. Jiang et al.\textsuperscript{62} observed the increase in separation between the polar and non-polar domains as the water concentration is increased, which is consistent with others studies on water-miscible ILs.\textsuperscript{62} Using MD simulations, Méndez-Morales et al.\textsuperscript{63} concluded that the water cluster size is independent of the cation chain length but strongly dependent on the hydrophobicity of the anion, which also determines the water network
formation and the miscibility of the ILs. Thus, the polar network established by strong
electrostatic interactions between cations and anions is gradually disrupted as the water
concentration is increased in the IL system.

Water can also be present in DESs as an unavoidable impurity or can be added in a
controlled way to modulate the physicochemical properties of the system, such as viscosity,
polarity, conductivity, and density.\textsuperscript{57} Water perturbs the hydrogen bonding network in DES by
establishing hydrogen bonding with the individual components of the DESs. For instance, in
choline chloride: polyols DESs, at water concentration <30% mole fraction, water molecules are
absorbed in the molecular matrix of the DESs by establishing hydrogen-bonding with ions and
HBDs (chloride ion-water, HBD-water, choline cation-water).\textsuperscript{64} At this water concentration, the
structure in the DES system is retained, and the diffusion of ions is not affected. For the most
common DESs (ChCl:G, ChCl:EG, and ChCl:U, 1:2 molar ratio) the thermophysical pro-
perties are not affected at low water concentration, however, at high water concentration (>50% mole
fraction) water dampens intermolecular and intramolecular interaction in these DESs, and the
properties are expected to change drastically.\textsuperscript{65}

Using a combination of neutron total scattering and empirical potential structure
refinement (EPSR), Hammond et al.\textsuperscript{66} observed that in ChCl:U (1:2 molar ratio), at low water
levels (\textit{i.e.}, \leq 1 mole fraction), water contributes slightly to the hydrogen bonding network, and
the majority of the original nanostructure (80\%) is maintained. Between 2 and 10 water mole
fraction, the DES system resists hydration by retaining their initial structure since water is
located into nanostructured domains around choline cations. At 15 water mole fraction
(50\%w/w), the DES structure is disrupted, where water-water and DES-water interactions
dominate. Thus, the system is described as an aqueous solution of the DES components.
However, further investigations are needed in a wider variety of DESs to determine the limit of water content that retains the original DES network and properties.

**Effect of Temperature**

Many ILs have been studied by experimental spectroscopy (IR, Raman, NMR, neutron scattering) and computational tools, and the results have indicated that the ions can array orderly over a broad range of temperatures. For instance, Triolo et al.\(^\text{40}\) found the existence of structural organization at the nanometer scale in the liquid and supercooled state in some alkylimidazolium-based (alkyl= butyl-, hexyl-, octyl-, and decyl-) ILs. Similarly, Aoun et al.\(^\text{67}\) observed that for 1-hexyl-3-methylimidazolium chloride, the low-Q peak intensity in the X-ray diffraction measurements decrease with increasing temperatures (-100 °C to 150 °C), indicating that the nanostructures exist even at higher temperatures. Li et al.\(^\text{68}\) observed a shift of the diffraction peaks to lower Q values in pyrrolidinium-based ILs, which suggests a decrease in the aggregate size as the temperature increases. They proposed that at ambient temperatures, the Van der Waals interactions are stronger for the ILs with longer alkyl chain, increasing chain association. However, at higher temperatures, Van der Waals interactions are reduced, and the polar group electrostatic interactions dominate. As a result, at higher temperatures, a cation tail diffuses from its nonpolar aggregate, which leads to a decrease in the size of these aggregates. In general, nanoscale ordering in the form of polarity (polar-nonpolar) alternation is expected to decrease with an increase in temperature. This is reflected with an increase in the first sharp diffraction peak in the X-ray scattering diffraction pattern (prepeak). However, the opposite trend has been observed by Araque et al.\(^\text{25}\) and Hettige et al.\(^\text{69}\) in tetraalkylphosphonium-based ILs. They suggested that the increase in the temperature leads to a more disorganized nonpolar subcomponent but with a better-defined polar component. Overall, the temperature has a
particular effect in the nanostructural organization of the ILs that depends on the chemical structure of the IL system.

The effect of temperature in the nanoscale structural organization has been studied in only a few DESs system. However, the effect of temperature on the physical properties, such as viscosity, density, and surface tension, has been studied by several research groups.\textsuperscript{70, 71} For example, the increase in temperature leads to a decrease in viscosity in the DES system due to a weaker interaction between the HBA and the HBD as the temperature increases. Kaur et al.\textsuperscript{72} reported an unusual effect of temperature in the nanoscale structure in lithium perchlorate salt + alkylamide-based DESs. The simulated X-ray and neutron scattering structure functions showed an increase in the prepeak intensity as the temperature increases. They suggested that the increase in nanoscale heterogeneity as the temperature increases in these DES is due to ion-pair segregation at higher temperatures.

**Confocal Fluorescence Correlation Spectroscopy for Measuring Dynamics and Heterogeneity**

Fluorescence correlations spectroscopy (FCS) has been commonly used to measure dynamics on a variety of heterogeneous systems, such as biological membranes and polymer samples. The heterogeneity is reflected in the deviation from the Brownian diffusion model. Particularly, FCS has been used as evidence for reporting structural heterogeneity and diffusional properties in ILs and DESs by measuring the translational diffusion properties of fluorescent molecules (cationic, neutral, and anionic).\textsuperscript{73-79} FCS is a rapid and sensitive tool that requires only a small amount of sample (microliters). For instance, Werner et al.\textsuperscript{76} studied the translational diffusion of fluorescent species (Rhodamine 6G (R6G), 4-(Dicyanomethylene)-2-methyl-6-(4-dimethylaminostyryl)-4H-pyran (DCM), and fluorescein) within 1-butyl-3-methylimidazolium...
hexafluorophosphate, [BMIM][PF₆]. Their results showed that fluorescein experiences the highest rate of diffusion in [BMIM][PF₆], while R6G experiences the lowest translational diffusion. Overall, the diffusion of the fluorescent probes followed the trend of $D_{\text{anionic}} > D_{\text{neutral}} > D_{\text{cationic}}$. The association of the anionic fluorophore with the bulky IL is expected. However, the measurements showed a different trend. It was hypothesized that the symmetrical distribution of negative charge on the anion allows the interaction with several surrounding cations, leading to ionic ‘cross-linking’. Guo et al.\textsuperscript{73} and Patra et al.\textsuperscript{74} observed a bimodal diffusion behavior in the FCS data on a series of pyrrolidinium- and imidazolium-based ILs. These results suggested the presence of a heterogeneous environment, in which the fluorophore diffuses at different rates in the non-aggregated and self-segregated domains.

Anomalous diffusion also has been observed in a series of DESs.\textsuperscript{80,81} Interestingly, Hossain et al.\textsuperscript{82} found that the position of the hydroxyl group has an insignificant effect on the variation of spatial and dynamic heterogeneity in a series of alcohol-based DESs. On the other hand, they found that the heterogeneity in a series of tetraalkylammonium bromide-based DESs becomes prominent when the alkyl chain in the cation of the HBD increases in length. This suggested that the heterogeneity arises from the segregation of ionic moieties and the hydrophilic alkyl chain of the HBA.\textsuperscript{83} Overall, FCS is a very versatile technique for measuring dynamic and structural heterogeneities in ILs and DESs. The fundamental principles of FCS are reviewed in the following sections.

**Fundamentals of Fluorescence Correlation Spectroscopy**

In the last decades, fluorescence correlation spectroscopy (FCS) has become one of the most commonly employed techniques for studying various dynamical molecular processes, especially translational diffusion and heterogeneity of systems. It has found applications in measuring local concentrations, diffusion coefficients, reaction rates, and detection of
intermolecular interactions, to mention a few.\textsuperscript{84} The principle of FCS is based on the statistical analysis of time-dependent intensity fluctuation of fluorescent molecules (at nanomolar concentration) diffusing in or out the detection volume, this causes fluorescence intensity fluctuations over time. These fluctuations are stochastic and differ one from another over time and position.\textsuperscript{85,86} To obtain quantitative information about the diffusion process, it is necessary to carry out a statistical analysis of the fluctuations. The fluorescence fluctuation $\delta F(\tau)$ caused by the change of fluorescence signal intensity at any time $t$ in the detection volume can be expressed as:

$$\delta F(t) = F(t) - \langle F(t) \rangle$$  \hspace{1cm} (1)

Here, $F(t)$ corresponds to the temporal fluctuations in the detected fluorescence intensity, $\langle F(t) \rangle$ is the average fluorescence intensity. Autocorrelation of recorded intensities leads to FCS curves, $G(\tau)$, which can be normalized according to:

$$G(\tau) = \frac{\langle \delta F(t) \cdot \delta F(t+\tau) \rangle}{\langle F(t) \rangle^2}$$  \hspace{1cm} (2)

In the equation above, the delay time, $\tau$, refers to the time the fluorescence molecules stay in the detection volume. $\delta F(t)$ is correlated with the delay time, $\tau$, and the $F(t + \tau)$ represents the fluorescence intensity at any time, $t$, after the delay time, $\tau$.

In a typical FCS experiment the parameters of the Gaussian beam are fixed and known beforehand from calibration of the instrument. The standard formula for the FCS autocorrelation functions for fluorescent molecules confined into either a three-dimensional (3D) or two-dimensional (2D) planes are shown in Eq. 3 and Eq. 4, respectively.

$$G(\tau) = \frac{1}{N} \cdot \left[ 1 + \left( \frac{\tau}{\tau_d} \right)^\alpha \right]^{-1} \cdot \left[ 1 + \left( \frac{\omega_{xy}}{\omega_x} \right)^2 \left( \frac{\tau}{\tau_d} \right) \right]^{-\frac{1}{2}}$$  \hspace{1cm} (3)
\[ G(\tau) = \frac{1}{N} \left[ 1 + \left( \frac{\tau}{\tau_d} \right)^\alpha \right]^{-1} \quad (4) \]

Where \( N \) corresponds to the number of fluorescent particles diffusing in the detection volume, \( \tau_d \) is the diffusion time, and \( \omega_{xy} \) and \( \omega_z \) are, respectively, the transversal and axial dimensions of the observation volume determined at the height of \( 1/e^2 \). Ordinarily in diffusion fluctuations, the mean-squared displacements (MSD), \( \langle r^2 \rangle \), grows linearly in time, \( \langle r^2 \rangle \propto t \). This behavior is considered free (Brownian) diffusion, with \( \alpha = 1 \). However, in disordered systems, the mean-squared displacement of the fluorescent molecules scales as a power law in time, \( \langle r^2 \rangle \propto t^\alpha \). This is considered anomalous diffusion and it is characterized by \( \alpha \neq 1 \). If the \( \alpha \) exponent takes the values of \( \alpha < 1 \) or \( \alpha > 1 \), the process is referred to as sub-diffusion or super-diffusion, respectively.\(^{87-89}\) The translational diffusion coefficient, \( D \), is calculated by employing the \( \tau_d \) value obtained from the fit to Eq. 3 or 4.

\[ \tau_d = \frac{\omega_{xy}^2}{4D} \quad (5) \]

According to Stokes-Einstein equation, the diffusion coefficient, \( D \), is related to the viscosity of the medium, \( \eta \), and the hydrodynamic radius of the molecule, \( R \), the Boltzmann’s constant, \( k_B \), and the absolute temperature, \( T \), as:

\[ D = \frac{k_B T}{6\pi\eta R} \quad (6) \]

**Instrumental Set-up of Confocal FCS**

Briefly, in an FCS experiment, the exciting radiation provided by a pulsed laser is increased in size by inserting a beam expander in the optical path. This expanded beam is directed into the microscope objective via a dichroic mirror. The expanded beam will overfill the back aperture of the objective in order to obtain a smaller focal volume. Then, the objective focuses the excitation beam into the sample, usually a high numerical aperture objective is used.
(1.49 NA). This ensures the largest collection efficiency (higher count rate/molecule), in addition to the highest spatial resolution. The fluorescence signal from the sample is collected by the same objective and passed through the dichroic mirror and the appropriate emission filter. A pinhole in the image plane blocks the fluorescence light not originated from the focal plane, thus providing axial resolution. Then, this fluorescence signal is focused onto the detector, and autocorrelated by a hardware correlator PC card. The resulting correlated data are fitted using the appropriate mathematical model. The home-build confocal FCS setup used for performing the FCS measurements is illustrated in Figure 3.

**Figure 3.** Instrumental schematic for a confocal fluorescence correlation spectroscopy instrument. A 532-nm and 590 nm are used as excitation lasers. Abbreviations: M mirror, DM dichroic mirror, BE beam expander, SPCM single photon counting module, QWP quarter phase plate, PH pinhole.

**Thesis Organization**

This dissertation is categorized into 4 chapters. In Chapter 1, a general introduction of ILs and DESs is presented, as well as a review of the factor affecting their nanosegregation. Additionally, the fundamentals for fluorescence correlation spectroscopy as a tool for measuring
dynamics and heterogeneity are presented. In Chapter 2, the diffusional dynamics of a polar and nonpolar fluorophore in tetraalkylphosphonium-based ionic liquid films measured by fluorescence correlation spectroscopy are discussed. The translational diffusion behavior of two fluorescent probes and their connection with nanosegregation in a series of DESs base on tetraalkylammonium-halide salts and carboxylic acids is presented in Chapter in Appendix A and B depicts the application of Raman spectroscopy for studying chemical structure and heterogeneity in carbonaceous nanostructured-based sensors. General conclusions and future insights are presented in Chapter 4.

References


CHAPTER 2. DIFFUSIONAL DYNAMICS OF TETRAALKYLPHOSPHONIUM IONIC LIQUID FILMS MEASURED BY FLUORESCENCE CORRELATION SPECTROSCOPY

Deyny L. Mendivelso-Pérez, Muhammad Qamar Farooq, Kalyan Santra, Jared L. Anderson, Jacob W. Petrich, and Emily A. Smith*

Department of Chemistry, Iowa State University, Ames, Iowa 50011, United States
The Ames Laboratory, U.S. Department of Energy, Ames, Iowa 50011, United States

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Abstract

Fluorescence correlation spectroscopy (FCS) is applied to investigate the diffusional dynamics of hydrophilic (Atto 590) and amphiphilic (DiD) fluorophores in a series of alkylphosphonium ionic liquid (IL) films ([P448][Cl], [P668][Cl], [P66614][Cl], and [P66614][NTf2]) in order to determine diffusional parameters and to elucidate nanoscale structural heterogeneities within the IL. From the measured correlation functions, the diffusion coefficients of the fluorescent molecules are estimated, rendering values that span from 0.39-1.2 and 0.146-5.2 µm²/s, for Atto 590 and DiD, respectively. An increase in the diffusion coefficient values is correlated to the increase in the alkyl chain length, which in turn is correlated with a decrease in their viscosity. Interestingly, deviations from Brownian diffusion behavior of the fluorescent probes in the ILs are observed, showing a time-dependent diffusion coefficient in most of the cases. These deviations can be attributed to the presence of nanoscale structural heterogeneities in the tetraalkylphosphonium ILs. These results experimentally confirm the presence of nanosegregation in tetraalkylphosphonium ILs, which has been previously observed in molecular dynamics studies.
Introduction

Ionic liquids (ILs) are generally defined as organic salts that have a melting point below 100 °C. They are typically composed of an organic cation paired with an inorganic or organic anion. These materials exhibit an assortment of useful and interesting physicochemical properties, such as low-to-negligible vapor pressure, wide solvating power, and good thermal stability. Moreover, some IL properties can be tuned by choosing the appropriate cationic and anionic components. Consequently, ILs have attracted considerable attention for the extraction and separation of organic compounds, biomolecules, and metal ions. ILs have been used as solvents for liquid-liquid and liquid-phase microextraction, buffer additives, capillary-wall-supported coatings in capillary electrophoresis, sorbents for solid-phase extraction, and sorbent coatings for solid-phase microextraction. The amphiphilic nature in combination with the good thermal stability of ILs can be particularly exploited in chromatographic techniques, in which they are used as stationary phases to separate both polar and nonpolar compounds. Furthermore, they have been employed as matrices for matrix-assisted laser desorption/ionization mass spectrometry.

Several experimental and computational studies have revealed that some of the properties observed in ILs at the macroscopic scale are the result of structural heterogeneities at the molecular level, which affect the solvation and rotational dynamics of a solute in ILs. This structural heterogeneity is a consequence of the aggregation of the alkyl tail of the cations, formed mainly through attractive van der Waals interactions (nonpolar domains), and charge ordering (polar domains). The formation of domains is facilitated by an increase in the alkyl chain length, and their presence significantly affects the transport behavior and ionic conductivity of solutes in IL matrices.
Using a multiscale coarse-grained model, Wang et al.\textsuperscript{15} explored the effect of the cation side-chain length in a series of alkylimidazolium nitrate ILs. These simulations showed that, with sufficient side-chain length (C≥4), neutral tail groups of cations aggregate to form nonpolar domains, while the charged head groups of cations and anions homogenously distribute due to the collective short-range electrostatic interactions forming polar domains. However, Russina et al.\textsuperscript{16} using small-wide angle x-ray scattering (SWAXS), accessed intermolecular dynamic features that suggest the existence of the nanoscale structure in [C\textsubscript{n}MIM][NTf\textsubscript{2}] for n ≥3. Furthermore, using molecular dynamics (MD) simulations, Canongia et al.\textsuperscript{10} and Pádua et al.\textsuperscript{17} observed nano-organization in 1-alkyl-3-methylimidazolium-based ILs. An important conclusion is that aggregation of nonpolar domains was observed for alkyl side chains as small as C\textsubscript{4}, in agreement with the results from Wang and Voth.\textsuperscript{15} Margulis and co-workers investigated the slow dynamics of an imidazolium IL by means of MD simulations and predicted the existence of heterogeneity.\textsuperscript{11,14,18} They concluded that this heterogeneity is the underlying microscopic cause of the red-edge effect, wherein the fluorescence emission wavelength shifts to longer wavelengths as the excitation wavelength also shifts to longer wavelengths.

Several experimental studies have been performed to study the diffusion of organic dyes in neat or IL-water systems using fluorescence correlation spectroscopy (FCS).\textsuperscript{19-25} To mentioned a few, Guo et al.\textsuperscript{20} provided experimental evidence for chain-length dependent self-aggregation in a homogeneous series of N-alkyl-N-methyl-pyrrolidinium bis(trifluoromethylsulfonyl) imide ILs of varying alkyl chain length (n= 3, 4, 6, 8, 10) using Rhodamine 6G. Similarly, Patra and Samanta\textsuperscript{22} studied the translational diffusion of charged (R123) and neutral (DCM, 4NBD) fluorescent probes via FCS. Their study showed evidence for chain length-dependent self-aggregation in a series of 1-alkyl-3-methylimidazolium ILs. The
FCS experiments indicated that the fluorescent probes exhibited an anomalous diffusion behavior in the ILs tested, suggesting the presence of two distinct domains. Additionally, they found that by increasing the alkyl chain length in the cation, the fast and slow diffusion rate decreased; and the contribution of the slow diffusion increased, suggesting an increased in the domain size as the alkyl chain in the imidazolium ring increased. Interestingly, a biomodal diffusion behavior of the fluorescent probes was observed even in a small alkyl chain length ([EMIM][NTf₂]) in agreement with previous studies.¹⁶

Recently, there has been an increasing interest in employing phosphonium ILs due to their lower cost of synthesis, higher thermal stability, and solubilization of low polarity compounds in comparison to analogous ILs with nitrogen-based cations.²⁶ These features render them excellent materials for separation applications.²⁷ In the specific case of quaternary phosphonium salts, the existence of structural heterogeneities has been observed through MD simulations. Gontrani et al.²⁸ showed the presence of nanostructures in trihexyl(tetradecyl)phosphonium chloride ([P₆₆₆₆₁₄][Cl]) by MD simulations and X-ray scattering data, where the charged domains are formed by phosphorous and chloride atoms while the nonpolar regions correspond to the alkyl chains. Their studies described the morphology of the segregated domains as a sponge-like structure rather than a lamellar organization. Furthermore, Hettige et al.²⁹ studied the structural behavior of the same cation ([P₆₆₆₁₄⁺]) coupled with six different anions, and found that the structure of the ILs are defined by charge and polar alternation. They observed that a temperature increase in the IL system results in a better defined polar network. Wang et al.³⁰ applied MD simulations to a series of tetraalkylphosphonium cations coupled with a chloride anion in order to study the effect of the aliphatic chain in the microscopic ionic structures. They reported that for ILs consisting of cations with shorter alkyl
chains, the liquid morphologies are characterized by sponge-like interpenetrating polar and nonpolar networks. When the aliphatic chain length is increased, however, the polar network is partially broken or totally segregated within the nonpolar framework due to the progressive expansion of the nonpolar domains.

Nanoconfinement, or short-range ordering, has been reported by several authors for an IL film coated on a solid substrate. This short-range ordering can extend from 0.25 to 60 nm away from the substrate. Additionally, Anaredy et al. reported long-range ordering (ca. 1000 nm) in a set of IL films under the influence of shearing. By studying the rotational diffusion dynamics of anionic, cationic, and neutral fluorophores as a function of distance of a silica surface, Ma et al. reported organization within the IL over ca. 100 μm in a [BMIM][BF₄] IL film supported on a negatively-charged silica substrate. They raised the question if this long-range ordering could be observed in a wider range of ILs, where more bulky cations and a different set of anions are present in the IL structure.

In order to gain further insight into the structural heterogeneities observed by molecular simulations in tetraalkylphosphonium ILs, we used fluorescence correlation spectroscopy to study molecular diffusion in a series of tetraalkylphosphonium ILs films. The basic motivation is to understand how the nanostructural organization affects the diffusion behavior of fluorophores of different polarity, polar (Atto 590) and nonpolar fluorophore (DiD), when the cation and anion in tetraalkylphosphonium ILs are altered.
Scheme 1. Structure of fluorophores and constituent cations and anions of ILs used in this study. (a) Tributyl(octyl)phosphonium cation, \([\text{P}_{4448}^+]\); (b) trihexyl(octyl)phosphonium cation, \([\text{P}_{6668}^+]\); (c) Trihexyl(tetradecyl)phosphonium cation, \([\text{P}_{66614}^+]\); (d) bis[(trifluoromethyl)sulfonyl]imide, \([\text{NTf}_2^-]\); (e) chloride, \([\text{Cl}^-]\); (f) Atto 590 (mixture of 5- and 6-isomers); (g) DiD (1,1'-Dioctadecyl-3,3,3',3'-Tetramethylindodicarbocyanine, 4-Chlorobenzenesulfonate Salt)

Experimental Section

Sample Preparation

Scheme 1 shows the structure of the phosphonium ILs and fluorophores. Tributyl(octyl)phosphonium chloride, \([\text{P}_{4448}]\text{[Cl]}\), and trihexyl(octyl)phosphonium chloride, \([\text{P}_{6668}]\text{[Cl]}\), were supplied as a gift by CYTEC (West Paterson, NJ, USA).

Trihexyl(tetradecyl)phosphonium chloride, \([\text{P}_{66614}]\text{[Cl]}\), (98%) was purchased from Iolitec (Tuscaloosa, AL, USA). Trihexyl(tetradecyl)phosphonium bis[(trifluoromethyl)sulfonyl]imide, \([\text{P}_{66614}]\text{[NTf}_2\text{]}\), was synthesized as described in the Supporting Information. The ILs used in this study do not absorb light at 594 nm (Supporting Information, Figure S1). They were rigorously
dried under high vacuum for 48 h at 40 °C before use, rendering their water content to be ~1000 ppm. The water content was determined by coulometric titration using a DL39 Karl Fischer Coulometric Titrator (Mettler Toledo, Columbus, OH). The position of the bands in the OH region of the IR spectrum is sensitive to the water environment and extent of the H-bonding network, however, attenuated total reflection Fourier transform infrared (ATR-FTIR) spectra (Supporting Information, Figure S2) showed no peaks in the region 3000 – 3800 cm\(^{-1}\), where the antisymmetric (\(\nu_1\)) and symmetric (\(\nu_3\)) stretching modes of water are expected, at the low water concentrations used in this study. Viscosity measurements of the dry ILs were performed at 25 °C by the cone-plate method using a DV1 Brookfield digital viscometer with a CPE-51 spindle at 6 rpm. The water content and viscosity of the dried ILs are presented in Table 1.

Table 1. Viscosity, water content, and film thickness for the indicated samples.

<table>
<thead>
<tr>
<th>Ionic Liquid</th>
<th>Viscosity (cP)</th>
<th>Water content (ppm)(^a)</th>
<th>Film Thickness (nm)(^b)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glycerol</td>
<td>734</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>[P4448][Cl]</td>
<td>3961</td>
<td>1110 ± 90</td>
<td>1150 ± 60</td>
</tr>
<tr>
<td>[P6668][Cl]</td>
<td>3228</td>
<td>1120 ± 5</td>
<td>1040 ± 30</td>
</tr>
<tr>
<td>[P66614][Cl]</td>
<td>2270</td>
<td>1080 ± 60</td>
<td>940 ± 50</td>
</tr>
<tr>
<td>[P66614][NTf(_2)]</td>
<td>304</td>
<td>570 ± 10</td>
<td>840 ± 60</td>
</tr>
</tbody>
</table>

\(^a\) Average of 3 measurements  
\(^b\) Average of 15 measurements (3 measurements for each film)

To avoid the sorption of environmental moisture, all handling and sample preparation were performed in a nitrogen-filled glove box (relative humidity <5%). A 100 mg/mL solution of dried IL was prepared in dichloromethane, and an appropriate amount of the stock fluorophore solution (Atto 590 and DiD, Sigma Aldrich, prepared in dichloromethane) was added to reach a final concentration of 15 nM. For all reported results, the fluorophore concentration in the
samples was maintained at 15 nM. This provided sufficient signal with an average of approximately 10 molecules in the confocal volume at any time. The experimental conditions ensured that the fluctuations in the fluorescence intensity are dominated by the diffusion of molecules through the confocal volume.

To prepare the film of the IL containing fluorophore, 200 µL of solution was deposited on a 18 mm² amino-modified (full details are provided in the Supporting Information) cover glass (No. 1.5, Corning Inc., Corning, NY) and then spun for 180 s at 8500 rpm using a KW-4A spin coater (Chemat Technology, Northridge, CA). A homogeneous film was obtained and transferred to a home-made sample holder to avoid water intake from the environment during the FCS measurements. Figure S3A shows an optical image of one IL film. Additionally, Raman spectroscopy was used to confirm the formation of the IL film on the glass substrate (Supporting Information, Figure S3B). To compensate for cover glass thickness and refractive index mismatch, careful adjustment of the objective correction collar was performed for each measurement by finding the maximum signal (i.e., counts). In order to minimize effects from short-range layering due to surface confinement, FCS experiments were performed approximately 100-200 nm above the IL-glass interface. This was accomplished by moving the laser focus within the sample using a motorized microscope stage. Using a high numerical aperture objective, it is not possible to move the focus further away from the substrate, and it is possible that long-range organization may be present within these IL films.

The thickness of the IL films was measured using a spectroscopic ellipsometer (J.A. Woollam α-SE, J.A. Woollam Co. Inc., Lincoln, NE, USA), operating at the wavelength range of 380-900 nm at 70°. The IL film thickness was tracked as a function of time by acquiring Psi(ψ) and Delta (Δ), values at different time points. Data analysis was performed using
CompleteEase software. Figure S4 shows example ellipsometry data for one IL film. The thickness of the IL film was approximated using the Cauchy model. Thickness measurements were performed at five different locations on the sample, and an average thickness and standard deviation of the IL films were computed (Table 1).

**Instrumentation**

The FCS apparatus was based on an inverted confocal microscope (Nikon, Ti) equipped with a 594 nm pulsed picosecond (fwhm = 500 ps, repetition rate 20 MHz) diode laser (BDS-SMY, Becker & Hickel) as the excitation beam. The output of the laser (0.7 mW) was coupled to a polarization-maintaining single-mode optical fiber (KineMATIXP2, Qioptiq). The laser beam was expanded to overfill slightly the back aperture of the 100x oil-immersion microscope objective (Apo TIRF, NA. 1.49, Nikon) with a correction collar (0.13–0.20), and a neutral density filter (OD 1.5) was used to reduce the laser power at the sample. Fluorescence generated by the focused laser beam was collected by the same objective and isolated by a dichroic mirror (zt488/594rpc-uf5, Chroma Technology) and a long-pass emission filter (ET 645/75m, Chroma Technology) located in front of the detector. Fluorescence was then guided through a 100 μm pinhole to block the out-of-focus light. Photons were detected with a single photon counting detector (SPCM-AQR-16, Excelitas). A piezo stage (Mad City Labs) was used to control the movement of the sample. The output signal from the detector was sent to a time-correlated single photon counting board (SPC-830, Becker & Hickel). Data were analyzed with the built-in functions of IgorPro 6.37 (Wavemetrics, Lake Oswego, OR). All of the FCS measurements were conducted at 22 °C.
Data Analysis

The time-dependent fluorescence intensity, \( F(t) \), is analyzed in terms of its autocorrelation function \( G(\tau) \), which compares the fluorescence intensity at time \( t \) with the intensity after a time delay \( (\tau) \), eq 1:

\[
G(\tau) = \frac{\langle \delta F(t) \delta F(t+\tau) \rangle}{\langle F(t)^2 \rangle}
\]  

where \( \delta F = F(t) - \langle F(t) \rangle \); \( \langle F(t) \rangle \) is the time-average of the fluorescence intensity. One advantage of using FCS is that different models can be used to extract diffusion parameters. For example, for measurements in solution, the probe volume is defined by a 3D Gaussian profile, in which a 3D Brownian diffusion model (eq 2) can be used. The calibration measurement using a solution of fluorescent polymeric beads and glycerol used the 3D diffusion model.

\[
G(\tau) = \frac{1}{N} \left[ 1 + \left( \frac{\tau}{\tau_d} \right)^\alpha \right]^{-1} \left[ 1 + \frac{1}{\kappa^2} \left( \frac{\tau}{\tau_d} \right)^\alpha \right]^{-1/2}
\]  

For a 2D diffusion model, eq 3 can be applied. All measurements of IL films used the 2D model.

\[
G(\tau) = \frac{1}{N} \left[ 1 + \left( \frac{\tau}{\tau_d} \right)^\alpha \right]^{-1}
\]  

\( N \) is the average number of fluorescence molecules in the confocal volume, \( \tau_d \) is the characteristic diffusion time, \( \kappa \) describes the confocal volume and is given by \( \kappa = \omega_z / \omega_{xy} \), where \( \omega_z \) and \( \omega_{xy} \) correspond to the 1/e\(^2\) values in the axial and longitudinal directions, respectively. For Brownian diffusion, the exponent \( (\alpha) \) is unity. For anomalous diffusion, however, the mean-squared displacement of the fluorophore is no longer proportional to time, \( t \), but rather to \( t^\alpha \), where \( \alpha \) may assume values less than 1 or greater than 1, for sub-diffusion and superdiffusion, respectively.
The structure parameter, $\kappa$, of the excitation volume was calibrated using 20 nm fluorescent beads (580/605 fluorescent FluoSpheres 0.02 μm, Invitrogen/Molecular Probes, Eugene, OR) in water with a diffusion coefficient of 2.17 μm$^2$/s. Experimentally measured correlation data on the diffusion of FluoroSpheres were fit with the autocorrelation function for a 3D diffusion model (eq 2) rendering a $\omega_{xy}$ value of 450 nm, $\kappa = 3$, and an effective focal volume of 1.5 fL. The confocal volume in the longitudinal direction, $\omega_{xy}$, was corroborated by collecting confocal images of 100 nm fluorescent beads (580/605 fluorescent FluoSpheres 0.1 μm, Invitrogen/Molecular Probes, Eugene, OR) in different media with varying refractive indexes ($n = 1.5150$ (immersion oil), $1.49551$ ([P$_{4448}$][Cl]), $1.49245$ ([P$_{6668}$][Cl]), $1.48326$ ([P$_{66614}$][Cl]), and $1.45164$ ([P$_{66614}$][NTf$_2$]). The lateral cross-sections of the beads were fit using a Gaussian function and rendered an average $\omega_{xy}$ value of 450 ± 30 nm, which is in good agreement with the value obtained from the aforementioned instrument calibration. This shows that the refractive index difference among the IL samples as well as the calibration medium did not affect the accurate measurement of the diffusion parameters.

Correlation data for ILs films were fit using eq 2, and the obtained $\tau_d$ was used to calculate the diffusion coefficient ($D$) using eq 4. When $\alpha < 1$, the diffusion coefficient is time dependent. All reported diffusion coefficients correspond to a time of 1 s.

$$\tau_d = \frac{w_{xy}^2}{4D}$$  \hspace{1cm} (4)

**Results and Discussion**

Using FCS, the effects of alkyl chain length and anion on the diffusion of the cationic fluorophore (Atto 590) and amphiphilic fluorophore (DiD) in alkylphosphonium ILs were measured. Specifically, the $\alpha$-exponent in eqs 2 and 3 was used to quantify the degree of deviation from Brownian diffusion, which is measured in homogeneous media. Figure 1 depicts
the normalized fluorescence autocorrelation curves of the fluorophores in the [P_{4448}][Cl], [P_{6668}][Cl], and [P_{66614}][Cl] films. The quality of the fits was assessed by the distribution of the residuals. Summarized in Table 2 are the average values of the diffusion parameters obtained from the best fit of the autocorrelation curves for glycerol and tetraalkylphosphonium chloride ILs.

**Figure 1.** Normalized autocorrelation curves for (left) Atto 590 and (right) DiD in (red) [P_{4448}][Cl], (green) [P_{6668}][Cl], and (blue) [P_{66614}][Cl] tetraalkylphosphonium ILs. The corresponding fits to equation 3 are shown as black lines. The lower panels show the residuals between the experimental data and fit using the same color scheme.
Table 2. Translational diffusion parameters measured by FCS for the indicated tetraalkylphosphonium ILs and for the homogenous glycerol sample.

<table>
<thead>
<tr>
<th>Ionic Liquid</th>
<th>Atto 590 Diffusion Coefficient ($\mu$m$^2$/s)</th>
<th>Atto 590 $\alpha$</th>
<th>DiD Diffusion Coefficient ($\mu$m$^2$/s)</th>
<th>DiD $\alpha$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glycerol</td>
<td>2.9 ± 0.2</td>
<td>0.96 ± 0.03</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>[P$_{4448}$][Cl]</td>
<td>0.39 ± 0.05</td>
<td>0.72 ± 0.05</td>
<td>0.146 ± 0.009</td>
<td>0.50 ± 0.01</td>
</tr>
<tr>
<td>[P$_{6668}$][Cl]</td>
<td>0.73 ± 0.05</td>
<td>0.73 ± 0.04</td>
<td>0.90 ± 0.03</td>
<td>0.62 ± 0.01</td>
</tr>
<tr>
<td>[P$_{66614}$][Cl]</td>
<td>1.2 ± 0.1</td>
<td>0.97 ± 0.04</td>
<td>0.98 ± 0.09</td>
<td>0.65 ± 0.04</td>
</tr>
<tr>
<td>[P$_{66614}$][NTf$_2$]</td>
<td>3.2 ± 0.3</td>
<td>0.87 ± 0.04</td>
<td>5.2 ± 0.6</td>
<td>0.65 ± 0.04</td>
</tr>
</tbody>
</table>

Figure 2 plots the diffusion coefficients of Atto 590 and DiD as a function of the IL’s viscosity. Generally, the fluorophore’s diffusion coefficient decreases due to the increase in viscosity. However, the diffusion of fluorophores within the ILs of varying viscosity does not follow the Stokes–Einstein equation (dotted line within Figure 2). This deviation is expected because the Stokes law was generated for macroscopic particles in motion in a continuous medium. The diffusion coefficients of Atto 590 are smaller than the diffusion coefficients of DiD, which is unexpected, given the smaller size of Atto 590. As reported by Kaintzet et al.$^{40}$ “the dependence on relative size alone is insufficient to provide useful quantitative predictions of diffusion coefficients in ILs”. In addition, the differences in the diffusion coefficients between Atto 590 and DiD are not consistent across all the ILs. This would be expected for a homogenous medium when the only factors influencing diffusion are the size of the fluorophore and viscosity of the IL. When combined, these observations suggest that the ILs have nanoscale heterogeneities and that the two fluorophores are diffusing in different environments within the ILs. These experimental results highlight the complexity of the IL system, in part due to the presence of different local environments as the fluorophores diffuse in the focal volume. The presence of nanoscale heterogeneities is consistent with several computational studies of tetraalkylphosphonium ILs, including [P$_{4448}$][Cl], showing polar entities consisting of chloride...
anions and central P(CH$_2$)$_4$ groups and nonpolar domains composed of the remaining alkyl chain.\textsuperscript{29} The solvation properties of ILs are quite complex, and it is not possible to state the chemical nature of the domains in which the fluorophores are diffusing within these ILs with the available data. Additionally, it is not possible to state that the fluorophore has access of all domain within the ILs framework.

![Graph](image)

**Figure 2.** Atto 590 and DiD diffusion coefficients as a function of IL viscosity (red symbols, left axis). The error bars are standard deviation from 20 replicate measurements. The dotted line and black symbols (right axis) correspond to the expected diffusion coefficient values calculated based on the Stokes-Einstein equation and assuming a fluorophore diameter of 1 nm.

As expected for a homogenous sample that does not contain structural heterogeneities, the diffusion of Atto 590 in glycerol is Brownian, with $\alpha \sim 1$ (Supporting Information, Figure S5, Table 1). This demonstrates the sensitivity of the FCS measurements to distinguish homogeneous environments in high viscosity media. On the other hand, $\alpha$ for [P$_{4448}$][Cl] and [P$_{6668}$][Cl] are less than 1 for both fluorophores, and diffusion is not Brownian. In general, the deviation of the $\alpha$ value from unity suggests that the mean-squared displacement of the fluorophores follows an anomalous subdiffusive behavior (with $\alpha < 1$) because of the structural heterogeneity present in these complex media. The data in Table 2 show that the $\alpha$ value is
lower for DiD than Atto 590 within the same type of IL, indicating that DiD experiences less-Brownian diffusion. For DiD, \( \alpha \) reaches a constant value of 0.65 as the alkyl chain length in the IL increases. Given the amphiphilic nature of the DiD molecules, it might interact with both polar and nonpolar entities of the ILs. The characteristic size of nanoscopic structural heterogeneities in pyrrolidinium, pyridinium, and imidazolium ILs extend over a few nanometers and linearly scale with the aliphatic chain length.\(^{16, 41, 42} \) Given the presence of four aliphatic alkyl chains in the alkylphosphonium cation, the nonpolar nanostructures are expected to be larger in comparison with the nitrogen-based ILs (i.e. pyrrolidinium, pyridinium and imidazolium), as demonstrated by MD simulations.\(^{30} \)

Surprisingly, \( \alpha \) for Atto 590 in \([P_{66614}]\)[Cl] is similar to that in glycerol and is approximately 1. This indicates that the fluorophore experiences Brownian diffusion in this IL. Several computational studies, however, have demonstrated the presence of polar and nonpolar domains in \([P_{66614}]\)[Cl]. The volume fraction of the nonpolar domains is significantly larger in comparison with the volume fraction occupied by the polar parts of the \([P_{66614}]\)[Cl] IL, as Wang et al.\(^{30} \) demonstrated by MD simulations. Wang’s studies revealed that as the alkyl chain length in the cation increases, the nonpolar domain expanded, whereas the polar network lost part of its connectivity. This resulted in a segregated distribution of polar domains within the nonpolar structure. The discontinuity of the polar network might prohibit Atto 590 molecules from moving among different domains present in \([P_{66614}]\)[Cl] while producing Brownian diffusion within the domains it can access.

The effects of the same cation, \([P_{66614}]^+\), coupled with a different anion on the diffusion properties of Atto 590 and DiD was analyzed (Figure 3). When the anion is changed from \([\text{Cl}^-]\) to \([\text{NTf}_2^-]\), the diffusion coefficient increases with the reduction in the viscosity of the IL. On the
other hand, $\alpha < 1$ when the cation is kept constant and the anion size is increased. Hettige et al.\textsuperscript{29} observed by MD simulations that there is a more accentuated positive-negative alternation of charge, as well as a more polar-nonpolar alternation, in the $[P_{66614}]\text{NTf}_2$ in comparison with the $[P_{66614}]\text{Cl}$. Furthermore, Gardas et al.\textsuperscript{43} estimated the cation and anion volume for a series of ILs, where the predicted volume for $[P_{66614}^+], [\text{Cl}^-], \text{and} [\text{NTf}_2^-]$ was 947, 47, and 248 Å, respectively. Thus, the relative volume fraction of the polar domain is larger for $[P_{66614}]\text{NTf}_2$, and the alpha value smaller, compared to $[P_{66614}]\text{Cl}$. Comparing all ILs in this study, this indicates that the alpha value of Atto 590 is smaller for the ILs with the largest relative volume fraction of the polar domains.

\textbf{Figure 3.} Experimental normalized autocorrelation curves of Atto 590 (left) and DiD (right) in (blue) $[P_{66614}]\text{Cl}$ and (pink) $[P_{66614}]\text{NTf}_2$. The corresponding fits to equation 3 are shown as black lines. The lower panels show the residuals between the experimental data and fit using the same color scheme.
Conclusions

The translational diffusion dynamics of Atto 590 and DiD in alkylphosphonium ILs films was studied using FCS. The results are in agreement with several experimental and MD simulations for a variety of phosphonium-based and nitrogen-based ILs that have demonstrated that the IL structure defines solute dynamics.\textsuperscript{11,16} Anomalous diffusion of the fluorophores was observed in most of the IL systems under study, which can be attributed to the presence of structural heterogeneities that result from the phase segregation of the alkyl carbon chains. These experimental findings are in agreement with previous MD simulations for this class of ILs. Interestingly, however, it was observed that when the size of the nonpolar domains became significantly larger in comparison to the polar entities (\textit{i.e.,} [P\(_{66614}\)][Cl]), FCS experiments using a hydrophilic Atto 590 fluorophore may not give insight about the presence of these heterogeneities. These results provide new experimental understanding into the structural complexity of tetraalkylphosphonium ILs and will be useful in as a stepping stone for stimulating theoretical and computational investigations of the factors that affect this structural heterogeneity.

Acknowledgements

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References


Supporting Information

**Synthesis of [P₆₆₆₁₄][NTf₂]**

[\text{P₆₆₆₁₄}[\text{NTf₂}]] was synthesized by dissolving 1 g (1.925 mmol) of [\text{P₆₆₆₁₄}[\text{Cl}]] in 20 mL of acetone, then 3 equivalents 1.65 g (5.7762 mmol) of LiNTf₂ were dissolved in 10 mL of water and added dropwise to the [\text{P₆₆₆₁₄}[\text{Cl}]] solution. The reaction mixture was allowed to stir for 5 hours at room temperature. After the synthesis, the solvent was evaporated via rotoevaporation, and the product was dissolved in 30 mL of diethyl ether and washed with 5-mL aliquots of deionized water until the aqueous fraction yielded no precipitates using the silver nitrate test. The purified product was dried overnight in a vacuum oven.

**Glass Surface Modification Procedure**

Spin-coating tetraalkylphosphonium films directly onto a glass slide resulted in isolated patches of the ILs over time. Homogeneous and stable films were formed on an amino functionalized glass substrate. The amino silanization procedure was adapted from the literature.¹ 18×18-mm glass slides (no. 1.5) were cleaned by immersing them in isopropanol and sonicating for 30 minutes. Then, the slides were dried under a nitrogen stream. The surface activation was performed by immersing the cleaned glass slides in a freshly prepared piranha solution (a mixture of 30:70% by volume hydrogen peroxide (H₂O₂) and concentrated sulfuric
acid (H$_2$SO$_4$) and sonicated for 30 min (Caution: Piranha solution is extremely corrosive, reactive, and potentially explosive. Never mix piranha waste with organic solvents. Check precautions before using it). They were rinsed thoroughly with DI water, and sonicated for 5 minutes. This rinsing and sonicating procedure was repeated three times in order to ensure the complete removal of the piranha solution. The activated glass slides were then dried with nitrogen gas. The amino-silanization was realized by immersing the activated glass slides in a 3% (v/v) 3-Aminopropyltriethoxysilane (APTES, NH$_2$(CH$_2$)$_3$Si(OC$_2$H$_5$)$_3$) solution in acetone for 12 h. The amino-modified substrates were sonicated twice in APTES-free solvents (acetone, ethanol, DI water) for 5 min in order to remove unreacted APTES. This procedure was repeated twice for each solvent. Then, the amino-modified glass slides were dried using a stream of nitrogen gas and stored at 100 °C for 24 h or until use.

**Figure S1.** Absorption spectra of neat tetraalkylphosphonium ILs
Figure S2. ATR-FTIR spectra of water and dried tetraalkylphosphonium ILs. Signal from the antisymmetric ($\nu_1$) and symmetric ($\nu_3$) stretching modes of water in the region of 3000 – 3800 cm$^{-1}$ is not detected above the noise in this spectral region for the dried ILs (water concentration less than 1000 ppm).

Figure S3. (A) Optical microscope image of a $\sim$1 $\mu$m [P$_{66614}$][Cl] film on an amino-coated glass slide. (B) Raman spectrum at the center of the optical image. The strongest Raman signals are located in the C-H stretching region (2760–3050 cm$^{-1}$), assigned to the CH stretching vibrations of the alkyl chain. Additional bands at 1300 and 1440 cm$^{-1}$ are attributed to C-H bending and CH$_3$ bending.$^3$
Figure S4. Ellipsometry spectra (in Ψ and Δ) for a ~1 μm [P_{6614}][Cl] film. The solid lines are fits to the experimental data. The incident angle was 70°.

Figure S5. Normalized autocorrelation curve for Atto 590 in glycerol. The corresponding fit to equation 2 is shown as a solid black line. The lower panel presents the residuals between the experimental data and fit.
References


CHAPTER 3. NANOSCALE SPATIAL HETEROGENEITIES IN CARBOXYLIC ACID-BASED DEEP EUTECTIC SOLVENT (DES) FILMS

Deyny L. Mendivelso-Pérez, Muhammad Qamar Farooq, Jared L. Anderson, Jacob W. Petrich, and Emily A. Smith*

Department of Chemistry, Iowa State University, Ames, Iowa 50011, United States.
The Ames Laboratory, U.S. Department of Energy

Corresponding Author: *Email: esmith1@iastate.edu Tel: (515)-294-1424 ORCID ID 0000-0001-7438-7808

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Abstract

Deep eutectic solvents (DESs) are attractive materials that have some advantages over other solvents due to their tunable polarity, good thermal stability, easy synthesis, and generally lower price. However, there is a need to understand the nanoscale heterogeneity and dynamics in these novel solvents in order to fully exploit DESs in a range of applications. In this work, the translational diffusion of hydrophilic (Atto 590) and hydrophobic (DiI) fluorophores is investigated in a series of DESs based on tetraalkylammonium halide salts with carboxylic acids by means of fluorescence correlation spectroscopy (FCS). These studies show the presence of nanoscale heterogeneities in all DESs as the fluorophores follow an anomalous diffusion behavior, in other words a time-dependent diffusion coefficient was measured. No significant variation in heterogeneity could be detected by FCS with an increase in the alkyl chain length of the hydrogen bond donor (HBD). However, a significant enhancement in the diffusion coefficient of the fluorescent molecules was observed as type of halogen is changed in the structure of the tetraalkylammonium halide salts.
Introduction

Smith et al.\(^1\) in 2003 adopted the term deep eutectic solvents (DESs) to describe a novel class of solvents obtained by mixing two or more compounds that exhibit a eutectic point much lower than the melting point of any of the individual components.\(^2\) Recently, Coutinho et al.\(^3\) proposed a new definition for DESs as eutectic mixtures for which the eutectic point temperature is lower than that of an ideal liquid mixture, and they stated that the solid-liquid phase diagram of the system should be known to identify the liquid phase region and the stoichiometry of the eutectic mixture. DESs have emerged as promising solvents for a variety of applications due to that their physicochemical properties can be tailored by selecting the proper combination of individual components. These characteristics make DESs desirable substitutes to replace conventional organic solvents as well as some ILs in many applications such as organic synthesis,\(^4\) catalysis,\(^5\) extraction,\(^6, 7\) other separation processes,\(^8\) nanotechnology,\(^9\) and electrochemistry.\(^10\)

The most reported DESs (type III DESs) are prepared by mixing a hydrogen-bond acceptor (HBA) (\textit{i.e.} quaternary ammonium salt) with a hydrogen-bond donor (HBD) at an appropriate molar ratio. Abbot et al.\(^2, 11\) postulated that the formation of DESs is generally attributed to charge delocalization, where hydrogen-bonding interactions between the HBD with the anion of the HBA causes an increase in its effective size, reducing the anion interaction with the cation. These strong hydrogen-bonding interactions between DESs’ constituents are responsible for the significant depression in the melting temperature of the mixture.\(^12\) Spectroscopy experiments mainly support the existence of hydrogen-bonding;\(^13-15\) Abbot et al.\(^2\), employing NMR spectroscopy, observed the presence of hydrogen bonding between urea and fluoride in a choline fluoride DES. This hydrogen-bonding network is not only essential for the DES formation but also strongly influences the local structure and dynamics within DESs.\(^16, 17\)
Computational chemical tools have been used in several studies to gain insight into the structure and dynamic aspects in DESs at the nanoscopic level.\textsuperscript{15,18-23} Sun et al.\textsuperscript{24} studied choline chloride (ChCl) and urea (U) using classical molecular dynamic (MD) simulations. It was found that the long-range ordered structure in the chlorine chloride crystal lattice is interrupted by the intercalation of urea molecules, resulting in an entropy increase and low melting point of the mixture. Garcia et al.\textsuperscript{25} and Hammond et al.\textsuperscript{26} revealed the presence of a hydrogen bonding network using density functional theory (DFT) and atomic simulations, respectively. They concluded that, although HBD-halogen hydrogen bonding has a prevailing role for DES formation, other types of hydrogen bonds (HBD-anion, HBD-cation, cation-anion, HBD-HBD) should be considered to obtain a more accurate picture of the intermolecular interactions in DESs. Additionally, the authors found that these interactions contribute to the formation of a cage-like structure in the DESs. Stefanovic et al.\textsuperscript{27} studied structure-property relationships for ChCl:urea (ChCl:U), ChCl:ethylenglycol (ChCl:EG), ChCl:glycerol (ChCl:Gly). Their simulations showed that HBD acidity, HBD structure/conformation and the extent of HBD self-interaction contribute to the nanostructure observed. Thus, the extensive hydrogen bond network in ChCl:U leads to substantially higher viscosity, compared to ChCl:EG and ChCl:Gly.

McDonald et al.\textsuperscript{28} using MD simulations showed that amphiphilic nanostructured DESs can be prepared by combining alkylammonium cations (alkyl: ethyl-, propyl-, and butyl) and glycerol at a 1:2 molar ratio. Their results revealed cation alkyl chain aggregation, which is better defined as the alkyl chain length increases. Cui et al.\textsuperscript{29}, using IR spectroscopy and MD simulations, demonstrated the presence of nanoscopic heterogeneities in amide-based DESs, where the heterogeneity in the studied DES increases with the asymmetry in the structure and
interactions of the HBA cations. Alizadeh et al.\textsuperscript{30} found strong microheterogeneity in long side-chain DES systems composed of choline chloride and some of its derivates mixed with ethylene glycol. Their MD simulations showed a clear distinction of polar and nonpolar domains, where polar groups form a completely connected domain for cations with a short side chain (less than six carbons), while the non-polar groups tend to be highly dispersed within the polar domain. Additionally, alkyl chains with more than eight carbons units result in an increase in the size of the nonpolar domains. This leads to increased heterogeneity in the systems with longer side chains. Computational studies have shown that DESs possess unique spatial heterogeneity on the molecular length scale and that the presence of this heterogeneities influences their physicochemical properties.\textsuperscript{15, 31, 32}

In addition to computational studies, fluorescence-based studies have demonstrated the presence of heterogeneity in a variety of DESs. To mention a few, Turner et al.\textsuperscript{33} observed a red-edge effect in a series of DESs based on ChCl with either urea, glycerol, ethylene glycol, or malonic acid, and using coumarin 153 as a fluorescent probe. These observations suggested spatial heterogeneity in the DESs. Using excitation-wavelength-dependent fluorescence, time-resolved fluorescence anisotropy, and fluorescence correlation spectroscopy (FCS) measurements Hossain et al.\textsuperscript{34, 35, 36} showed spatial and dynamic heterogeneity in ChCl:EG (1:2),\textsuperscript{34} and ChCl:alcohol-based,\textsuperscript{35} and tetraalkylammonium bromide-based DESs.\textsuperscript{36} Their studies revealed an anomalous diffusional dynamics for the fluorophores in the studied DESs, which they explained in terms of the presence of dynamic heterogeneity of the media.

Hydrophobic DESs have gained popularity due to their high extraction efficiency for non-polar organic and inorganic analytes.\textsuperscript{37, 38} Thus, they have been suggested as a substitute for conventional hydrophobic organic solvents such as ether and n-hexane \textsuperscript{39} or expensive
hydrophobic ILs. As it has been previously mentioned, the presence of heterogeneity in DESs can affect microscopic properties and in turn how they perform in various applications. For instance, the presence of heterogeneities in DESs at the nanoscale can affect the efficiency of a separation process. However, experimental studies that give insight in the dynamic properties and elucidate structural heterogeneities in DES systems are still in their early stages. Therefore, there is a need for gaining an understanding of the molecular structure in DES systems. To this end, we performed fluorescence correlation for studying the presence of heterogeneities in a series of hydrophobic DESs obtained by mixing tetraalkylammonium salts (HBA) with carboxylic acids (HBD). Atto 590 and DiI were used as fluorescent probes. The insights obtained from this study will be helpful for understanding the presence of structural heterogeneities in these novel series of DESs.

**Experimental Section**

**Materials**

Tetraethylammonium chloride ([N\textsubscript{2222}][Cl\textsuperscript{−}], >98%), tetrabutylammonium chloride ([N\textsubscript{4444}][Cl\textsuperscript{−}], >97%), tetrabutylammonium bromide ([N\textsubscript{4444}][Br\textsuperscript{−}], >99%), octanoic acid (Oct. A., >99%), decanoic acid (Dec. A., >99%), and dodecanoic acid (Dod. A., >99%), Atto 590, and 1,1'-dioctadecyl-3,3,3',3'-tetramethylindo carbocyanine perchlorate (DiI) were purchased from MilliporeSigma (St. Louis, MO, USA).

**Preparation and Characterization of DESs**

Eight DESs were prepared using quaternary ammonium halide salts as a HBA and carboxylic acids as HBD species: [N\textsubscript{2222}][Cl\textsuperscript{−}]:Dec. A. [1:2 mole ratio], [N\textsubscript{4444}][Cl\textsuperscript{−}]:Oct. A. [1:1 and 1:2 mole ratio], [N\textsubscript{4444}][Cl\textsuperscript{−}]:Dec. A. [1:2 mole ratio], [N\textsubscript{4444}][Cl\textsuperscript{−}]:Doc. A. [1:1 and 1:2 mole ratio], [N\textsubscript{4444}][Br\textsuperscript{−}]:Oct. A. [1:2 mole ratio], and [N\textsubscript{4444}][Br\textsuperscript{−}]:Dec. A. [1:2 mole ratio]. The two components were weighed to prepare mixtures of known molar ratio and transferred to a 20
mL scintillation vial equipped with a magnetic stirring bar. The mixture was stirred for six hours to provide an ample time for the formation of a eutectic mixture. After six hours, a uniform homogeneous transparent liquid was obtained. It was found that heating the samples during their preparation often results in the formation of a yellowish color, which significantly contributes towards the fluorescence background; therefore, the preparation of DESs was done at room temperature. Then, DESs were rigorously dried under vacuum, and the dried samples were stored in a desiccator until used. Further DESs handling and sample preparation were performed under a humidity chamber (relative humidity < 5%) to avoid water intake from the environment. Water content and viscosity were determined in the dried DESs and the measured values are reported in Table 1. The prepared DESs were characterized using Fourier transform infrared spectroscopy (FTIR) (Supporting Information, Figure S1) and $^1$H NMR (Supporting Information, Figure S2) and. The NMR and FTIR spectra indicate that not side reactions took place during the DESs preparation. The DESs’ chemical structures are presented in Scheme 1.

**Scheme 1.** Structures of quaternary ammonium halide salts (HBAs) and carboxylic acids (HBDs) used for the preparation of the DESs and fluorescent probes investigated in this work: (a)
tetraethylammonium chloride ([N$_{2222}^+$][Cl$^-$]), (b) tetrabutylammonium chloride ([N$_{4444}^+$][Cl$^-$]), (c) tetrabutylammonium bromide ([N$_{4444}^+$][Br$^-$]), (d) octanoic acid (Oct. A.), (e) decanoic acid (Dec. A.), (f) dodecanoic acid (Dod. A.). Fluorophores: (g) Atto 590, and (h) 1,1'-dioctadecyl-3,3',3'-tetramethylindo carbocyanine perchlorate (DiI).

Table 1. Viscosity and water content for the dried deep eutectic solvent samples.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Water content (% w/w) $^a$</th>
<th>Viscosity $^a$ (cP)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glycerol</td>
<td>-</td>
<td>1400 ± 9</td>
</tr>
<tr>
<td>[N$_{2222}^+$][Cl$^-$]: Dec. A. (1:2)</td>
<td>0.14 ± 0.01</td>
<td>297 ± 1</td>
</tr>
<tr>
<td>[N$_{4444}^+$][Cl$^-$]: Oct. A. (1:1)</td>
<td>0.09 ± 0.02</td>
<td>1536 ± 2</td>
</tr>
<tr>
<td>[N$_{4444}^+$][Cl$^-$]: Oct. A. (1:2)</td>
<td>0.040 ± 0.001</td>
<td>465 ± 1</td>
</tr>
<tr>
<td>[N$_{4444}^+$][Cl$^-$]: Dec. A. (1:2)</td>
<td>0.033 ± 0.003</td>
<td>475 ± 1</td>
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<tr>
<td>[N$_{4444}^+$][Cl$^-$]: Dod. A. (1:1)</td>
<td>0.09 ± 0.01</td>
<td>1945 ± 3</td>
</tr>
<tr>
<td>[N$_{4444}^+$][Cl$^-$]: Dod. A. (1:2)</td>
<td>0.035 ± 0.002</td>
<td>665 ± 2</td>
</tr>
<tr>
<td>[N$_{4444}^+$][Br$^-$]: Oct. A. (1:2)</td>
<td>0.021 ± 0.005</td>
<td>457 ± 1</td>
</tr>
<tr>
<td>[N$_{4444}^+$][Br$^-$]: Dec. A. (1:2)</td>
<td>0.037 ± 0.005</td>
<td>554 ± 2</td>
</tr>
</tbody>
</table>

$^a$ Average of 3 measurements

**DESs Film Preparation**

An appropriate amount of the stock fluorophore (Atto 590 and DiI prepared in methanol) solution was added to a 1.5 mL Eppendorf conical tube and dried under vacuum until complete solvent evaporation. The fluorophore concentration in the final prepared samples was maintained at 10 nM. This provided enough fluorescence signal while having few molecules (<10 molecules) in the detection volume. Then, 200 µL of a 100 mg/mL solution of dried DESs in dichloromethane was added. To prepare the film of the DESs containing fluorophore, 200 µL of solution was deposited on a 18-mm$^2$ amino-modified glass cover slip (No. 1.5, Corning Inc., Corning, NY) and then spun at 8500 rpm for 240 s using a KW-4A spin coater (Chemat Technology, Northridge, CA). The glass surface modification procedure was described by Mendivelso-Perez et. al.$^{41}$ The resulting homogeneous film was transferred to a home-made sample holder to avoid the absorption of water from the environment during the FCS
measurements. DESs film thickness was determined by means of spectroscopic ellipsometry (SE), where the film preparation conditions were selected to render a final film thickness of approximately 1 µm (Supporting Information, Figure S3, Table S1). Additionally, an optical image and Raman spectra of the film were obtained to confirm the formation of a homogeneous film on the glass substrate (Supporting Information, Figure S4).

**Instrumentation**

Water content measurements were performed using a Karl Fischer Coulometric Titrator (Mettler Toledo, Columbus, OH), where Hydranal-Imidazole (Honeywell) was used as a buffer. The viscosities of the dried DESs were measured at 20 °C by the cone-plate method using a DVI Brookfield digital viscometer with a CPE-51 spindle at 20 rpm, which was equipped with a circulating bath with standard digital temperature controller. \(^1\)H NMR spectra were collected on a Bruker 600 operating at 600 MHz, all samples were dissolved in CDCl\(_3\). FTIR spectra were recorded on an Agilent Cary 630 FTIR spectrometer at a resolution of 4.0 cm\(^{-1}\) using 128 interferometer scans. SE measurements (J.A. Woollam \(\alpha\)-SE, J.A. Woollam Co. Inc., Lincoln, NE, USA) were collected at the wavelength range of 380-900 nm at 70°. The DESs’ film thickness was tracked as a function of time by acquiring \(\Psi(\psi)\) and Delta (\(\Delta\)), values at different time points. Data analysis was performed using CompleteEase software, and the thickness of the film was approximated using the Cauchy model (Supporting Information, Figure S3). Thickness measurements were performed at three different locations on the sample and an average thickness and standard deviation of the DESs films were computed. The Raman spectra of the DES films were collected using an XploRa Plus confocal Raman upright microscope equipped with a Synapse EMCCD camera (Horiba Scientific, JY, France). A 785-nm laser excitation source (5 mW at the sample) and a 100× air objective (0.9 numerical aperture, LMPlan N,
Olympus) were employed. The spectra were collected from 900 to 3300 cm\(^{-1}\) with a 1200 grooves-mm\(^{-1}\) grating, and the reported spectra were averaged over three replicates, each with 30s acquisition time and 2 accumulations.

The FCS measurements were performed in a home-build FCS instrument which consists of an inverted confocal microscope (Nikon, Ti) equipped with a 532-nm pulsed picosecond (fwhm=600 ps, repetition rate 20 MHz, LDHPicoQuant, Berlin, Germany) and 594-nm pulsed picosecond (fwhm=500 ps, repetition rate 20 MHz, BDS-SMY, Becker & Hickel, Berlin, Germany) diode laser as the excitation beams. Briefly, the laser output (0.5 mW 532-nm laser and 0.7 mW 594-nm laser) was coupled to a polarization-maintaining single-mode optical fiber. The slight overfilling of the back aperture of the 100× oil-immersion microscope objective (Apo TIRF, NA. 1.49, Nikon, correction collar 0.13-0.20) was achieved by expanding the laser beam. An appropriate density filter was used to reduce the laser power at the sample and to avoid photobleaching. Fluorescence generated by the focused laser beam was collected by the same objective and isolated by a dichroic mirror (LDP02=532RU-25-Semrock and zt488/594rpc-uf5-Chroma Technology) and a long-pass emission filter (D605/55m and ET 645/75m, Chroma Technology) located in front of the detector. A 100 µm pinhole was used to block the out-of-focus light. Photons were detected by a single photon counting module (SPCM) (APD; SPCM-AQR-16, Excelitas). Control movement of the sample was performed using a piezo stage (Mad City Lab). The output signal from the SPCM was sent to a time-correlated single-photon counting board (SPC-830, Becker&Hickel). Autocorrelation data were analyzed with the built-in functions of IgorPro 6.37 (Wavemetrics, Lake Oswego, OR). All the FCS measurements were performed at room temperature (22 °C).
FCS Data Analysis

Assuming the excitation intensity profile follows a 3D Gaussian distribution and that the dynamics of the fluorophore solely occur from its diffusion through the detection volume, the fit of the fluorescence correlation function, $G(\tau)$, will be obtained by using the normalized autocorrelation function described in equation 1:

$$G(\tau) = \frac{1}{N} \left[ 1 + \left( \frac{\tau}{\tau_d} \right)^{\alpha} \right]^{-1} \left[ 1 + \frac{1}{\kappa^2 \left( \frac{\tau}{\tau_d} \right)^{\alpha}} \right]^{-1/2}$$  \hspace{1cm} (1)

For a 2D diffusion model, equation 2 can be applied.

$$G(\tau) = \frac{1}{N} \left[ 1 + \left( \frac{\tau}{\tau_d} \right)^{\alpha} \right]^{-1}$$  \hspace{1cm} (2)

In equation 1 and 2, N is the average number of molecules in the detection volume, $\tau_d$ is the characteristic diffusion time, $\kappa$ describes the detection volume and is given by $\kappa = \omega_z / \omega_{xy}$, where $\omega_{xy}$ and $\omega_z$ correspond to the 1/e2 values in the axial and longitudinal directions, respectively. These terms describe the spatial properties of the detection volume and they are determined experimentally by calibration measurements. This calibration can be performed using a solution of polymer beads of known diffusion coefficient, and the value of $\omega_{xy}$ and $\omega_z$ are determined from the best-fitting curve. For Brownian diffusion, the mean-squared displacement (MSD), $\langle r^2 \rangle$, of the fluorescent molecules follows the classical linear law $\langle r^2 \rangle \propto t$. For anomalous diffusion, the MSD of the molecules follows a power law in the time variable: $\langle r^2 \rangle \propto t^\alpha$ with $0 < \alpha < 1$.

The detection volume for the 532-nm and 594-nm lasers was calibrated using 40-nm (540/560 fluorescent FluoSpheres 0.02 μm, Invitrogen/Molecular Probes, Eugene, OR) and 20-nm (580/605 fluorescent FluoSpheres 0.02 μm, Invitrogen/Molecular Probes, Eugene, OR)
fluorescent beads diffusing in water, respectively. The diffusion coefficient for the 20-nm and 40-nm fluorescent beads were 2.17 and 1.11 μm²/s, respectively, as calculated by the Stokes-Einstein equation. The experimentally measured correlation data on the diffusion of FluoroSpheres were fit with the autocorrelation function for a 3D diffusion model (Equation 1) rendering a $\omega_{xy} = 400$ nm and $\kappa = 3.5$ for the 532-nm excitation laser, and $\omega_{xy} = 450$ nm and $\kappa = 3$ for the 594-nm excitation laser. The effective focal volume was determined to be 0.8 fL and 1.2 fL for the 532-nm and 594-nm excitation lasers, respectively. Correlation data for all DES films were fit using the 2D model (equation 2), and the obtained $\tau_d$ was used to calculate the translational diffusion coefficient (D) using equation 3.

$\tau_d = \frac{w_{xy}^2}{4D}$

Results and Discussion

To further understand the nature of the DESs formed from quaternary ammonium halide salts (HBA) with carboxylic acid (HBD), we have studied the translational diffusion of Atto 590 (hydrophilic) and DiI (hydrophobic) at a 10 nM concentration using FCS. To the best of our knowledge, this is the first report of the translational diffusion in these DES systems. Figure 1 shows the normalized fluorescence autocorrelation curves of the fluorophores in [N$_{2222}^+$][Cl$^-$]: Dec. A. (1:2), [N$_{4444}^+$][Cl$^-$]: Oct. A. (1:1), [N$_{4444}^+$][Cl$^-$]: Oct. A. (1:2), [N$_{4444}^+$][Cl$^-$]: Dec. A. (1:2), [N$_{4444}^+$][Cl$^-$]: Dod. A. (1:1), and [N$_{4444}^+$][Cl$^-$]: Dod. A. (1:2) films. The autocorrelation data was fit using the 2D model (equation 2) with anomalous diffusion, this approach rendered the best fit base on the residual values and the translational diffusion parameter are reported in Table 2.
Table 2. Translational diffusion parameters measured by FCS for the studied DES systems and for the homogenous glycerol sample.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Atto 590</th>
<th></th>
<th>DiI</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Diffusion</td>
<td>α</td>
<td>Diffusion</td>
<td>α</td>
</tr>
<tr>
<td></td>
<td>Coefficient</td>
<td></td>
<td>Coefficient</td>
<td></td>
</tr>
<tr>
<td></td>
<td>(μm²/s)</td>
<td></td>
<td>(μm²/s)</td>
<td></td>
</tr>
<tr>
<td>Glycerol</td>
<td>2.8 ± 0.3</td>
<td>0.95 ± 0.03</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>[N2222][Cl]:Dec. A. (1:2)</td>
<td>8.1 ± 0.4</td>
<td>0.83 ± 0.02</td>
<td>5.5 ± 0.4</td>
<td>0.80 ± 0.01</td>
</tr>
<tr>
<td>[N4444][Cl]:Oct. A. (1:1)</td>
<td>0.9 ± 0.1</td>
<td>0.78 ± 0.02</td>
<td>1.1 ± 0.2</td>
<td>0.72 ± 0.01</td>
</tr>
<tr>
<td>[N4444][Cl]:Oct. A. (1:2)</td>
<td>2.4 ± 0.3</td>
<td>0.79 ± 0.02</td>
<td>2.5 ± 0.1</td>
<td>0.76 ± 0.01</td>
</tr>
<tr>
<td>[N4444][Br]:Dec. A. (1:2)</td>
<td>3.2 ± 0.3</td>
<td>0.80 ± 0.06</td>
<td>3.3 ± 0.2</td>
<td>0.77 ± 0.02</td>
</tr>
<tr>
<td>[N4444][Cl]:Dod. A. (1:1)</td>
<td>0.77 ± 0.05</td>
<td>0.78 ± 0.02</td>
<td>1.1 ± 0.3</td>
<td>0.82 ± 0.03</td>
</tr>
<tr>
<td>[N4444][Br]:Dod. A. (1:2)</td>
<td>2.0 ± 0.2</td>
<td>0.80 ± 0.02</td>
<td>2.5 ± 0.2</td>
<td>0.77 ± 0.02</td>
</tr>
<tr>
<td>[N4444][Br]:Oct. A. (1:2)</td>
<td>3.0 ± 0.5</td>
<td>0.78 ± 0.03</td>
<td>1.87 ± 0.03</td>
<td>0.78 ± 0.04</td>
</tr>
<tr>
<td>[N4444][Cl]:Dod. A. (1:2)</td>
<td>3.5 ± 0.3</td>
<td>0.79 ± 0.04</td>
<td>2.75 ± 0.03</td>
<td>0.77 ± 0.02</td>
</tr>
</tbody>
</table>

*Average of 40-50 measurements*

Figure 1. Fluorescence correlation curves for the diffusion of Atto 590 (left) and DiI (right) in ([N2222][Cl]: Dec. A. (1:2), [N4444][Cl]: Oct. A. (1:1), [N4444][Cl]: Oct. A. (1:2), [N4444][Br]: Dec. A. (1:2), [N4444][Cl]: Dod. A. (1:1), [N4444][Br]: Dod. A. (1:2]). The solid lines represent fits to the data using an anomalous diffusion model (equation 2). The excitation wavelength is 532-nm for DiI and 594-nm for Atto 590. The lower panels show the residuals between the experimental data and fit using the same color scheme. The depicted
residual corresponds to $[\text{N}_{2222}^2\text{Cl}]^{-}$: Dec. Ac. (1:2), the residuals for the remaining data are shown in the supplemental information (Supporting Information, Figure S5).

All measured $\alpha$-values were <1, indicating that the mean-squared displacement (MSD) of the fluorescent molecules follows an anomalous diffusion instead of the Brownian diffusion model ($\alpha$ =1). Brownian diffusion is expected in a homogenous medium; and an anomalous diffusion suggests there are different environments, or heterogeneities, in the DES nanostructure. In order to test the sensitivity of the FCS technique to distinguish diffusion in homogeneous and heterogeneous media, the translational diffusional of the hydrophilic fluorophore (Atto 590) was measured in a homogenous glycerol solvent. Atto 590 does exhibit Brownian diffusion ($\alpha \sim 1$) in glycerol (Supporting Information, Figure S6). Hence, the anomalous diffusion can be attributed to the presence of different environments or heterogeneities in the studied DES systems. Similar anomalous diffusion behavior has been reported in DES using FCS.\(^{33-36, 41, 42}\) For instance, Hossain et al.\(^{34, 36}\) observed an anomalous behavior for coumarin 153 (C153) and 4-aminophthalimide (4-AP) in DESs prepared using tetraalkylammonium bromide-based (HBA) and glycerol (HBD). The anomalous translational diffusion of the fluorescent molecules was attributed to the presence of heterogeneity in the DES systems. However, the $\alpha$-values for 4-PD are not affected by the alkyl chain length of the cation.

Interestingly, in our data the $\alpha$-values obtained for Atto 590 and DiI do not exhibit a noticeable difference. This could suggest that both fluorescent probes experience similar local heterogeneity, and the alkyl chain length in the HBD does not affect the $\alpha$-values. Hossain et al.\(^{36}\) reported almost constant $\alpha$-values for 4-aminophthalimide (4-PD) in a series of tetraalkylammonium bromide-based DESs. They attributed this result to strong association of the fluorescent probes to the components of the DESs. However, when they used coumarin 153 as a
fluorophore, the deviation from the Brownian model is more prominent as the alkyl chain length increases in the cation in the HBA.

Diffusion coefficient values for both fluorophores decrease with an increase in viscosity of the system, which is expected. However, the diffusion coefficients are similar for both fluorophores when the DESs have an alkylammonium-based chloride salt as HBA, which is unexpected given the smaller size of Atto 590. This could suggest that the size of the fluorescence probe is not the only parameter governing the diffusion. In this regard, interactions between the fluorophore molecules with the constituents of the DES might play an important role in the diffusional behavior of Atto 590 and DiI in the studied DES systems. However, it is not possible to state the nature of these interactions from the FCS data.

Figure 2. Fluorescence correlation curves for the diffusion of Atto 590 (left) and DiI (right) in in (●) [N₄₄₄₄⁺][Br⁻]: Oct. A. (1:2), (●) [N₄₄₄₄⁺][Br⁻]: Dec. A. (1:2). The solid lines represent fits to the data using an anomalous diffusion model (eq. 2). The excitation wavelength is 532-nm for DiI and 594-nm for Atto 590. The lower panels show the residuals between the experimental data and fit using the same color scheme. DiI and 594-nm for Atto 590. The lower panels show the residuals between the experimental data and fit using the same color scheme.
The effect of a different halogen in the structure of the anion in the HBA on the diffusion properties of Atto 590 and DiI in \([\text{N}4\text{444}^+][\text{Br}^-]: \text{Oct. A.}\) and \([\text{N}4\text{444}^+][\text{Br}^-]: \text{Dec. A.}\) (1:2) were analyzed (Figure 2). When the anion is change from [Cl] to [Br], an interesting diffusion behavior of the fluorescent molecules was observed. In these systems, the diffusion coefficients for both fluorophores are significantly different for the two aforementioned DESs, which could indicate that the presence of bromide in the structure of the DESs, to some extent, enhances the dynamics of the fluorophores. Notably, the diffusion coefficient is higher for Atto 590 that for DiI for the same HBA: HBD combination. The presence of bromide in the structure weakens hydrogen-bonding in the components of the DESs, given its lower electronegativity in comparison with chloride, which in turn disrupt the hydrogen-bonding network. This might facilitate the diffusion of the hydrophilic fluorophore in comparison to the hydrophobic fluorophore. Additionally, for the same DES systems, it was observed that as the alkyl chain length in the HBD increases, the diffusion coefficient increases, following an opposite trend in viscosity. Thus, the same fluorescent molecules exhibit a higher translational diffusion in \([\text{N}4\text{444}^+][\text{Br}^-]: \text{Dec. A.}\) \((\eta = 554 \, \text{cP})\) than in \([\text{N}4\text{444}^+][\text{Br}^-]: \text{Oct. A.}\) \((\eta = 457 \, \text{cP})\). It has been reported that the presence of long alkyl chains in the HBD or HBA tends to perturb hydrogen-bonding formation in DESs due to the steric effect of the bulky carbon chains.\(^{16}\) This weakening effect in the hydrogen-bonding network could enhance the diffusion of the fluorescent probes given that the molecular structure in the DES is less rigid. Further experiments in which the diffusion behavior of the fluorophores in alkylammonium bromide-based DESs with different alkyl chain lengths must be pursued to confirm the observed trend.
Conclusions

In summary, we have studied the translational diffusion dynamics of hydrophilic (Atto 590) and hydrophobic (DiI) fluorophores in eight carboxylic-based DESs films to gain insight about the presence of heterogeneities in these novel materials. Anomalous diffusion behavior was observed in all studied DES system when using a hydrophilic and a hydrophobic fluorophore. This anomalous behavior is related with the presence of nanosegregation in the media. Given that studies for the identification of nanoscale heterogeneities in deep eutectic solvents are in the early stages, our work presented here will contribute towards the understanding of deep eutectic solvents at the nanoscale level.

Acknowledgements

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References


Figure S1. FTIR spectra of dried DESs and their individual components. Hydrogen bond acceptor (black), hydrogen bond donor (HBD), and DES (blue). (a) [N\textsubscript{2222}\textsuperscript{+}][Cl\textsuperscript{-}]: Dec. A. (1:2), (b) [N\textsubscript{4444}\textsuperscript{+}][Cl\textsuperscript{-}]: Oct. A. (1:1), and (c) [N\textsubscript{4444}\textsuperscript{+}][Cl\textsuperscript{-}]: Oct. A. (1:2). (d) [N\textsubscript{4444}\textsuperscript{+}][Cl\textsuperscript{-}]: Dod. A. (1:2), (e) [N\textsubscript{4444}\textsuperscript{+}][Cl\textsuperscript{-}]: Dod. A. (1:2), (f) [N\textsubscript{4444}\textsuperscript{+}][Br\textsuperscript{-}]: Oct. A. (1:2), and (g) [N\textsubscript{4444}\textsuperscript{+}][Br\textsuperscript{-}]: Dec. A. (1:2). Dotted line shows the shift to higher frequencies of the carbonyl group (C=O) located at 1700 cm\textsuperscript{-1} after incorporation of the individual components.
Figure S1 (continued)
Figure S2. $^1$H NMR spectra of dried DESs.
[N$_{4444}^{4+}$][Br$^-$]: Dec. A. (1:2)

[N$_{4444}^{4+}$][Br$^-$]: Oct. A. (1:2)

Chemical Shift (ppm)

Figure S2 (continued)
Figure S3. Ellipsometry spectra (in $\Psi$ and $\Delta$) for a 900 nm $[N_{4444}^+][Cl^-]$: Oct. A. (1:1) film. The solid lines are fits to the experimental data. The incident angle was 70°.

Table S1. Sample preparation conditions and measured thickness for DESs films.

<table>
<thead>
<tr>
<th>Sample</th>
<th>RPM$^a$</th>
<th>Time (s)$^b$</th>
<th>Film Thickness (nm)$^c$</th>
</tr>
</thead>
<tbody>
<tr>
<td>$[N_{2222}^+][Cl^-]$: Dec. A. (1:2)</td>
<td>5000</td>
<td>120</td>
<td>920 ± 40</td>
</tr>
<tr>
<td>$[N_{4444}^+][Cl^-]$: Oct. A. (1:1)</td>
<td>8500</td>
<td>240</td>
<td>1100 ± 10</td>
</tr>
<tr>
<td>$[N_{4444}^+][Cl^-]$: Oct. A. (1:2)</td>
<td>8500</td>
<td>240</td>
<td>970 ± 50</td>
</tr>
<tr>
<td>$[N_{4444}^+][Cl^-]$: Dec. A. (1:2)</td>
<td>8500</td>
<td>240</td>
<td>860 ± 10</td>
</tr>
<tr>
<td>$[N_{4444}^+][Cl^-]$: Dod. A. (1:1)</td>
<td>8500</td>
<td>240</td>
<td>990 ± 50</td>
</tr>
<tr>
<td>$[N_{4444}^+][Cl^-]$: Dod. A. (1:2)</td>
<td>8500</td>
<td>240</td>
<td>920 ± 30</td>
</tr>
<tr>
<td>$[N_{4444}^+][Br^-]$: Oct. A. (1:2)</td>
<td>8500</td>
<td>180</td>
<td>8790 ± 10</td>
</tr>
<tr>
<td>$[N_{4444}^+][Br^-]$: Dec. A. (1:2)</td>
<td>8500</td>
<td>180</td>
<td>890 ± 20</td>
</tr>
</tbody>
</table>

$^a$ Spin-coating speeds, rotation per minute; $^b$ Spin-coating duration; $^c$ Average of 9 of measurements (3 measurements for each film)
Figure S4. (a) Optical microscope image of a ∼1 μm [N₄₄₄₄][Cl]: Octa. A. (1:1) film on an amino-modified glass slide. (b) Raman spectrum at the center of the optical image. The characteristic band of carboxylic acid (C=O) is located at 1660 cm⁻¹. Additional bands at 2760–3050 cm⁻¹ correspond to the CH stretching vibrations of the alkyl chains.

Figure S5. Panels show the residuals from the best fit of the autocorrelation functions obtained from the FCS experiments for (a) Atto 590 and (b) DiI. The best fit was obtained by fitting the data to an anomalous diffusion model (Equation 2). (Red) [N₄₄₄₄⁺][Cl]: Oct. A. (1:1), (light blue) [N₄₄₄₄⁺][Cl]: Oct. A. (1:2), (Pink) [N₄₄₄₄⁺][Cl]: Dec. A. (1:2), (blue) [N₄₄₄₄⁺][Cl]: Dod. A. (1:1), (purple) N₄₄₄₄⁺][Cl]: Dod. A. (1:2).
**Figure S6.** Normalized autocorrelation curve for Atto 590 in glycerol. The corresponding fit to equation 2 is shown as a solid black line. The lower panel presents the residuals between the experimental data and fit.
CHAPTER 4. GENERAL CONCLUSIONS

The primary goal of the work presented in this dissertation was to study the translational diffusion of fluorescent probes as a tool to identify the presence of nanostructural organization in tetraalkylphosphonium-based ILs and carboxylic-based DESs films. To this end, fluorescence-based techniques such as fluorescence correlation spectroscopy (FCS) were employed. Nanosegregation in ILs and DESs is known to play a vital role in the unique chemical properties of these novel materials and more importantly determines their possible applications.

In Chapter 2, the translational diffusion dynamics of fluorophores in tetraalkylphosphonium ionic liquid films by fluorescence correlation spectroscopy was studied. It was observed that the translational diffusion of fluorescent molecules presents deviation from the Brownian diffusion behavior. The deviation from the free diffusion model is attributed to the presence of nanostructural heterogeneities in tetraalkylphosphonium ionic liquid films. These experimental results were in agreement with previous molecular dynamic simulations studies that predicted the presence of nanostructural heterogeneities/domains in tetraalkylphosphonium ionic liquids. The domain structures are believed to be formed of polar entities consisting of anion and central P(CH$_2$)$_4$ groups and nonpolar domains composed of the remaining alkyl chain. These findings were the first step in determining the implications that nanoscale heterogeneity has for the applications of this type of ionic liquids, which includes separation applications.

In Chapter 3, fluorescence correlation spectroscopy (FCS) was employed to gain insight into the presence of nanostructural heterogeneities in eight DESs composed of tetraalkylammonium halide salts and carboxylic acids. These type of DESs have received increase attention due to their high extraction efficiency for nonpolar organic and inorganic analytes. Despite of the potential applications of these types of DESs, nanosegregation in these
class of materials has not been reported experimentally. FCS results confirmed the presence of nanodomains in the studied DESs, as Atto 590 and DiI fluorophores follow an anomalous diffusion model, which is characteristic of inhomogeneous systems. The effect of the alkyl chain length of the hydrogen bond in the $\alpha$-values was not noticeable for the experimental conditions. In summary, nanostructural heterogeneity was observed for eight carboxylic-based DESs.

Future work should strive to understand the impact of water content and temperature and their influence on the structural heterogeneities in ILs and DESs. Notably, the addition of water to these materials can disrupt the nanostructural network and affects physical properties such as density, viscosity, ionic mobility, which are influenced even by small water concentrations. In the case of DESs, water perturbs the hydrogen bonding network by establishing hydrogen bonding with the individual components of the DES system. Thus, water can be added in a controlled way to modulate the physicochemical properties of the system, such as viscosity and polarity. Therefore, studying the translational diffusion properties of fluorescent molecules can give further understanding of the impact that water could have in DESs nanosegregation.
APPENDIX A. AEROSOL JET PRINTED GRAPHENE ELECTROCHEMICAL HISTAMINE SENSORS FOR FOOD SAFETY MONITORING

Kshama Parate1,†, Cícero C. Pola1,†, Sonal V. Rangnekar2,‡, Deyny L. Mendivelso-Perez3,4, Emily A. Smith3,4, Mark C. Hersam2,* Carmen L. Gomes1,*, Jonathan C. Claussen1,*

1 Department of Mechanical Engineering, Iowa State University, Ames, IA, 50011, USA
2 Department of Materials Science and Engineering, Northwestern University, Evanston, IL 60208, USA
3 Department of Chemistry, Iowa State University, Ames, IA, 50011, USA
4 The Ames Laboratory, U.S. Department of Energy, Ames, IA, 50011, USA

* Corresponding authors: jcclauss@iastate.edu, carmen@iastate.edu, m-hersam@northwestern.edu

† Contributed equally to this work

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Abstract

Carbon nanomaterials such as graphene exhibit unique material properties such as high electrical conductivity, surface area, and biocompatibility that have the potential to significantly improve the performance of electrochemical sensors. Since in-field electrochemical sensors are typically disposable, they require materials that are amenable to low-cost, high-throughput, and scalable manufacturing. Conventional graphene devices based on low-yield chemical vapor deposition techniques are too expensive for such applications, while low-cost alternatives such as screen and inkjet printing do not possess sufficient control over electrode geometry to achieve favorable electrochemical sensor performance. In this work, aerosol jet printing (AJP) is used to create high-resolution (~40 µm line width) interdigitated electrodes (IDEs) on flexible substrates, which are then converted into histamine sensors by covalently linking monoclonal antibodies to
oxygen moieties created on the graphene surface through a CO\textsubscript{2} thermal annealing process. The resulting electrochemical sensors exhibit a wide histamine sensing range of 6.25-200 ppm (56.25 \textmu M – 1.8 mM) and a low detection limit of 3.41 ppm (30.7 \textmu M) within actual tuna broth samples. These sensor metrics are significant since histamine levels over 50 ppm in fish induce adverse health effects including severe allergic reactions (e.g., Scombroid food poisoning).

Beyond the histamine case study presented here, the AJP and functionalization process can likely be generalized to a diverse range of sensing applications including environmental toxin detection, foodborne pathogen detection, wearable health monitoring, and health diagnostics.

**Keywords:** graphene, aerosol jet printing, immunosensor, interdigitated electrode, electrochemical impedance spectroscopy, food safety

**Introduction**

Graphene is being implemented in a wide range of devices including transistors, sensors, and energy storage devices because of its high mechanical strength, electrical conductivity, electroactivity, and thermal conductivity [1-3]. Graphene used in such devices is typically synthesized through chemical vapor deposition (CVD) [4], epitaxial growth [5], laser ablation methods [6], and thermal decomposition of silicon carbide [7], which are generally expensive, high-energy, low-yield processes performed on rigid substrates (e.g., silicon). Moreover, the graphene has to be either transferred to a device-compatible substrate through wet chemical transfer steps or physical stamping processes [8, 9], or the graphene needs to be patterned on a growth substrate into a device through atomic layer etching [10], focused ion beam patterning [11], or block copolymer lithography [12, 13]. Although these techniques are capable of manufacturing high-resolution devices (< 50 \textmu m line resolution), they are energy intensive and low yield, and often require sophisticated cleanroom processing [14-16].
Printing of graphene and graphene oxide flakes acquired through bulk, high-yield exfoliation of graphite represents a low-cost and scalable alternative to creating graphene-based devices [17, 18]. However, these printing techniques generally produce low-resolution graphene devices with feature sizes that typically range between a few hundreds of microns and a few millimeters [19-22]. To attain higher resolution (< 50 µm), additional laborious steps such as lithographic patterning [23], high-resolution stencil fabrication [2, 24], or sacrificial mask layers [2, 24, 25] are needed. In addition, these manufacturing techniques are subtractive rather than additive and hence are less amenable to scalability.

Aerosol jet printing (AJP) offers an alternative high-yield and high-resolution printing technique for device fabrication. This direct-write, additive printing method eliminates the requirement of several fabrication steps and is capable of generating high-resolution features without the need of auxiliary patterning [26, 27]. In AJP, a printable ink is atomized by a pneumatic or ultrasonic mechanism, and then focused onto a substrate using a coaxial air sheath. This printing mechanism implies that AJP is amenable to a wide range of inks with relaxed viscosity tolerance (1-1000 mPa·s) compared to other additive manufacturing methods [28]. Furthermore, AJP is compatible with a variety of flexible and rigid substrates such as conductors, semiconductors, and dielectrics, including nonplanar surfaces and chemically or thermally sensitive samples [29]. AJP has been used to fabricate transistors, electrodes, supercapacitors, fuel cells, and solar cells, and also for a few electrochemical biosensing applications such as sensing biomolecules including glucose, proteins such as interferon-gamma and interleukin-10 for the detection of Johne’s disease, interleukin-8 and for cells differentiation studies [29-34]. However, its application for detecting food allergens such as histamine is yet to be shown.
Herein, we experimentally realize an AJP graphene-based histamine sensor that is suitable for rapid in-field monitoring. Histamine (2-(1H-imidazol-4-yl)ethanamine) is a biogenic amine associated with fish product spoilage and seafood allergies [35, 36] that currently is monitored for food safety through tedious laboratory techniques (e.g., fluorescence [37], high-performance liquid chromatography (HPLC) [38], thin layer chromatography [39], liquid chromatography with mass spectrometry [40], enzyme-linked immunosorbent assay (ELISA) [41], and impedimetric techniques [42]), which require hours to quantify histamine concentrations within a sample. While electrochemical sensing for histamine has been demonstrated previously, it usually involves depositing metallic nanoparticles [43, 44] or carbon-based materials on an electrode surface [45, 46], or the use of labeled enzyme mediated reactions [47, 48]. In contrast, our histamine biosensor circumvents the need for laboratory analysis and is capable of electrochemically quantifying histamine concentrations in food fluids with a response time of only 33 min (including incubation), which is appreciably faster than the traditional methods for histamine detection. The biosensor is aerosol jet printed in the form of an interdigitated electrode (IDE) on a polyimide sheet (Kapton®) with a graphene-nitrocellulose ink that results in high signal-to-noise ratios, fast response times, and enhanced reaction-diffusion kinetics during electrochemical sensing [49, 50]. The printed graphene IDE consists of 50 fingers of 40 μm width and 100 μm spacing, surpassing the resolution of traditional inkjet-printed electrodes, and are functionalized with oxygenated moieties through CO$_2$ thermal annealing [51]. These oxygen species are used to covalently link histamine antibody to the graphene surface through carbodiimide cross-linking chemistry. The resulting biosensor is capable of sensing histamine in buffer solution and real fish broth at biologically relevant concentrations with negligible interference or non-specific adsorption from competing proteins.
Results and Discussion

Fabrication of Aerosol Jet Printed (AJP) Graphene Interdigitated Electrode (IDE)

The AJP fabrication process consists of three main steps: graphene ink formulation, aerosol jet printing, and post-print baking (Figure 1a-c). Graphene ink formulation starts with the liquid phase exfoliation of graphite powder in acetone using nitrocellulose as a stabilizing polymer. A powder of few-layer graphene nanosheets coated in nitrocellulose was obtained after processing the slurry of exfoliated graphite, and an aerosol jet printable ink was formulated from the graphene-nitrocellulose powder using a solvent system of 9:1 ethyl lactate: dibutyl phthalate. This ink was aerosol jet printed into interdigitated electrode patterns using optimized printing conditions at a print speed of 5 mm·s⁻¹. The printed devices were baked at 350°C in air using a tube furnace to drive the decomposition of nitrocellulose into amorphous sp²-bonded carbonaceous residue, which improves the mechanical properties of the graphene film as well as increases the electrical conductivity to >10⁴ S·m⁻¹ [52, 53]. The devices were further annealed in CO₂ at 400°C in order to enhance antibody binding with the graphene surface by promoting surface functionalization with carboxyl and carbonyl groups [54].
Figure 1. Fabrication and biofunctionalization scheme of the AJP graphene biosensor including: (a) direct-write printing of graphene in an IDE pattern on a polyimide (Kapton®) sheet; (b) CO$_2$ thermal annealing to increase oxygenated species on the printed graphene surface; (c) immobilization of histamine antibodies on the IDE via carbodiimide cross-linking chemistry; (d) blocking the remaining unfunctionalized areas of the IDE with Superblock$^\text{TM}$ buffer to prevent non-specific adsorption during consequent biosensing; (e) histamine binding to the IDE and resulting Nyquist plot generated during electrochemical biosensing.

Image Analysis of the AJP Graphene IDE

The graphene film morphology and film thickness were characterized by optical microscopy, scanning electron microscopy (SEM), and atomic force microscopy (AFM) (Figure 2). The AJP graphene device was patterned as an IDE containing 50 fingers (25 per each finger-comb side), each with a width of 40 µm, length of 7 mm, and inter-finger spacing of 100 µm (Figure 2a-b, 2d). This patterning resulted in a geometric surface area of 14 mm$^2$, with an electrochemical surface area of 8.01 mm$^2$, contributing to a total of 57% active sites with respect to the total geometric area of the IDE (see Supplemental Information Figure S1). The graphene flakes showed a highly dense and aligned printed film that allows for efficient charge transfer across the material (Figure 2c) [52]. A more efficient charge transfer process is desired for biosensing applications as affinity-based sensors rely on the change of surface chemistry, which
is proportional to the available electrochemical surface area, due to target-capture probe binding and an associated change in the charge transfer behavior [55].

**Figure 2.** Optical characterization of the AJP graphene IDE. (a,d) Optical micrograph of the printed AJP IDE at 7x and 100x magnification, respectively, showing the high-resolution printed IDE fingers. (b-c) SEM micrographs displaying surface topography of the graphene sheets printed on the polyimide sheet at 150x and 15000x magnification, respectively. (e) Optical image of the AFM tip used for the measurement of the IDE finger height. AFM image of the graphene IDE finger showing the top area over which the AFM tip was scanned. Profile of the AFM micrograph showing the average film thickness of the graphene IDE finger is ~63 nm.

**Electrical Characterization of the AJP Graphene IDE**

The AJP process deposits graphene ink with smaller satellite droplets that diverge from the main stream beyond the focal point [56], generating aerosol printed lines with diffuse edges. The evaporation of the deposited graphene ink also exhibits a weak coffee-ring effect, resulting
in a printed line morphology that possesses a depression in the center and taller edges as shown by AFM imaging (Figure 2e). By averaging over the linewidth, the printed graphene film displayed a thickness of 63 ± 6 nm with a surface roughness of 8 ± 1 nm. AJP graphene printed features have a relatively low material consumption in comparison to other techniques that have shown similar printed line resolution since the AJP printed line thickness is significantly lower than previous reports (0.3 – 2 μm) [30, 56, 57]. Despite the exceptionally thin nature of the AJP graphene printed film, the electrochemical performance of these devices remains exemplary due to the high percentage of electrochemically active sites and resulting high sensitivity for biosensing (as will be shown in detail below).

It is also important for a functioning IDE that the electrode fingers are not shorted, which is a potential concern of the satellite droplet overspray of the graphene ink. Hence, the devices were tested for any electrical shorting before use. The AJP graphene IDEs showed a sheet resistance of 1.5 ± 0.3 kΩ/sq that is similar to solution-phase printed and annealed graphene inks based on cellulose-based binders that have been previously employed for inkjet printing (0.2 kΩ/sq. – 1.1 kΩ/sq.), but higher than thicker printed graphene films [51, 58, 59] that have been fabricated through spin coating (50-90 Ω/sq.) [25, 60] or screen printing (1 Ω/sq.) [61] or printed without binders through polymer casting (0.2 kΩ/sq.) [62], stamping (8 Ω/sq.) [63], or rolling compression (3.8 Ω/sq.) [64]. The higher sheet resistance for the AJP graphene IDEs is expected to the low thickness (63 nm) compared to the aforementioned techniques: 150 nm - 7 μm for inkjet printing [51, 58, 59], 0.8 μm for spin coating [25], and 2.5 μm for screen printing [61]. Nevertheless, the sheet resistance of the AJP graphene IDEs are sufficiently low for effective electrochemical sensing (as will be shown in detail below).
Spectral Characterization of the AJP Graphene IDE

The AJP graphene IDEs were further characterized by Raman spectroscopy and X-ray photoelectron spectroscopy (XPS). As shown in Figure 3a, the characteristic Raman D peak was observed at ~1311 cm\(^{-1}\), which is associated with the presence of defects in the hexagonal graphitic layers [65]. Similarly, a G peak that is indicative of sp\(^2\)-bonded carbon atoms in graphene was observed at ~1579 cm\(^{-1}\) [65]. Meanwhile, the 2D band generated by the double resonance electron-phonon scattering characteristic of graphene [66] was observed at ~2614 cm\(^{-1}\). In addition to the graphene bands, polyimide bands were observed at ~1392 cm\(^{-1}\) (corresponding to C-N stretching vibration of the imide system), ~1610 cm\(^{-1}\) (related to C=C stretching vibration of the 1,4-disubstituted phenyl ring), and ~1784 cm\(^{-1}\) (related to C=O stretching vibration of imide) [67-69]. The presence of polyimide bands on the graphene spectra (see Figure S2) is expected since the Raman laser penetrates through the thin graphene film. The intensity ratio of \(I_{2D}/I_G\) peaks of graphene spectra was measured to be 0.8 ± 0.2, which indicates a multilayer graphene film as expected [70]. Likewise, the intensity ratio of \(I_D/I_G\), which provides a measure of the presence of defects on the graphene surface, was measured to be 1.4 ± 0.1 [71, 72]. This defect level enhances heterogeneous charge transfer during electrochemical measurements and also facilitates the attachment of biorecognition agents, such as antibodies, required for the functionalization of the sensor [1, 50, 73].
Further characterization of the AJP graphene-based IDEs was performed with XPS to estimate the concentration of the functional groups present. Based on Figure 3b, the graphene surface possesses both sp² and sp³ bonded carbon atoms, represented by the peaks corresponding to binding energies of 284.6 eV and 285.6 eV with relative concentrations of 46.4% and 19.6%, respectively. In addition to carbon-carbon bonds, oxygen functional groups such as C-O-C at 286.5 eV (16.3%), carbonyl groups (C=O) at 287.5 eV (5.6%), and carboxyl groups (O-C=O) at 289.1 eV (6%) were observed [74]. The CO₂ annealing of AJP graphene-based electrodes is associated with the enhancement of oxygen-containing moieties, such as carbonyl and carboxyl, on the graphene surface [54]. Since antibodies are covalently attached to graphene via EDC/NHS esterification reaction, the presence of carbonyl and carboxyl moieties is crucial for device functionalization [75].
Electrochemical Sensing of Histamine with the AJP Graphene IDE Biosensor

Next, the AJP graphene IDEs were functionalized with histamine antibody via EDC/NHS covalent binding chemistry and imaged using AFM (see Methods, Figure S3) prior to subsequent histamine sensing. From the obtained Nyquist plots (Figure 4a), a consistent increase in the charge transfer resistance ($R_{ct}$) values (calculated as the diameter of the semi-circular shaped portion of the plot) was observed with increasing histamine concentration. During incubation, the histamine molecules bind to the antibody epitope creating an insulating layer on the electrode. As more histamine molecules are bound to the antibodies on the electrode, the insulating layer increases, decreasing the effectiveness of electron transfer between the electrolyte solution and the electrode, resulting in the increase of $R_{ct}$ values [76]. A calibration plot was obtained by normalizing the $R_{ct}$ with respect to the $R_{ct}$ value measured for zero concentration of histamine in the buffer solution (PBS), as shown in Figure 4b. The AJP graphene-based biosensor presented a linear sensing range from 6.25 to 100 ppm ($p_{\text{model}} = 0.000$, $p_{\text{lack-of-fit}} = 0.666$, $R^2 = 0.823$), a limit of detection of $2.52 \pm 0.92$ ppm, and a sensitivity of $2.9 \pm 1.2 \text{ k}\Omega \cdot \text{decade}^{-1}$ (Figure 4b). This high sensitivity and low detection limit can be attributed to the high surface area, charge transfer efficiency, faster steady state establishment, and signal-to-noise ratio of the comb-like structure associated with electrochemical IDEs [25, 77, 78].
Figure 4. Histamine detection using the AJP graphene IDE biosensor. (a) Nyquist plots for each histamine concentration added to PBS. (b) Calibration plot showing percent change of charge transfer resistance (R_{ct}) with respect to histamine concentration ranging from 6.25 to 800 ppm (56.25 µM to 7.2 mM) in PBS. Error bars represent the standard deviation calculated from 4 independently biofunctionalized electrodes (n = 4). (c) Nyquist plots for each histamine concentration added to fish broth. (d) Calibration plot showing percent change of charge transfer resistance (R_{ct}) with respect to histamine concentration ranging from 6.25 to 800 ppm (56.25 µM to 7.2 mM) in fish broth. Error bars represent the standard deviation calculated from 5 independently biofunctionalized electrodes (n = 5).

Next, the AJP graphene-based biosensor was evaluated in a real biological matrix (i.e., freshly prepared fish broth). Again, the increase of histamine concentration in spiked fish broth resulted in an increase of R_{ct}, as observed from the Nyquist plots in Figure 4c and the calibration plot in Figure 4d. Based on the calibration plot, the dynamic linear sensing range for histamine detection in fish broth is 6.25 to 200 ppm (p_{model} = 0.000, p_{lack-of-fit} = 0.955, R^2 = 0.884). A limit
of detection of 3.41 ± 1.42 ppm was observed for fish broth samples, which is similar to the detection limit obtained in buffer (p = 0.315; α = 0.05). In addition, the sensitivity of the AJP graphene-based biosensor in fish broth (4.5 ± 1.6 kΩ·decade⁻¹) was comparable to the sensitivity observed in buffer (p = 0.141; α = 0.05). These results emphasize the versatility and stability of the AJP graphene-based biosensor even when used in chemically complex samples such as fish broth, which is rich in amino acids, lipids, vitamins, and minerals [79].

Lower sensing ranges and limits of detection have been demonstrated in the literature using other sensing devices that also require much longer response times, such as electrochemiluminescence with a sensing range between 0.01 ppm and 1 ppm (overall response time: 8.5 hours) [80]; quartz crystal microbalance with a sensing range between 0.11 ppb and 11.22 ppm (overall response time: 4.5 hours) [81]; and photoluminescent quantum dots between 10.7 ppm and 63.36 ppm (overall response time: 67 min) [82]. However, these devices either present sensing ranges below the established FDA or European Food Safety Authority (EFSA) toxicity limits, which could lead to unnecessary rejection of fish samples, or they require sophisticated equipment, pre-labeling, and/or optical measurements, which are generally not suitable for in-field food sensing that requires rapid and low-cost sensor materials and operation in turbid field samples. A recent study by Vanegas et al [83] reported an in-field electrochemical biosensor comprised of laser induced graphene that was capable of monitoring total biogenic amines in fish samples with a sensing range of 5.56 to 177.84 ppm and with a detection limit of 1.29 ppm. However, this graphene-based biosensor required functionalization with metallic copper microparticles and enzymes, was not specific to histamine (a single type of biogenic amine) and required a 60-min current polarization prior to detection. Similarly, Gumpu et al. [84] reported an enzymatic electrochemical histamine biosensor consisting of a glassy carbon
electrode modified with polyaniline and ceria nanoparticles and showed a linear sensing range of 50 – 117 ppm. In contrast, the sensing range presented by the AJP graphene-based biosensor in fish broth confirmed its capacity for efficient detection of histamine that is compliant with FDA recommended levels (50 ppm) and EFSA (100 ppm) [85, 86] with an overall response time of 33 min (including incubation of electrode with the sample) and negligible interference from turbid biological sample conditions. According to the FDA, since there is a large variability of histamine distribution throughout fish, it is possible to find 50 ppm in certain tissues, while other parts of the same fish may present 500 ppm or more [85, 87]. Therefore, the detection of histamine levels around 50 ppm are critical to determine whether a fish is safe for consumption while avoiding both food poisoning and wastage.

*Non-specific Adsorption Testing with the AJP Graphene IDE Biosensor*

The AJP IDE biosensor was also tested in complex media consisting of large proteins in order to analyze the resiliency of the biosensor towards non-specific adsorption (Figure 5). The interferent proteins selected herein (i.e., bovine serum albumin (BSA), goat serum (GS), and whey protein (WP)) are either frequently applied as blocking agents for biosensor devices due to their large size and effectiveness in covering surfaces or commonly occur in food products [88, 89]. Non-specific adsorption of such large interferent proteins could possibly result in false positives and surface fouling, which would affect the effectiveness of the biosensor [90]. Hence, it is important to determine their possible non-specific adsorption on the sensor. A concentration of 50 ppm was used for the interfering proteins to determine their effect on detecting 50 ppm of histamine, which is the minimum toxic level recommended by the FDA [85]. For all the interfering proteins, the percentage change of $R_{ct}$ (less than 10%) was significantly smaller ($p = 0.000$, $\alpha = 0.05$) when compared to a change of 48% for the same concentration of histamine.
Furthermore, the percentage change of $R_{ct}$ by interfering proteins was lower than the selectivity test signal changes established by similar studies [91, 92]. These results indicate that large protein molecules do not have a significant effect on the sensor function, such as amplifying the blocking effect on the immunosensor or exhibiting cross-reactivity with the histamine antibody.

**Figure 5.** Non-specific adsorption test of AJP graphene IDE biofunctionalized with histamine antibody against bovine serum albumin (BSA), goat serum (GS), and whey protein (WP) to determine the effect of the incubation of the sensor in commonly found large protein molecules in food samples and used as blocking agents that can potentially interfere with the histamine antibody activity. All of the interfering proteins show a minimal change of $R_{ct}$ (< 10%). Error bars represent the standard deviation calculated from 3 independently biofunctionalized electrodes ($n = 3$).

**Conclusions**

In this work, an AJP graphene-based histamine biosensor was developed. The printed graphene IDE featured low-cost, one-step writing with high resolution fingers of 40 μm width and nanometer-scale thickness of 63 nm. AJP-printed IDEs were functionalized with monoclonal histamine antibody via EDC/NHS chemistry. Histamine detection was tested in buffer and in fish broth to validate the sensor performance. The AJP graphene biosensor platform could detect histamine in PBS and fish broth over toxicologically relevant ranges of 6.25 to 100 ppm and 6.25
to 200 ppm, respectively, with similar detection limits of 2.52 ppm and 3.41 ppm, respectively. The sensors also showed a quick response time of only 33 min without the need for pre-labeling and pre-treatment of the acquired fish sample. Furthermore, the biosensor sensitivity was not significantly affected by the non-specific adsorption of large protein molecules that are commonly found in food samples and used as blocking agents. Such a facile and rapid biosensor can thus find applications in food processing facilities, import and export ports, and supermarkets where continuous on-site monitoring of food samples is essential to determine and maintain the quality of every food item sold. This on-site testing will eliminate the need for sending the food samples for laboratory testing, which requires additional handling steps, increases time and cost to histamine analysis, and consequently increases the risk of foodborne illnesses and food wastage.

The AJP graphene IDE platform developed here can likely also be used in other biosensing applications where rapid monitoring of target molecules is desired, as the sample pre-treatment is eliminated using the developed immunosensing protocol. Apart from sensing small allergen molecules such as histamine, the immunosensor could be used to detect various targets such as cells and protein biomarkers. By switching the antibody immobilized on the sensor platform to one that is specific towards the detection of suitable biological target species, the sensor can further cater to specific applications. Some examples include food pathogens (Salmonella spp.) [93], fatal human diseases (cancer, HIV) [94, 95] or animal or plant diseases (avian influenza, Citrus tristeza) [96, 97]. Additionally, aerosol jet printing for manufacturing graphene IDEs is a highly scalable process in which a variety of metallic and non-metallic materials (i.e., graphene, silver) can be formulated into inks and printed in high-resolution patterns on substrates with different degrees of flexibility such as polyimide or silicon [29, 98]
without employing expensive patterning techniques such as photolithography. Such high-resolution printed electrochemical devices can be implemented for energy harvesting [99] or for producing supercapacitors [100] apart from biosensing, thus widening their scope of applications.

**Materials and Methods**

**Materials**

Graphite powder (grade 3061) was purchased from Asbury Graphite Mills (Asbury, NJ). Nitrocellulose was purchase from Scientific Polymer (Ontario, NY, USA). Acetone, ethyl lactate, dibutyl phthalate, N-(3-dimethylaminopropyl)-N’-ethylcarbodiimide (EDC), N-hydroxysuccinimide (NHS), ethanolamine, 2-(N-morpholino) ethanesulfonic acid (MES buffer), histamine ≥ 97.0%, potassium hexacyanoferrate (II) trihydrate, potassium ferricyanide, and goat serum were purchased from Millipore Sigma (Saint Louis, MO, USA). Potassium chloride was purchased from Fisher Scientific (Hampton, NH, USA). Superblock™ buffer was purchased from Thermo Fisher (Waltham, MA, USA). Whey protein was purchased from Equate (Walmart, Bentonville, AR, USA). Bovine serum albumin (BSA) was purchased from VWR International (Solon, OH, USA). Phosphate buffer saline was purchased from Alfa Aesar (Tewksbury, MA, USA). Mouse anti-histamine monoclonal antibody (Cat. No. MAB5408) was purchased from EMD Millipore (Burlington, MA, USA)

**Graphene Ink Formulation**

Graphene ink was prepared as previously described by Secor et al. [52]. First, graphite powder was mixed with nitrocellulose (1:1) and dispersed in acetone. The suspension was shear mixed for 4 hours at 10,230 rpm to promote the exfoliation of graphite flakes. Then, the suspension was centrifuged for 15 min at 4600 rcf, followed by 20 min at 6650 rcf, and the
supernatant was collected. The collected supernatant was flocculated with aqueous NaCl solution (0.04 g·mL$^{-1}$) and centrifuged for 6 min at 10,000 rcf. The obtained pellet was washed with DI (distilled) water, dried under a 60 W lamp, and crushed to form a powder. The graphene-based powder (30 mg·mL$^{-1}$) was dispersed in a 1:9 (v/v) mixture of dibutyl phthalate and ethyl lactate in bath sonication (110 W, 40 kHz) for 6 hours, thus forming the graphene ink. The prepared graphene ink was filtered through a 3.1 μm membrane for printing.

**Aerosol Jet Printing**

The graphene-based ink was aerosol jet printed in an interdigitated electrode (IDE) pattern on top of a polyimide (Kapton®, Dupont, MI, USA) substrate using an Optomec AJ200 Printer (Albuquerque, NM, USA). The substrate was maintained at 60 °C in order to reduce the coffee ring effect by cancelling the evaporation driven material flow using geometric surface tension material flow [101], and the printing speed was adjusted to 5 mm·s$^{-1}$ to print the devices with minimum thickness and appropriate electrical conductivity [34]. During the printing process, the sheath flow rates were kept between 40 – 60 sccm, with a carrier flow rate between 15 – 45 sccm. The flow rates were optimized at the beginning of every print session to deposit continuous lines presenting 40 μm width and minimal overspray, which were continually tuned throughout the printing process. Nitrogen was used as both sheath and carrier gas. The graphene IDEs were printed in a single pass (1 layer), which is sufficient to fabricate high quality conductive electrodes for electrochemical sensing, as we have shown previously [52]. After printing, the IDEs were heat treated in air in a box furnace (Lindberg Blue M, Thermo Scientific, Waltham, MA, USA) for 30 min at 350 °C to evaporate any residual solvent and to pyrolyze the nitrocellulose. At 350 °C, the maximum improvement to the electrical properties of the graphene film is obtained due to the decomposition of nitrocellulose into a carbonaceous sp$^2$-bonded
residue [52]. Finally, printed devices were carbon dioxide annealed in a tube furnace (OTF-1200X, MTI Corp, Richmond, CA, USA) at 400 °C for 2 hours to increase the amount of carboxyl groups on the graphene surface.

**Functionalization**

Graphene-based IDEs were functionalized using 0.4 M EDC and 0.1 M NHS prepared in 0.1 M MES buffer (pH 6.0) for 1 hour at room temperature. Then, the IDEs were incubated overnight with mouse anti-histamine monoclonal antibody solution (1:150) in sterile 1x PBS. After the incubation, 1 M ethanolamine was used to quench the unreacted EDC/NHS for 20 min, followed by blocking with Superblock™ buffer for 20 min to eliminate any non-specific binding of histamine.

**Scanning Electron Microscopy**

Scanning electron microscopy (SEM) images of the AJP graphene-based devices were obtained using a FEI Quanta 250 FE-SEM (ThermoFisher Scientific, OR, USA). Initially, the samples were coated with a 2-nm layer of Iridium using a turbo pump sputter coater. Then, the images were acquired using a working distance of ~10 mm, spot size of 3.0, and 10 kV of accelerating potential.

**Atomic Force Microscopy**

Atomic force microscopy (AFM) images were acquired using a Dimension Icon Scanning Probe Microscope (Bruker, Santa Barbara, CA, USA) in air. Tapping mode was used to obtain images of the IDE surface before and after the immobilization of antibodies (see Supplemental Information Figure S3). The IDE finger height measurements were carried out in Peak Force Tapping mode using ScanAsyst. The probes used were model ScanAsyst-Air (Bruker, Santa Barbara, CA, USA) or TESPA probes (Bruker, Santa Barbara, CA, USA). AFM
images were post processed using plane-fitting (second order) and/or flattening (zeroth order) within the Nanoscope software.

Raman Spectroscopy

Raman spectra of the AJP graphene-based electrodes were collected using an XploRa Plus confocal Raman upright microscope equipped with a Synapse EMCCD camera (Horiba Scientific, JY, France). A 785-nm laser excitation source (5 mW at the sample) and a 100× air objective (0.9 numerical aperture, LMPlan N, Olympus) were used to collect the Raman signal under ambient laboratory conditions. The spectra were collected with a 600 grooves·mm⁻¹ grating, and all reported spectra were averaged over six replicates, each with 60 s acquisition time and 3 accumulations. Igor Pro 6.36 (Wavemetrics, Inc., Lake Oswego, OR, USA) scientific analysis and graphing software was used to analyze the Raman data. A Lorentzian distribution with linear baseline correction was utilized to fit the data in order to extract peak intensity (height). \( I_D/I_G \) and \( I_{2D}/I_G \) reported values correspond to an average of 6 spectra.

X-ray Photoelectron Spectroscopy

X-ray photoelectron spectroscopy (XPS) evaluation was carried out using a Kratos Amicus/ESCA 3400 instrument. The sample was irradiated with 240 W unmonochromated Al Kα x-rays, and photoelectrons emitted at 0° from the surface normal were energy analyzed using a DuPont type analyzer. The pass energy was set at 150 eV, and a Shirley baseline was removed from all reported spectra. Raw data files were processed using CasaXPS software (v 2.3.19).

Electrochemical Measurement

Electrochemical measurements were carried out using a two-electrode set up on a CH Instruments potentiostat station (CHI 7081E). All measurements were conducted in 5 mM Fe(CN)₆³⁻/Fe(CN)₆⁴⁻ ferri/ferrocyanide (1:1) redox probe with 0.1 M KCl dissolved in 1x PBS.
Electrochemical impedance spectroscopy (EIS) measurements were carried out using a frequency range of 0.1 Hz – 100 kHz with an AC voltage amplitude of 10 mV and no DC bias. Nyquist plots were used to determine the charge transfer resistance ($R_{ct}$), which is the resistance for charge transfer across the electrode-electrolyte interface.

**Histamine Sensing**

Histamine standard solutions were prepared in 1x PBS in a range between 6.25 and 800 ppm (1 ppm = 1 mg L$^{-1}$ = 9 µM). IDEs were incubated with 100 µL of each standard solution for 30 min at 50 rpm to allow histamine to bind to the immobilized antibody on the IDE surface. Between each measurement, the electrodes were washed with 100 µL of 1x PBS thrice to remove unbound histamine molecules. Histamine calibration plots in 1x PBS were obtained by measuring the $R_{ct}$ for each successive concentration using the same EIS parameters as previously described.

**Fish Broth Sensing**

A homogeneous fish broth was initially prepared by blending (1000 W, 30 s) 25 g of fresh yellowfin tuna (*Thunnus albacares*) filet (Anova Food, LLC, San Diego, CA, USA) with 500 mL of sterilized 1x PBS. Then, the fish broth was filtered through a Whatman grade 1 qualitative filter paper (11.0 µm) (Millipore Sigma, Saint Louis, MO, USA) to remove the large particles, followed by a 0.45 µm syringe filter (Corning, Corning, NY, USA) and stored at 4 °C until use. Measurements were carried out as described on section 4.10.

**Non-specific Adsorption Test**

Bovine serum albumine (BSA), goat serum (GS), and whey protein (WP) were used as interferents to test the affinity of the AJP graphene-based biosensor to non-specific interactions. The biosensor was incubated with solutions containing 50 ppm (in 1x PBS) of each interferent in
the same conditions of histamine (30 min). Then, the EIS measurements were recorded, and the percentage change of $R_{ct}$ was calculated for each one of the interferents.

*Data Analysis*

A completely randomized design was used in this study and the results were reported as mean ± standard deviation. Calibration curves and non-specific adsorption test results were obtained by performing at least 3 independent experiments. Data analysis was performed using JMP Pro statistical software (version 15, SAS, Cary, NC). Qualitative comparisons were carried out using t-test with confidence level of 95%. Regression analysis with confidence level of 95% was performed to determine the linear sensing range and the functional correspondence among quantitative variables. The limit of detection for the developed biosensors was calculated using three times the standard deviation (3σ) of the zero-concentration measurement [102, 103].

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Cyclic voltammetry (CV) was carried out at different scan rates (5, 10, 25, 50, 100 mV·s\(^{-1}\)) using a three-electrode set up on a CH Instruments potentiostat station (CHI 7081E). All measurements were conducted in 5 mM Fe(CN)\(_6\)\(^{3-}/\)Fe (CN)\(_6\)\(^{4-}\) ferri/ferrocyanide (1:1) redox probe with 0.1 M KCl dissolved in 1x PBS. From the CV of the aerosol jet printed (AJP) graphene interdigitated electrode (IDE) (Figure S1a), the peak separation voltage varies between ~ 0.6 to 1.2 V. Hence, this system is irreversible (\(\Delta E_p > 200\) mV) and first the charge transfer coefficient (\(\alpha\)) needs to be calculated [1]. The value of \(\alpha\) can be calculated from the plot of peak
voltages versus logarithm of scan rate (Figure S1b). For anodic peak voltage, α can be determined from the slope of the line as $slope = \frac{2.3 RT}{(1-\alpha) n F}$ [1]. From the Figure S1b, the regression equation obtained was $y = 224.16x + 1021.4$ with $R^2 = 0.96$, which yields an $\alpha = 0.74$. Next, the electrochemical surface area was calculated from the Randles – Ševčík equation and slope of the Randles – Ševčík plot (Figure S1c) for anodic current using the equation:

$slope = 2.99 \times 10^5 \alpha^{1/2} A C_o D_o^{1/2}$ [2]. Here, $C_o$ is the concentration of redox probe (5 mM), $D_o$ is the diffusion coefficient of the redox probe ($7.2 \times 10^{-6}$ cm$^2$·s$^{-1}$), $A$ is the electrochemical surface area of the electrode and the regression equation for the anodic peak current was obtained as $y = 2.76 \times 10^{-4} x + 1.48 \times 10^{-5}$ with $R^2 = 0.99$. The forward (anodic) peak current rather than cathodic peak current for the calculation was used due to the reduced amount of product formation that might hinder the reverse peak values of the CV [3]. The electrochemical surface area of the aerosol jet printed (AJP) interdigitated electrode (IDE) was calculated as 8.01 mm$^2$. 
Figure S1. Calculation of electrochemical surface area (ESA) for the AJP IDE. (a) CV of the AJP graphene IDE at various scan rates (5, 10, 25, 50, 100 mV·s\(^{-1}\)). (b) Plot of the peak voltages versus logarithmic value of the scan rate to calculate the charge transfer coefficient, \(\alpha\); and (c) Randles–Sevcik plot showing a linear variation of the peak current with square root of the scan rate and using the resulting slope to calculate the ESA.
Raman Spectroscopy of Kapton® (Polyimide) Substrate

Raman spectroscopy yields a plot as shown in Figure S2, due to the large spot size (1 μm) of the laser as compared to the finger width (40 μm) of the AJP graphene IDE. The polyimide bands obtained are restricted to the first order region of graphene spectrum (D and G regions). The resulting background needs to be subtracted from the raw data in order to get the spectrum pertaining to graphene only.

Figure S2. Raman spectrum of the Kapton® substrate used in the aerosol jet printed graphene IDE, showing the peaks that correspond to the polyimide bands.
Qualitative Analysis of Antibody Immobilization on AJP Graphene IDE

The histamine antibody immobilized on the graphene IDE was characterized by AFM. As shown in the Figure S3, the micrograph was recorded for an untreated graphene (Figure S3a) and after the antibody was attached to the graphene via EDC/NHS chemistry (Figure S3b). As the graphene surface is relatively uneven on the nanometer scale and the size (height) of antibody is only about 4 nm [4], the antibodies can be difficult to visualize against a rough background. Nevertheless, due to the antibody attachment, there is an overall increase in the rough texture of the electrode surface. The boxes indicate areas that show prominent texture development on Figure S3b as compared to Figure S3a due to antibody attachment. Furthermore, the change in roughness of the surface as analyzed by the AFM software, Nanoscope Analysis (v. 1.9), was 3.7 nm which correlates with the antibody height.

Figure S3. Surface characterization of the AJP graphene IDE using AFM showing (a) untreated graphene surface, and (b) graphene surface functionalized with histamine antibody. The color coded dashed boxes represent corresponding areas where increase of surface texture is prominent with an increase in roughness of 3.7 nm after antibody immobilization.
References


APPENDIX B. FLEXIBLE LASER-INDUCED GRAPHENE FOR NITROGEN SENSING IN SOIL

Nate T. Garland¹, Eric S. McLamore², Nicholas D. Cavallaro², Deyny Mendivelso-Perez⁴,⁶, Emily A. Smith⁴,⁶, Dapeng Jing⁵, Jonathan C. Claussen*¹,⁶

¹ Mechanical Engineering Department, Iowa State University, Ames, Iowa 50011, USA
² Agricultural and Biological Engineering Department, Institute of Food and Agricultural Sciences, University of Florida, Gainesville, Florida 32611, USA
⁴ Chemistry Department, Iowa State University, Ames, Iowa 50011, USA
⁵ Materials Analysis and Research Laboratory, Iowa State University, Ames, Iowa 50011, USA
⁶ The Ames Laboratory, U.S. Department of Energy, Ames, Iowa 50011, USA

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Abstract

Flexible graphene electronics are rapidly gaining interest, but their widespread implementation has been impeded by challenges with ink preparation, ink printing, and post-print annealing processes. Laser-induced graphene (LIG) promises a facile alternative by creating flexible graphene electronics on polyimide substrates through a one-step laser writing fabrication method. Herein we demonstrate the use of LIG for electrochemical ion selective sensing of plant-available nitrogen (i.e., both ammonium and nitrate ions: NH₄⁺ and NO₃⁻) in soil samples. The laser used to create the LIG was operated at distinct pulse rates (10, 20, 30, 40, and 50 ms) in order to maximize the LIG electrochemical reactivity. Results illustrated that a laser pulse rate of 20 ms led to a high percentage of sp² carbon (77%) and optimal peak oxidation current of 120 uA during ferricyanide cyclic voltammetry. Therefore, LIG electrodes created with a 20 ms pulse rate were consequently functionalized with distinct ionophores specific to
NH$_4^+$ (nonactin) or NO$_3^-$ (tridodecylmethylammonium nitrate) within polyvinyl chloride (PVC)-based membranes to create distinct solid contact ion selective electrodes (SC-ISEs) for NH$_4^+$ and NO$_3^-$ ion sensing respectively. The LIG SC-ISEs displayed near Nernstian sensitivities of 51.7 ± 7.8 mV/decade (NH$_4^+$) and -54.8 ± 2.5 mV/decade (NO$_3^-$), detection limits of 28.2 ± 25.0 uM (NH$_4^+$) and 20.6 ± 14.8 uM (NO$_3^-$), and linear sensing ranges within 10$^{-5}$-10$^{-1}$ M. Moreover, soil slurry sensing was performed and recovery percentages of 96% and 95% were obtained for added NH$_4^+$ and NO$_3^-$, respectively. These results, combined with a facile fabrication that does not require metallic nanoparticle decoration, make these LIG electrochemical sensors appealing for a wide range of in field or point-of-service applications for soil health management.

**Introduction**

Graphene-based electronics offer great promise for a wide variety of applications, including supercapacitors and batteries,\textsuperscript{1} graphene tattoo sensors,\textsuperscript{2} and other flexible electronics,\textsuperscript{3} due to the unique material properties of graphene such as high flexibility, electrical and thermal conductivity, and tensile strength. Challenges with realizing graphene-based electronics lie in relatively complex fabrication procedures, which have generally included chemical vapor deposition (CVD) and/or complex substrate transfer techniques. CVD synthesized carbon nanomaterials (carbon nanotubes or graphene) often require pre-patterning a surface with metal catalysts (e.g., nickel, cobalt or iron) and carbon growth within a high temperature (up to 1000°C), low pressure vacuum environment injected with carbon precursor gases.\textsuperscript{4-7} However, alternative scalable manufacturing protocols for graphene-based electrical circuits are beginning to emerge, including solution-phase printing (inkjet printing,\textsuperscript{8-9} screen printing,\textsuperscript{10} dispenser printing\textsuperscript{11}) and direct laser scribing,\textsuperscript{12-13} among others. Solution phase printing techniques generally require an additional post-print processing step such as thermal,\textsuperscript{14} laser,\textsuperscript{15} or photonic
annealing\textsuperscript{16-17} to improve electrical conductivity. High-temperature thermal annealing (250-400° C\textsuperscript{9,18}) of printed graphene is used to burn off non-conductive cellulose-based binders and solvents as well as to weld or sinter printed graphene flakes. However, thermal annealing is not conducive for use on thermally sensitive polymer or paper substrates. Others have used photonic annealing\textsuperscript{17} or rapid-pulse laser annealing\textsuperscript{14} that is conducive for thermally sensitive polymers, but similar to any annealing technique these methods require an additional post-print processing step that increases the electrode fabrication complexity. However, a recently discovered, promising alternative to printed graphene circuits is laser inscribed graphene (LIG).\textsuperscript{13,19-20}

LIG is a one-step lithography-free process upon which a laser converts sp\textsuperscript{3} hybridized carbon found in substrates such as polyimide into sp\textsuperscript{2} hybridized carbon—the carbon allotrope found in graphene. LIG is a versatile technique that has been used to produce graphene films that are superhydrophobic,\textsuperscript{21} doped with metal oxide nanocrystals,\textsuperscript{19} functionalized with polymer,\textsuperscript{20} or developed into vertically-aligned graphene fibers.\textsuperscript{22} Typically, a rapid pulse (femtosecond), infrared laser is used to selectively transform distinct regions or patterns of polyimide film (Kapton tape) into sp\textsuperscript{2} hybridized carbon, \textit{viz.}, porous graphene. This laser processing technique can be performed in air at room temperature without the need for prior patterning of the substrate with a metal catalyst or the need for chemical etching/cleaning techniques associated with CVD graphene fabrication. This facile LIG manufacturing protocol also eliminates the need for ink preparation, ink printing, and post-print annealing processes associated with solution-phase printed graphene. The utility of LIG has been displayed in numerous applications including supercapacitors,\textsuperscript{13,21,23-24} non-biofouling surfaces,\textsuperscript{25} transparent heaters\textsuperscript{26}, and more recently, electrochemical sensors.\textsuperscript{27-30} For example, a non-enzymatic amperometric glucose sensor comprised of LIG functionalized with Cu nanocubes was used to
selectively measure glucose over a wide concentration range (25 µM–4 mM), which is physiologically relevant to glucose levels in saliva, tears, and blood.\(^{27}\) In another report, a LIG electrode was used to monitor electroactive small molecules, including ascorbic acid (AA), dopamine (DA), and uric acid (UA) \textit{via} cyclic voltammetry and differential pulse voltammetry.\(^{28}\) Other recent biosensor examples include a LIG aptasensor developed for thrombin\(^{29}\) and a LIG biosensor for biogenic amines based on a nanocopper-diamine oxidase hybrid material\(^{30}\). However, ion sensing using LIG has yet to be demonstrated.

In this work, we demonstrate the first use of LIG for ion sensing by developing a solid-contact ion-selective electrode (SC-ISE). Cyclic voltammetry (CV), X-ray photoelectron spectroscopy (XPS), Raman spectroscopy, and Scanning Electron Microscopy (SEM) characterization reveal that the laser scribing pulse rate (10, 20, 30, 40, 50 ms) significantly alters the structure and electroactive nature of the LIG-based electrodes. The developed LIG electrodes are converted into SC-ISE by functionalization with a PVC membrane containing an ionophore specific to NH\(_4^+\) (nonactin) or NO\(_3^-\) (tridodecylmethylammonium nitrate). The resulting nitrogen SC-ISE sensors display a near Nernstian sensitivity of 51.7 ± 7.8 mV/decade (NH\(_4^+\)) and -54.8 ± 2.5 mV/decade (NO\(_3^-\)), detection limit of 28.2 ± 25.0 uM (NH\(_4^+\)) and 20.6 ± 14.8 uM (NO\(_3^-\)), linear sensing range of 10\(^{-5}\)-10\(^{-1}\) M, and relatively low long term drift (0.93 mV/hr). The LIG ion sensors were used to measure NH\(_4^+\) or NO\(_3^-\) in a complex matrix (soil slurry) with an average signal recovery of 96 ± 14 %. In soil column tests, transport of NH\(_4^+\) and NO\(_3^-\) followed expected trends, where higher retention was obtained for soils with low ion content and vice-versa. The recovery rate for LIG sensors was higher than 95% for all studies in complex soil matrices (nitrogen rich, and nitrogen poor soils), paving the way for future development of bury-and-forget soil sensors.
Materials and Methods

LIG Electrode Fabrication

All chemicals used in the ISE membrane were obtained from Sigma-Aldrich. LIG electrodes were prepared with a standard high temperature Kapton tape adhered to Epson printer paper (Kapton tape was placed on the emulsion side of the photo paper) followed by treatment with a 405 nm 1W pulsed laser on two-dimensional stepper motors (HTPOW). The overall geometry (25.8 mm in length with a circular working area with diameter of 5.3 mm) of each electrode was first developed using computer aided design. The laser pulse rate governed the laser energy density per software provided with the equipment. The pulse rate is defined by the manufacturer as the length of the laser pulse at each raster step. Laser pulse rates of 10, 20, 30, 40, and 50 ms were tested in an effort to maximize the electronic and electrochemical properties of the LIG electrodes. After laser induced graphitization of the polyimide, the LIG electrodes were passivated with an insulating lacquer, except for the 5.3 mm diameter region that acted as the working electrode (see Figure S1 in the Supplemental Information). Passivation prevented electrode short circuiting and non-specific binding during electrochemical sensing.27-29

LIG Functionalization for Ion Selective Sensing

After graphitization of the polyimide, LIG working electrodes were oxygen plasma treated using a Dyne-A-Mite IT Plasma Treater (Enercon) for 2 s in air to normalize surface chemistry prior to functionalization with a SC-ISM (unless otherwise noted, see Results and Discussion). The subsequent general steps for functionalization with solid-contact ion selective membranes (SC-ISM) include: (i) LIG conditioning, (ii) membrane deposition/functiona

2fization, and (iii) membrane conditioning. Plasma treated LIG electrodes were conditioned with primary ion for 30 minutes by drop casting a 100µL aliquot of electrolyte on the working electrode. The
electrolyte for NH$_4^+$ electrodes was 0.1 mM NH$_4$Cl and the electrolyte for NO$_3^-$ electrodes was 0.05 mM NaNO$_3$ + 0.05 mM NaCl. Next, the working electrode was functionalized with either a NH$_4^+$- or NO$_3^-$-selective SC-ISM. NH$_4^+$ ionophore cocktail was prepared by mixing nonactin, tetrakis-(4-chlorophenyl)-borate, dioctyl sebacate, and polyvinyl chloride (PVC) (1/0.5/33.2/65.3 % wt, respectively) for a total mass of 100 mg. The NO$_3^-$ cocktail contained 0.25% (wt) methyltriphenylphosphonium bromide, 1.93% (wt) nitrocellulose dissolved in 35% 2-propanol, 16.25% (wt) 2-nitrophenyl octyl ether, 5.75% (wt) PVC, 74.32% (wt) tetrahydrofuran (THF), and 1.5% (wt) tridodecylmethylammonium nitrate. SC-ISM cocktails were dissolved in 1 mL THF followed by vortex mixing for ~1 min and overnight refrigeration to ensure homogeneity. To prepare SC-ISM on LIG, a 30 µL aliquot of the ion-selective cocktail was drop cast onto the working electrode and allowed to dry overnight at room temperature in air. The dried SC-ISE were then conditioned in either 1mM NH$_4$Cl (NH$_4^+$-selective electrodes) or 1mM KNO$_3$ (NO$_3^-$-selective electrodes) for 24 hours and stored dry at room temperature when not being tested. The reference electrodes were comprised of either a commercial 3M Ag/AgCl reference electrode (BASi, Inc.) or Ag/AgCl paint$^{30}$ as noted.

**Spectroscopic Characterization**

A range of material characterization tests were performed to determine the laser energy density that led to the largest increase in the electroactive nature of the LIG. Laser pulse rates of 10, 20, 30, 40, and 50 ms were tested. Scanning electron microscopy (SEM) images were taken using a FEI Quanta 250 FE-SEM (Thermo Fischer) at a magnification of 50X, 150X, and 500X. LIG samples were imaged after metal sputter coating. X-ray photoelectron spectroscopy (XPS) measurements were performed using a Kratos Amicus/ESCA 3400 instrument. The sample was irradiated with 240 W umonochromated Mg Kα x-rays, and photoelectrons emitted at 0° from the
surface normal were energy analyzed using a DuPont type analyzer. The pass energy was set at 150 eV and a Shirley baseline was removed from all reported spectra. CasaXPS was used to process raw data files. Raman spectroscopy measurements were performed using an XploRa Plus confocal Raman upright microscope equipped with a 532nm excitation source (2 mW at the sample) and a Synapse EMCCD camera (Horiba Scientific/NJ, France). A 50X air objective (Olympus, LMPlanFL) with a 0.5 numerical aperture was used to collect Raman images in the epi-direction. Raman mapping was performed over an area of 20×20 μm² with a step size of 2 μm. The Raman spectrum collected at each pixel was recorded over a spectral range of 600-3300 cm⁻¹ with a 1200 grooves/millimeter grating that corresponds to an average of 2 measurements with a 15s acquisition time. All data were processed using Igor Pro 6.37 scientific analysis and graphing software (Wavemetrics, Lake Oswego, OR, USA). The spectra were fit to a Gaussian function with a linear baseline using the multi-fit peak function in order to extract peak intensity (height).

**Electrochemical Characterization**

Electrochemical tests were conducted using a CHI6273E electrochemical workstation (CHI Instruments, USA) or a National Instruments potentiostat³¹ where the LIG electrode acted as the working electrode and an external Ag/AgCl electrode (saturated in 3 M KCl) or a Ag/AgCl tape as the reference electrode, as noted. Cyclic voltammetry (CV) scans were obtained to characterize the electroactive nature of the distinct LIG electrodes with a 3-electrode set-up with a platinum wire acting as the counter electrode. CV scans were performed from -0.6 V to 0.8 V in 5mM ferro/ferricyanide solution at a scan rate of 25 mV/s. The LIG electrodes were first conditioned by running the CV scans at least 5 times, after which peak oxidation and reduction currents/potentials were stable at 25 mV/s before data was obtained.
Bending tests were conducted according to our previous work\textsuperscript{32}, using a VEECO FPP-5000 4-point probe to conduct sample measurements. Average sheet resistance was calculated for 9 replicate tests, and percent change in sheet resistance reported for each material tested. Contact angle measurements were made using a custom-built goniometer, and image analysis was conducted according to our previous work.\textsuperscript{32}

**Nitrogen Sensing and Soil Analysis**

LIG SC-ISEs were first characterized for NH$_4^+$ or NO$_3^-$ sensing in DI water (pH 7) using calibration procedures defined by the International Union of Pure and Applied Chemistry.\textsuperscript{33} Stock NH$_4$Cl or KNO$_3$ solution was added to increase the cell concentration in 0.5 log steps over the concentration range 10$^{-7}$ to 10$^{-1}$ M. Calibration tests were performed 1 to 3 days after conditioning in NH$_4$Cl. To determine response time, an empirical model was fit to the time series data for each step addition using the Chi$^2$ method; response time ($t_{95}$) was calculated as the time necessary for the sensor to reach 95\% of the total signal (Figure S9 in Supplemental Information). In addition, a water layer test\textsuperscript{34-36} was performed by exposing LIG SC-ISE to a 0.01 M solution of target ion (NH$_4$Cl or KNO$_3$) for 4 hours, followed by a 0.01 M solution of weakly interfering ion (NaCl) for 2 hours, and finally returned to the original solution for 18 hours while recording potential. Limit of detection (LOD) was calculated using the 3-sigma method (3\sigma) as previously reported.\textsuperscript{30}

After calibration in DI water, soil slurries were prepared using a 1:10 (w/w) ratio of soil (NAPT Reference Sample 2011-109) to DI water. After mixing and under continuous stirring conditions, the LIG SC-ISE and reference electrode were placed into the slurry. Stable sensor readings were obtained under baseline conditions (no NH$_4$Cl/KNO$_3$ added), and after spiking the slurry with exogenous primary ion solution (10$^{-2}$ to 10$^{-4}$ M). This procedure was repeated three
times with new soil slurries for each replicate and the calibration curves prepared. Next, a single point measurement was made in a new soil slurry (n=3) by adding $10^{-3}$ M NH$_4$Cl or KNO$_3$ to the sample. Calibration curves were used to calculate the observed NH$_4^+$ or NO$_3^-$ concentration and then the value was compared to the expected reading. Recovery was calculated based on the observed and predicted difference in potential following methods common to the literature. After validating calibration of LIG in soil slurries, soil column studies were conducted as a proof of concept application.

Soil samples for column studies were obtained from either a field supplemented with human urine (Rich Earth Institute, REI), or from a coastal mixed hardwood site (Hapludalfs) according to our previous work.$^{37}$ Hardwood soils were collected from a protected area at the University of Florida Whitney Laboratory for Marine Biosciences (Marineland, FL). Five soil cores (15 cm deep) were sampled and homogenized to create a composite sample. Soil samples were packed into a 5 cm diameter, 25 cm tall clear PVC soil column (490 mL) fitted with a commercial pH probe (Fisher), NO$_3$ probe (Vernier, Beaverton, OR) and a NH$_4^+$ electrode (Thomas Scientific, Swedesboro, NJ). Jumper wires (Arduino) were fixed to LIG electrodes using a conductive glue (0.5:1 g-graphite powder:g-liquid tape). LIG sensors were fixed adjacent to commercial probes for all soil column studies (see Figure S2-S3 in the Supplemental Information). Finally the columns were packed with soil samples and lightly tamped. Different water samples (either DI, 1.0 mM NH$_4$Cl, or 0.5 mM KNO$_3$) were added to the inlet manifold at the top of the soil column as noted, and output from each electrode was recorded at a sample frequency of 300 Hz.
Results and Discussion

Sensor Development & Material Characterization

All sensors were fabricated using adhesive Kapton polyimide sheets adhered to Epson printer paper (see Materials and Methods). The polyimide/paper substrate was treated with the pulsed UV laser at different pulse rates (10, 20, 30, 40, and 50 ms) to determine how laser energy density affected the material properties of LIG and consequently the electroactive nature of the material. First, the morphology of the LIG fabricated at distinct laser pulses was analyzed via SEM and displayed a general progressive smoothing of the graphene structure with increasing laser pulse rate (Figure 1a). More specifically, LIG samples created with a laser pulse rate of 10 ms were characterized by distinct rounded periodic features with high surface roughness. At laser pulse rates of 20 ms and above, these round nodule features flatten, becoming well-fused or continuous at 40 and 50 ms. Such changes in surface morphology are most likely due to the discrete, reduced heat transfer at lower pulse rates versus continuous, enhanced heat transfer at higher pulse rates. LIG created with higher pulse rates resulted in a relatively smooth, flat structure with lower surface roughness ($R_q=14.4\mu m$), while LIG created with lower pulses rates in stochastic formation of high surface area nodule structures ($R_q=14.4\mu m$) (also see Figure S4 in the Supplemental Information). These periodic features and fused surfaces each featured a high surface area structure, which is similar to other porous carbon materials used as transduction surfaces in SC-ISEs\textsuperscript{28}. At laser pulse rates of 30 ms and above, surface cracks were observed, which consequently may have impeded electronic transport (see 500x magnification SEM images in Figure 1 and cyclic voltammograms in Figure S8). LIG created with laser pulse rates of 20 ms exhibited a relatively well fused surface with periodic raised patterning without noticeable cracking.
Figure 4. Scanning electron microscopy (SEM) micrographs, Raman spectograms, and bending tests for LIG created with distinct laser pulse rates. Color coding refers to LIG that was created with laser pulse rates of 10 ms (black), 20 ms (orange), 30 ms (blue), 40 ms (green), and 50 ms (brown). (a) SEM images show smoother LIG surfaces with longer pulse rates but cracking above 20 ms pulse rates. (b) Raman spectra indicate a transition from sp\(^3\) to sp\(^2\) carbon with longer pulse rates. (c) Bending tests show low deviation from unbent state at bending radii under 30º for pulse rates lower than 40 ms.

Raman spectroscopy and X-ray photoelectron spectroscopy (XPS) were next used to analyze the material changes of the LIG with the distinct laser pulse treatments. Raman spectroscopy was employed to analyze the degree of graphitization and sp\(^3\) to sp\(^2\) carbon transformation of the polyimide after laser processing at the distinct pulse rates (Figure 1b). The Raman spectra displays a prevalent D, G, and 2D band: the D band (~1343 cm\(^{-1}\)) corresponds to the disordered-active Raman mode, the G band (~1574 cm\(^{-1}\)) due to C-C stretching modes is present for all graphitic materials, and the second order Raman feature called the 2D band (~2675 cm\(^{-1}\)), originated from second order zone-boundary phonons.\(^{38}\) The signal is spatially
heterogeneous, as revealed by Raman maps collected over 20 μm × 20 μm sample regions (see Figure S5 in the Supplemental Information). The average intensity ratio \( I_D/I_G \) is a measure of the disorder and defects and was calculated for each LIG sample (see SI). The \( I_D/I_G \) (~1) values analyzed from the Raman spectra indicate a high degree of disorder in all the LIG electrodes. Additionally, a clear 2D band is observed for 10 ms and 20 ms LIG samples. The presence of this 2D band in the Raman spectra suggests more complete graphitization of polyimide substrate and formation of graphene layers \(^{22,26}\) at shorter pulse rates. \( I_{2D}/I_G \) average ratios of 0.2 ± 0.1 and 0.3 ± 0.1 were calculated using a Lorentzian fitting function for 10 ms and 20 ms samples, respectively. These values are consistent with Wang et al., where they use a pulsed laser was for graphitization of a polyimide substrate. At longer laser pulse rates, the 2D band is not visible in the Raman spectra in combination with a broadened D and G peak with low intensity. Thus, further increase in pulse rates degrades the quality of the LIG samples, suggesting a laser-induced amorphization as the sample is exposed to longer pulse rates.\(^{39,40}\) XPS was performed to confirm graphene formation and measure sp\(^2\) carbon atom hybridization percentages for the distinct LIG substrates (see Figure S6 in the Supplemental Information). All laser pulse times displayed similar sp\(^2\) percentages, ranging from 76.2-78.3%. In particular, the 20 ms laser pulse rate sample was 77% sp\(^2\). These results are on par with previous reports that have converted sp\(^3\) hybridized polyimide to graphitic sp\(^2\) via laser processing.\(^{27-28,40}\)

Bending tests (concave) were conducted to investigate sensor flexibility and ensure proper sensor function with concave soil column tests (Figure 1c). Other than the sensors fabricated with a 50 ms laser pulse time, all sheet resistance changed by less than 20% for bending radii ≤ 30°. For the soil column studies used herein, the bending radius was ~5° (see Figure S7 in the Supplemental Information). Based on these results, any of the LIG sensors with
a laser pulse time \( \leq 40\text{ms} \) are valid for the soil column studies regarding flexibility. The lowest sheet resistance (15-20 kΩ/square) was obtained for laser pulse times of 30-40 ms, followed by resistance of 30-40 kΩ/square for laser pulse times of 10-20 ms; and \( \sim 60\text{ kΩ/square} \) for 50 ms. For laser pulse times of 40 ms and 50 ms, cracking was observed at a bending radii of 30° and 15° respectively. For laser pulse times below 40 ms, no cracking was observed below a bending radius of 45°.

To further analyze the effect of laser pulse time on electrode electroactivity, cyclic voltammetry was used to determine peak current in the presence of a negatively charged redox probe (KFeCN₆). Cyclic voltammograms (see Figure S8 in the Supplemental Information) displayed a quasi-reversible behavior for all LIG electrodes, with the peaks most distinct at a laser pulse time of 20 ms. Similarly, highest peak current (120 µA) and lowest peak potential separation (250 mV) were measured for LIG created with a laser pulse time of 20 ms. Peak current was lowest for LIG created with laser pulse times above 30 ms (\( \sim 40\mu\text{A} \)), and oxidative/reductive peaks were poorly defined, indicating poor electron transport.

Based on the analysis of morphology, surface roughness, Raman/XPS spectra, sheet resistance during bending, cracking behavior, and peak oxidative current, a laser pulse rate of 20 ms was used throughout the remainder of this study for developing SC-ISE. This laser pulse rate produced flexible, crack-free, high surface area graphene (77% \( \text{sp}^2 \)), with the highest peak current (120 µA) among all conditions tested.

**Sensor NH₄⁺ and NO₃⁻ Ion Calibration**

In an effort to quantify individual sensor variability, multiple electrodes from multiple fabrication batches were fabricated using the 20 ms laser pulse rate and functionalized with NH₄⁺- or NO₃⁻ selective SC-ion selective membranes (ISMs) (see Materials and Methods).
Chronopotentiometry plots (Figure 2a,c) were used to construct calibration plots (inset of Figure 2a,c) using an averaged sampling of steady state values at a given concentration. Sensitivity and detection limit (3σ) were calculated from the linear range of calibration plots for each sensor, and average response time \( t_{95} \) was calculated by taking the mean of \( t_{95} \) for individual steps in the linear region. The average sensitivity for NH\(_4^+\) electrodes in DI water was 51.7 ± 7.8 mV/decade, and the average sensitivity for NO\(_3^-\) electrodes in DI water was -54.8 ± 2.5 mV/decade. The near-Nernstian behavior for NH\(_4^+\) electrodes was likely a result of a phase boundary equilibrium between LIG functional groups and ions in solution, wherein the concentration of charged groups on the LIG induce a change in local activity that is sufficiently large to reduce the net potential; this thermodynamic effect has been previously described for other electrode assemblies. Amemiya et al\(^{42}\) noted that this sub-Nernstian behavior can be caused by primary and secondary ions of any charge that complex with the ionophore, but this effect is not likely for the calibration experiments conducted in DI water where there are no secondary ions present. Given the heterogeneous nature of the LIG surface after graphitization of polyimide, thermodynamic interactions with charged groups on LIG is the most likely cause for the near-Nernstian behavior. The average limit of detection (LOD) in DI water for NH\(_4^+\) electrodes was 28.2 ± 25.0 uM and 20.6 ± 14.8 uM for NO\(_3^-\) electrodes. The average \( t_{95} \) for all sensors was 5.9 ± 4.1s (see Figure S9 in the Supplemental Information).

In preparation for soil column testing, SC-ISEs were further calibrated by measuring NH\(_4^+\) or NO\(_3^-\) in a complex soil slurry matrix spiked with NH\(_4\)Cl or KNO\(_3\) (Figure 2b, d). The average slope of the NH\(_4^+\) LIG SC-ISE calibration equation in soil slurry for three different tests was 55.2 mV ± 3.6 mV/decade and the average slope of the NO\(_3^-\) LIG SC-ISE calibration equation for five different tests was -53.4 mV ± 1.1 mV/decade. Using this equation, observed
values for a single point measurement (10^{-3} \text{ M NH}_4\text{Cl or KNO}_3) were compared to potential values expected at this concentration. The average recovery percentage was 96% for NH$_4^+$ and 95% for NO$_3^-$ (Table S2), which demonstrates excellent sensor accuracy in a complex sensing matrix.

Figure 5. Chronopotentiometry plots for NH$_4^+$ and NO$_3^-$ calibration in DI water and soil slurry solutions for the LIG SC-ISEs. NH$_4^+$ electrodes in (a) DI (calibration curve shown in inset) and (b) soil slurries. NO$_3^-$ electrodes in (c) DI (calibration curve shown in inset) and (d) soil slurries.

Experiments were performed to investigate the susceptibility of the LIG ISEs in developing an inner water layer (Figure 3). In SC-ISEs, a detrimental water layer can accumulate at the interface of the ion selective membrane and transduction layer (LIG in this study). Previous work$^{34}$ has suggested that a hydrophobic surface can prevent the formation of a water layer. The measured contact angle of the LIG electrodes created with a 20 ms laser pulse
rate was 80.0 ± 0.65 degrees (indicating a surface that is nearly hydrophobic, see Figure S10), and the LIG SC-ISE did not display characteristic detrimental drift\textsuperscript{36} associated with a water layer upon change of solution from primary (NH\textsubscript{4}\textsuperscript{+}/NO\textsubscript{3}\textsuperscript{-}) to weakly interfering (Na\textsuperscript{+}/Cl\textsuperscript{-}) ions. Moreover, both NH\textsubscript{4}\textsuperscript{+} and NO\textsubscript{3}\textsuperscript{-} LIG SC-ISEs showed minimal long term drift (0.93 mV/hr for NH\textsubscript{4}\textsuperscript{+} sensors and -5.3 μV/hr for NO\textsubscript{3}\textsuperscript{-} sensors) in 0.01 M NH\textsubscript{4}Cl or 0.01M KNO\textsubscript{3}, respectively.

Figure 3. Representative water layer test for NH\textsubscript{4}\textsuperscript{+} (a) and NO\textsubscript{3}\textsuperscript{-} (b) LIG SC-ISEs. Concentration for each indicated solution was 0.01 M.

NH\textsubscript{4}\textsuperscript{+} and NO\textsubscript{3}\textsuperscript{-} Ion Sensing in Soil Columns

To validate the LIG sensors, soil column studies were conducted with NH\textsubscript{4}\textsuperscript{+} and NO\textsubscript{3}\textsuperscript{-} SC-ISEs adhered to the inside of a 5 cm column and placed adjacent to corresponding NH\textsubscript{4}\textsuperscript{+} and NO\textsubscript{3} commercial ISE probes (Figure 4). Electrodes were within 1 cm of commercial electrodes and at the same column depth. A sterilized Florida soil (Hardwood) was packed into the column, and DI water was first flushed in the column (100 mL) to obtain a baseline measurement. Baseline NH\textsubscript{4}\textsuperscript{+} for LIG and commercial electrodes (0.05 mM, and 0.03mM, respectively) were not significantly different; similarly, baseline NO\textsubscript{3}\textsuperscript{-} for both electrodes (≈ 0.01 mM) was not different (p < 0.0001, α = 0.05). Next, 1mM NH\textsubscript{4}Cl or 0.5 mM KNO\textsubscript{3} was added to the column in two separate pulses (Figure 4a-b). After spiking nitrogen solution, a peak signal was obtained,
indicating a stable reading for each electrode (noted as “peak”), and then 100mL of DI water was used to flush the column while recording sensor output (noted as “flush”). For all tests, the peak response and flush response occurred within 2.5 min of adding solution, and the signal was stable for at least 60 seconds (in some tests the electrode began to dry after reaching steady state). The average baseline, peak, and flush for two multiple cycles are shown in Figure 4c-d (n=3 replicate tests). For all tests, the LIG SC-ISE was slightly lower than the commercial electrodes, and in most tests the commercial NO₃⁻ electrodes overpredicted the NO₃⁻ concentration. Based on an ANOVA test, there was no significant difference (p < 0.001, α = 0.05) between LIG and commercial electrodes in any test. In addition, there was no significant difference between the response times for each electrode in the column studies.

**Figure 4.** Soil column studies with LIG SC-ISEs compared to commercial ion probes. Representative real time plots of (a) NH₄⁺ and (b) NO₃⁻ ions during soil column flush.
experiments. Average nitrogen measured with LIG and commercial electrodes for (c) \( \text{NH}_4^+ \) and (d) \( \text{NO}_3^- \) ions. Error bars represent standard deviation of the arithmetic mean (n=3).

As a final proof of concept, nitrogen soil column studies were repeated using LIG SC-ISEs for a nitrogen augmented soil (noted as Rich Earth) and a N-deficient soil (noted as Hardwood). Each column study was conducted for a load of 100 mg-N/kg-soil, which is on the high end of fertilizer loads for these types of soil.\(^{44}\) For the \( \text{NH}_4^+ \) column studies (Figure 5a), both REI soils (138 ± 20 µM) and Hardwood soils (31 ± 10 µM) had baseline levels which were not significant, although the REI soil was significantly higher than the Hardwood soil, as expected given that the REI soil is irrigated with human urine.\(^{45}\) After adding a load of 50mg-N/kg-soil (5 g of soil), a characteristic pulse was recorded similar to the representative plots in Figure 4, and the mean of the steady state value used to represent peak runoff nitrogen. For REI soil, the peak \( \text{NH}_4^+ \) (1.6 ± 0.1 mM) was significantly higher than Hardwood soil (0.8 ± 0.1 mM) and represented only 57% of the input N on a mass basis. This is common for \( \text{NH}_4^+ \) column studies, as the \( \text{NH}_4^+ \) ion readily binds with particulate matter and biological materials within the column; thus, the mobility of soil \( \text{NH}_4^+ \) is relatively low.\(^{46}\) After flushing the column with 100mL of DI water, the \( \text{NH}_4^+ \) for REI (180 ± 80 µM) was higher than Hardwood (60 ± 40 µM), but only slightly higher than baseline values. This effect is also expected, as the desorption rate of \( \text{NH}_4^+ \) is not homogenous as the ion is washed from the column, given the large number of functional groups which can interact with \( \text{NH}_4^+ \).

For the \( \text{NO}_3^- \) column studies (Figure 5b), both REI soils (19 ± 4 µM) and Hardwood soils (7 ± 3 µM) had insignificant baseline levels, with REI slightly higher than Hardwood. After adding a 50 mg-N/kg-soil, the peak \( \text{NO}_3^- \) from REI soil (589 ± 55 µM) was not as high as the Hardwood soil (727 ± 61 µM). These levels represent 75% and 87% recoveries of N on a mass
basis for REI soil and Hardwood soil, respectively, which is expected, as soil NO$_3^-$ is highly mobile. After a DI water flush, the NO$_3^-$ concentration for REI soil (85 ± 23 µM) was not significantly different than Hardwood soil (73 ± 25 µM), and this remaining fraction accounts for approximately 10% of the total N load. The unaccounted fraction of NO$_3^-$ that remained in the column from the REI soils (approximately 10-15%) was assumed to be trapped within interstitial voids or adsorbed to biological material, although further studies are investigating this hypothesis in detail.

Figure 5. Soil column studies with LIG nitrogen electrodes. (a) Average NH$_4^+$ concentration during column studies with addition of 1 mM NH$_4$Cl. (b) Average NO$_3^-$ concentration during column studies with addition of 1 mM KNO$_3$. Error bars represent standard deviation of the arithmetic mean (n=3).

Conclusions

LIG electrodes fabricated on polyimide/Epson printer paper with distinct laser pulse rates (10, 20, 30, 40, and 50 ms) were demonstrated. The simple fabrication method employed represents the first example of a SC-ISE on LIG. LIG samples created with a laser pulse rate of 20 ms were determined to be the most effective electrode based on SEM, XPS, Raman Spectroscopy, and CV analysis. Building on this result, LIG electrodes were functionalized with
an NH$_4^+$ or NO$_3^-$ selective ionophore membrane, resulting in a SC-ISE used to measure soil nitrogen. ISEs were characterized in DI water and showed near Nernstian sensitivity of 51.7 ± 7.8 mV/decade (NH$_4^+$) and -54.8 ± 2.5 mV/decade (NO$_3^-$), detection limit of 28.2 ± 25.0 uM (NH$_4^+$) and 20.6 ± 14.8 uM (NO$_3^-$), and a linear sensing range of 10$^{-5}$-10$^{-1}$ M. A water layer test indicated the absence of a detrimental water layer known to form in SC-ISEs. The LIG SC-ISE also employed within a complex sensing matrix (soil) and exhibited 96% recovery of added NH$_4^+$ and 95% recovery of added NO$_3^-$. Soil nitrogen tests were conducted alongside commercial NO$_3^-$ or NH$_4^+$ electrodes, and the flexible LIG SC-ISEs were not significantly different, validating the use of this new low cost electrode for measuring soil nitrogen. In a proof of concept study, the LIG electrodes were used to study the first flush response of nitrogen rich soils and nitrogen poor soils in column studies, and the results demonstrate that the sensors are accurate for in field testing, paving the way for future development of bury-and-forget soil sensors.

On a broader scale these LIG based electrodes are comparable to the recent trend of creating low-cost carbon-based electrodes via screen printing$^{47-48}$ and inkjet printing$^9,49$ solution-phase flakes in lieu of developing electrodes from more costly CVD graphene synthesis processes. However, these solution-phase graphene printing techniques require metal screen masks, expensive printing equipment, development of a jettable ink, or post-print annealing processes that have complicated their fabrication. The approach described herein represents a one step, low cost manufacturing pathway for LIG electrodes and is the first example using LIG for use in ion sensing. Additionally, no metals such as metallic nanoparticles were needed to improve the electroreactive nature of the electrode, hence these LIG electrodes are amenable to scalable roll-to-roll manufacturing and suitable for use in disposable sensor technologies.
Further work with these sensors could result in improved methods of in field precision agriculture monitoring\textsuperscript{50}, water quality testing\textsuperscript{51}, wearable sweat biosensing and energy harvesting\textsuperscript{52-55}, and point of care diagnostics\textsuperscript{56-58}.

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Author Information

Corresponding Author: *E-mail: jcclauss@iastate.edu. Phone: 515-294-4690

ORCID

Jonathan C. Claussen: 0000-0001-7065-1077

Notes

The authors declare no competing financial interest.

References


**Supporting Information**

The Supporting information is available free of charge on the ACS Publications website at DOI: schematics of electrodes used in all experiments, photograph of soil column sensor arrangement without soil, photograph of soil column with soil, optical profilometry of LIG surfaces at various laser pulse rates, Raman spectroscopy maps of the $I_D/I_G$ ratio of a 20 × 20 $\mu$m$^2$ area of LIG for all laser pulse rates, Table of $I_D/I_G$ ratios collected from Raman spectrograms of LIG at all laser pulse rates, XPS C1s peak deconvolution for 20 ms LIG sample,
bending tests for LIG electrodes produced at all laser pulse rates, soil slurry recovery tests for NO$_3^-$ and NH$_4^+$ LIG SC-ISEs, representative chronopotentiometry plots (experimental and fitted model) for calculation of t95, photograph of water droplet on LIG electrode used for contact angle measurement at a laser pulse rate of 20 ms

Supplemental Information

**Figure S1.** *(Left)* Photograph of five LIG SC ISEs on a single polyimide swatch. *(Right)* Illustration of SC-ISE ion sensing. *(Bottom)* Representative electrode used in soil column studies. Passivated regions are shown, as well as bonding pads, working electrode, and reference electrode.
**Figure S2.** Photograph of LIG-ISE and commercial nitrogen probe. Both probes were for measuring nitrate in soil column studies.

**Figure S3.** Soil column fitted with commercial soil sensors for measuring pH and nitrogen to validate LIG nitrogen sensors.
Figure S4. Optical profilometry of LIG surfaces at various laser pulse rates. (a-b) 2D profile and representative line scan for a laser pulse rate of 10 ms, respectively. (c-d) Profile and line scan for a pulse rate of 50 ms. The following equation was used to calculate the $R_q$ for each line profile. $R_q = RMS = \sqrt{\frac{1}{n} \sum z^2}$
Figure S5. Raman maps of the $I_D/I_G$ ratio of a $20 \times 20 \mu m^2$ area of LIG created with 10, 20, 30, 40, and 50 ms distinct laser pulse rates. A higher $I_D/I_G$ ratio indicates more disorder and defects. Raman spectra at specific locations of the scanned area are shown in the right panel. White colored pixels correspond to locations where spectral peaks were not observed.
Table S1. Average $I_D/I_G$ ratios collected from Raman spectrograms of LIG created with 10, 20, 30, 40, and 50 ms laser pulse rates. A higher $I_D/I_G$ ratio indicates more disorder and defects.

<table>
<thead>
<tr>
<th>Laser Pulse Rate [ms]</th>
<th>Average $I_D/I_G$ [-]</th>
</tr>
</thead>
<tbody>
<tr>
<td>10</td>
<td>1.0 ± 0.1</td>
</tr>
<tr>
<td>20</td>
<td>1.1 ± 0.1</td>
</tr>
<tr>
<td>30</td>
<td>0.99 ± 0.03</td>
</tr>
<tr>
<td>40</td>
<td>1.1 ± 0.1</td>
</tr>
<tr>
<td>50</td>
<td>1.0 ± 0.1</td>
</tr>
</tbody>
</table>

Figure S6. XPS C1s peak deconvolution for 20 ms LIG sample, showing (dark red) sp$^2$ carbon, (light green) C-O bond, (light blue) C=O bond, and (light purple) satellite components.
Figure S7. Bending tests for LIG electrodes produced at different laser pulse rates show low deviation from an unbent state at bending radii under 30° for pulse rates lower than 40 ms.

Figure S8. CV data for LIG electrodes fabricated with 10, 20, 30, 40, and 50 ms laser pulse rates using a ferri/ferrocyanide redox probe in DI at a scan rate of 20 mV/s.
Table S2. Soil slurry recovery tests for nitrate and ammonium LIG SC-ISEs.

\[
\text{NO}_3^- \\
\begin{array}{cccc}
\text{Potential (mV)} & \text{Measured NO}_3^- (\text{mM}) & \text{Actual NO}_3^- (\text{mM}) & \text{Recovery (\%)} \\
108.2 & 0.049 & 0.05 & 97.8 \\
112.6 & 0.048 & 0.05 & 96.5 \\
146.3 & 0.042 & 0.05 & 84.8 \\
119.7 & 0.049 & 0.05 & 98.8 \\
109.2 & 0.048 & 0.05 & 96.2 \\
\end{array}
\]

\[
\text{NH}_4^+ \\
\begin{array}{cccc}
\text{Potential (mV)} & \text{Measured NH}_4^+ (\text{mM}) & \text{Actual NH}_4^+ (\text{mM}) & \text{Recovery (\%)} \\
139.9 & 9.72E-04 & 0.001 & 97.2 \\
135.9 & 8.24E-04 & 0.001 & 82.4 \\
142.8 & 1.10E-03 & 0.001 & 109.8 \\
\end{array}
\]

Figure S9. Representative chronopotentiometry plots for calculation of t95 displaying actual and fitted model data with a 0.5 log increase in target ion concentration.
Figure S10. Photo of water droplet on LIG electrode used for contact angle measurement at a laser pulse rate of 20 ms