# Fluorescence Measurements of Denatured Proteins within Electrospray Droplets

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# OVERVIEW

**Purpose** To investigate the conformation of biomolecules within ESI droplets.

**Method** Fluorescence measurements within the electrospray plume. Fluorophore: single tryptophan residue (Trp-59) in the protein cytochrome *c.*

**Results** Different solution and droplet denaturation curves, attributed to differences between bulk and droplet surface environments.

# INTRODUCTION

Spectroscopic measurements can help to investigate the conformation and structural changes that biomolecules undergo during their transfer from solution to the gas phase via electrospray ionization (ESI). Efforts in our laboratory have recently been directed toward using fluorescence spectroscopy to investigate the effects that the ESI process may have on the structure and conformation of peptides and proteins.

In this work, the fluorescence of horse heart cytochrome *c* (HHCC) during ES was investigated. HHCC is a 104-amino acid protein containing a single tryptophan residue on position 59 (Trp-59) and a heme group covalently attached to Cys-14 and 17. In its native conformation, the indole side chain of Trp-59 is hydrogen bonded to the heme. The proximity of these two residues results in the quenching of the Trp-59 fluorescence via resonance energy transfer to the heme.

As the protein unfolds, the effective distance between Trp and the heme increases and a subsequent increase in the emitted fluorescence results. These characteristics make of HHCC a good probe for protein folding studies using fluorescence spectroscopy.

The conformation of HHCC molecules within ES droplets is investigated as a function of alcohol concentration. By varying the amount of alcohol in the electrospray solutions, protein denaturation is induced and the resulting conformational changes are studied by monitoring the fluorescence of Trp-59. For comparison, bulk-solution fluorescence experiments were also performed under similar denaturation conditions. The fluorescence of the amino acid derivative N-acetyl-L-tryptophan-amide (NATA) was also investigated. The onset and extent of protein fluorescence is evaluated, and differences between solution and electrospray results are discussed.

# METHODS

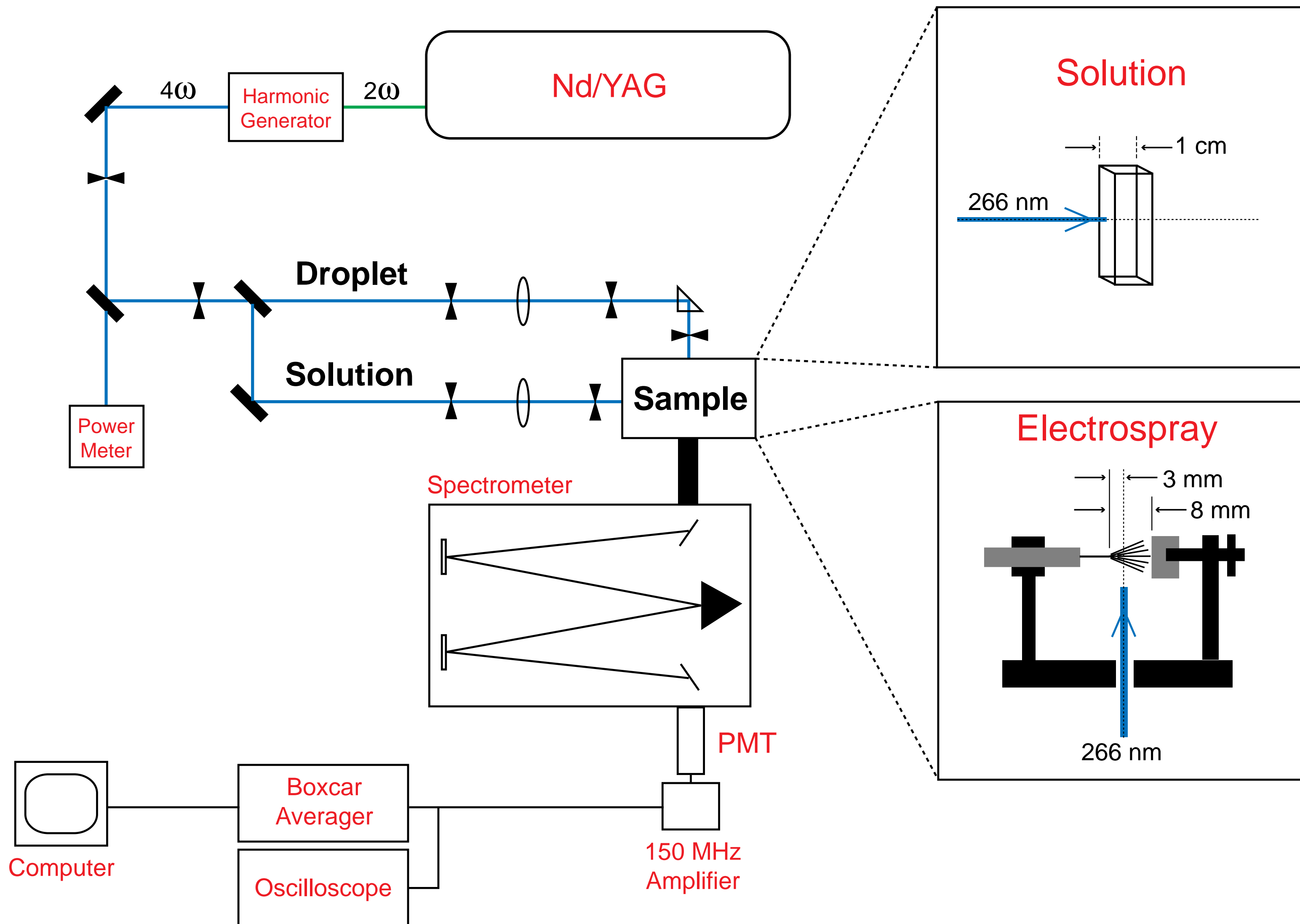
## Instrumentation

- The instrumental setup (Figure 1) is designed so that both solution and electrospray fluorescence measurements can be performed using the same excitation/detection assembly.
- Light source: 4th harmonic of Nd:YAG laser (266 nm)
- Laser intensity (solution): 20 $kW/cm^2$ (3 μJ/pulse)
- Laser intensity (electrospray): 0.9 $MW/cm^2$ (0.6 mJ/pulse)

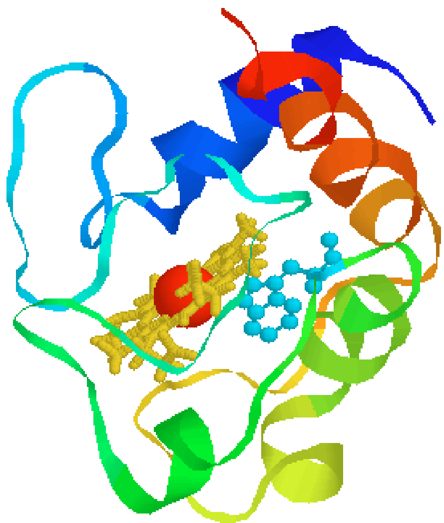
## Fluorescence measurements

The conformation of cytochrome *c* within ES droplets was investigated by measuring the protein fluorescence while electrospraying solutions of different alcohol content. Electrospray results were then compared with similar denaturation experiments in solution. The fluorescence of the amino acid derivative NATA was also measured both within the spray plume and in solution.

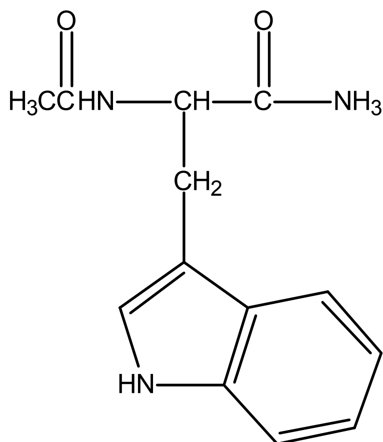
Figure 1: Fluorescence Setup



Cytochrome *c*



NATA




## Analytes

cytochrome *c*
NATA

## Denaturants

Methanol
1-Propanol

## Electrospray

Flow rate = 2 μL/min
Concentration = $3 \times 10^{-5}$ M
pH = 4

## Solution

10 mm path length cuvette
Concentration = $3 \times 10^{-6}$ M
pH = 4

# RESULTS

Figure 2 shows the electrospray and solution fluorescence spectra obtained for cytochrome *c* in different $H_2O$/MeOH solutions. At low alcohol concentrations, no fluorescence is detected indicating protein molecules within droplets and in bulk solution have folded conformations. At high methanol content, the protein losses its native conformation. Under these conditions, fluorescence is detected and the characteristic spectra are observed.

By measuring the extent of fluorescence at different solvent compositions, the protein denaturation process can be followed. Figures 3 and 4 show plots of total fluorescence as a function of MeOH and 1-PrOH, respectively. Electrospray and solution fluorescence results for cytochrome *c* and NATA are presented.

Figure 5 and 6 compare electrospray and solution protein denaturation curves for MeOH and 1-PrOH, respectively.

Comparison of electrospray and solution results reveals three distinct features:

- NATA exhibits higher fluorescence intensity than cytochrome *c* at all solvent compositions investigated for both electrospray and solution measurements.

  → Incomplete protein unfolding.

- Different cytochrome *c* denaturation curves obtained from electrospray and solution measurements.

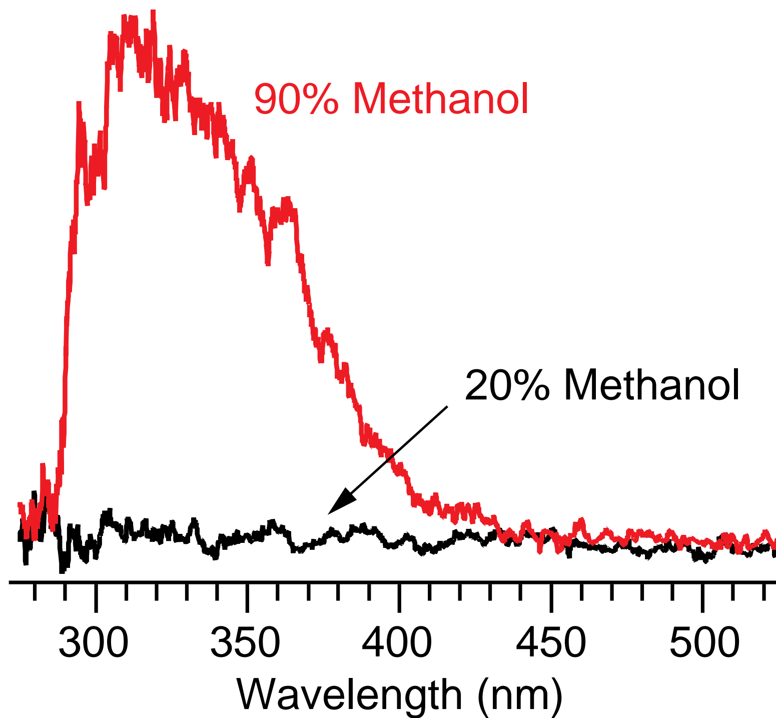
  → Different droplet and solution conformations for similar alcohol content solutions.

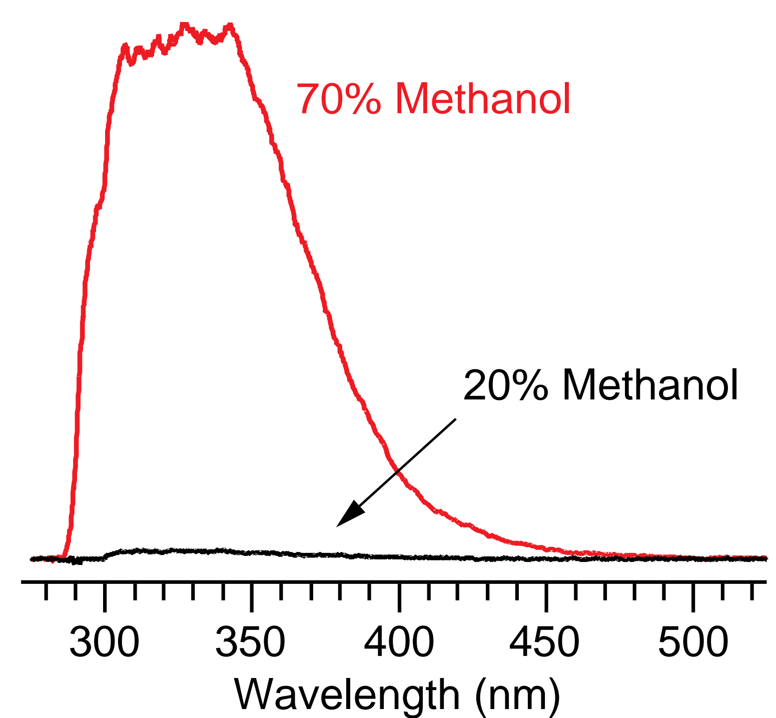
- Fluorescence of NATA during electrospray increases dramatically with alcohol concentration.

  → Different droplet and solution environments.

# Figure 2

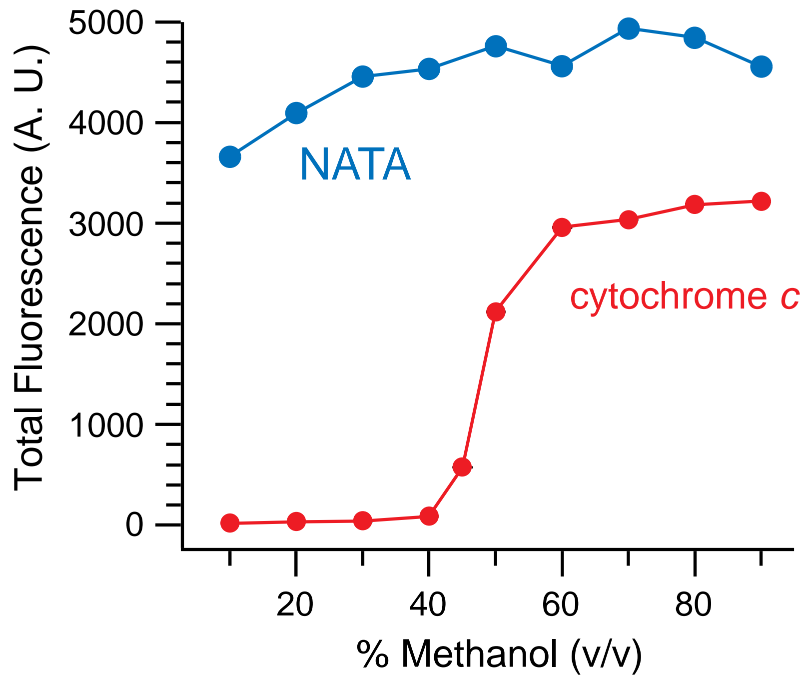
## Cytochrome *c* Fluorescence Spectra

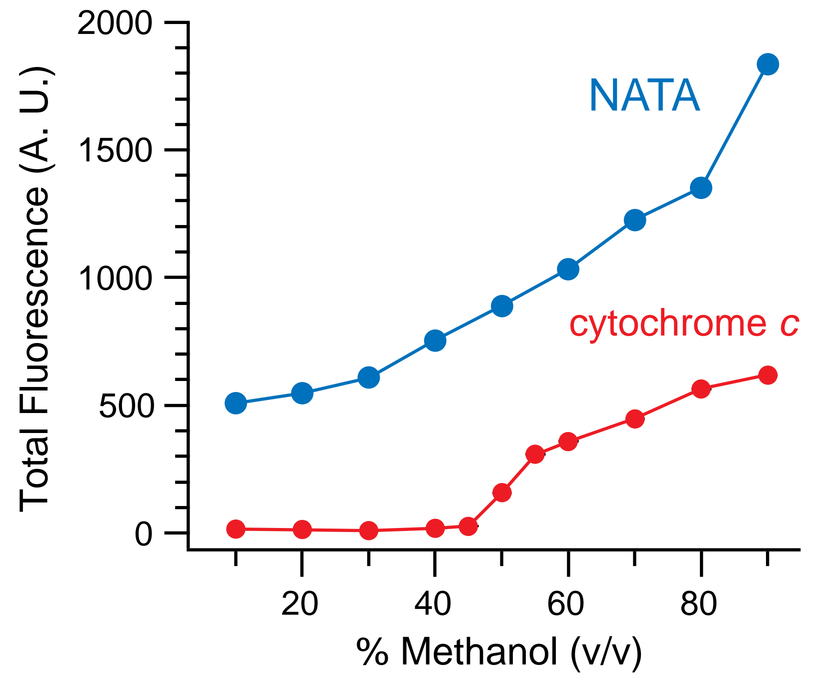


# Figure 3

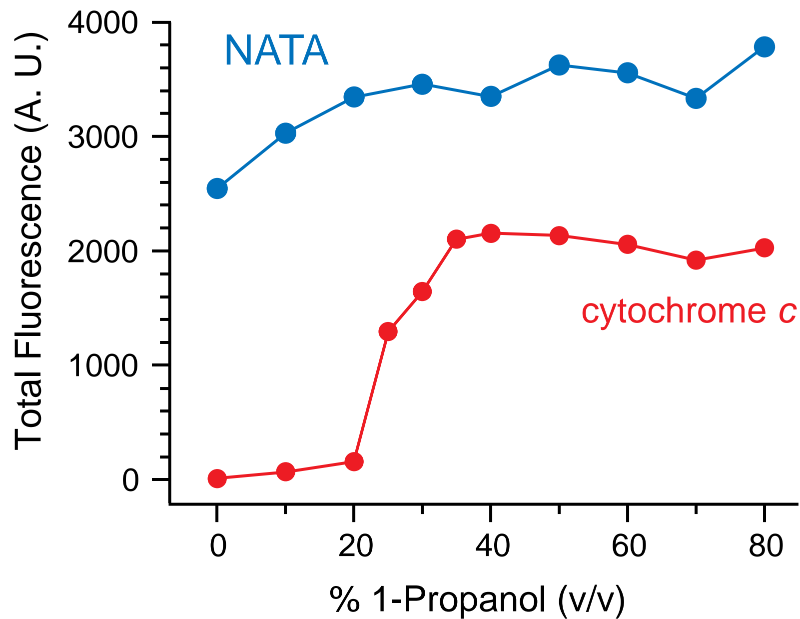
## Cytochrome *c* and NATA Fluorescence as a function of Methanol Content



# Figure 4

## Cytochrome *c* and NATA Fluorescence as a function of 1-Propanol Content



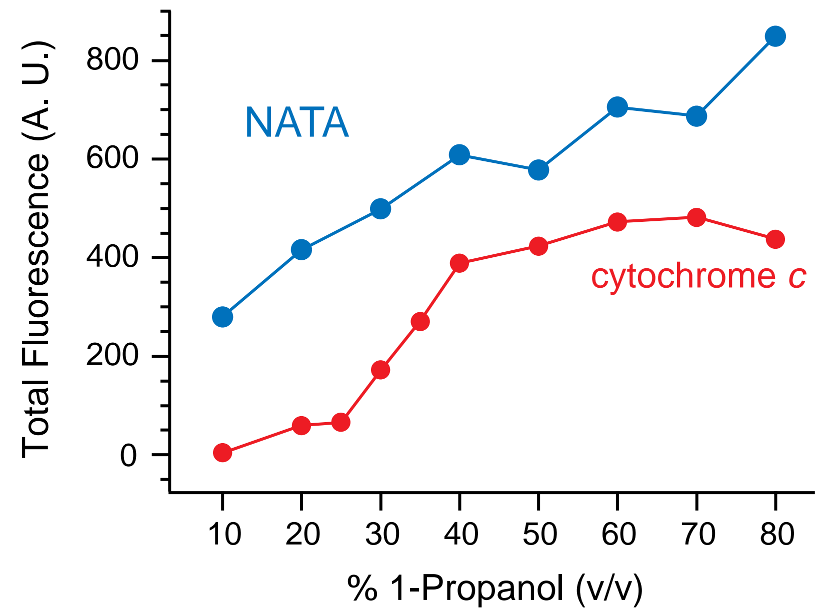



Figure 5

Cytochrome *c* Methanol-Denaturation Curves
Solution vs Electrospray

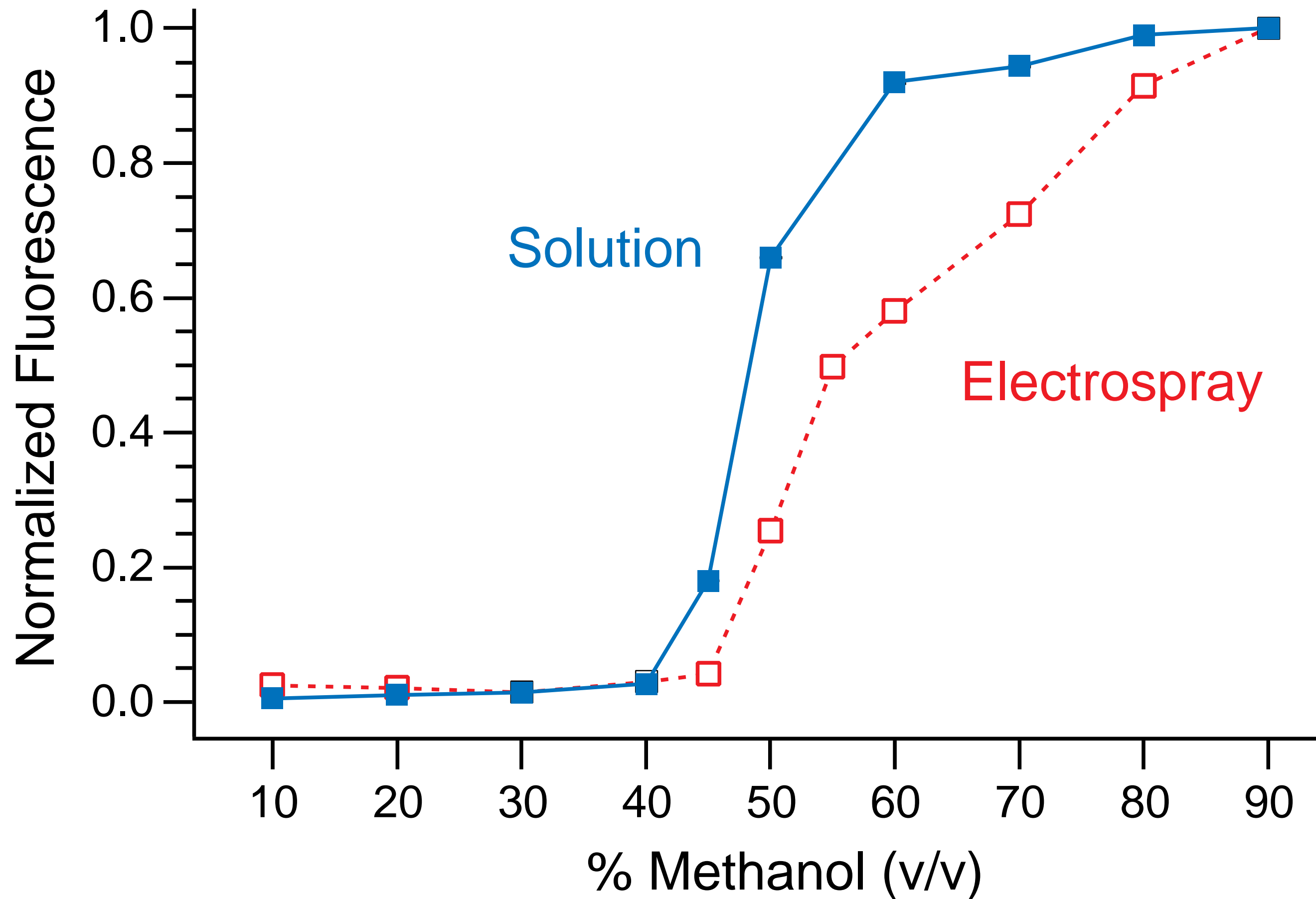
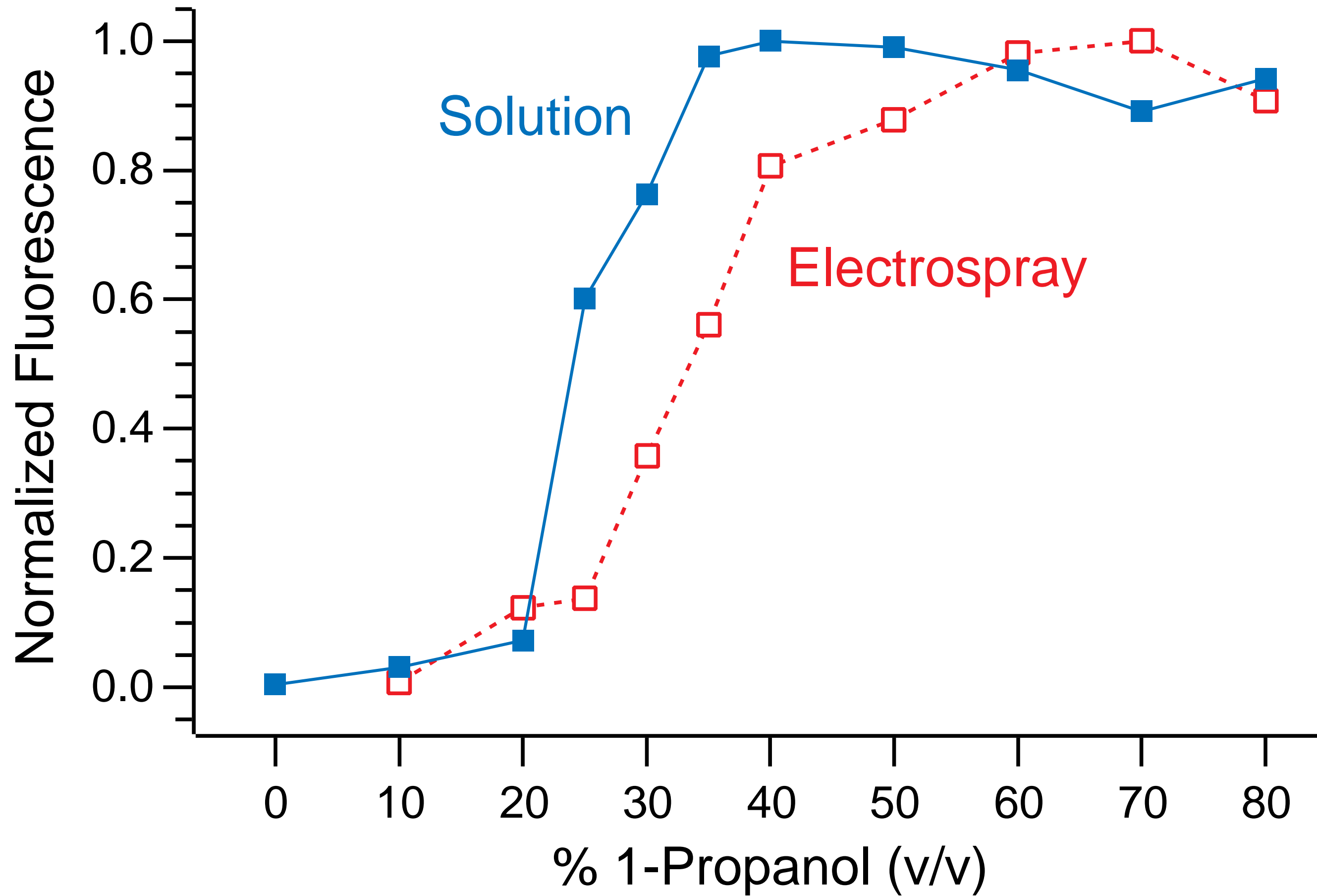


Figure 6

Cytochrome *c* 1-Propanol-Denaturation Curves
Solution vs Electrospray



# Droplet Fluorescence Model

Discrepancies between electrospray and solution fluorescence experiments on cytochrome *c* suggest the protein has adopted a conformation within the droplets that is distinct from that in bulk solution. Here, we present a model that describes the fluorescence from droplets. This model suggests that the experimental differences observed can result from the effect the droplet/air interface has on denatured cytochrome *c* molecules.

Figure 7 shows results from measurements of cytochrome *c* fluorescence, $F$, from electrospray droplets. The data display a dependence on the protein concentration, $M$, characterized by

$$F \propto \frac{1}{1+\left(31R_o^{6}\right)M^{3}}$$

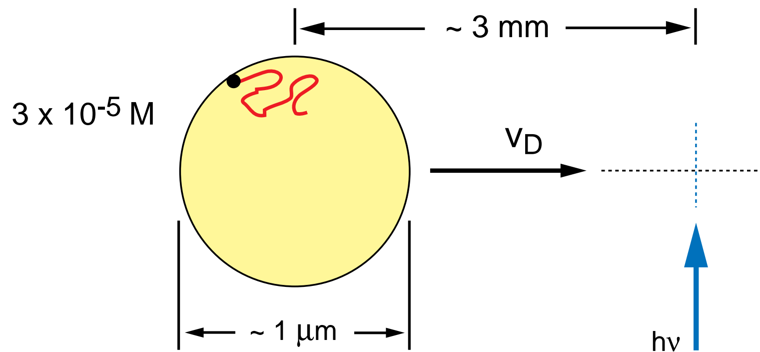
where $R_o$ is the distance characterizing the range of dipole-dipole interaction. This form is characteristic of a singlet-singlet energy transfer process. A value of $R_o$ = 50 Å is obtained from the cyto-

chrome *c* data. This intermolecular separation will only be sufficient to quench the fluorescence by singlet-singlet energy transfer if the molecules remain adsorbed at the droplet/air interface as a result of the increased surface density. Quenching is not expected to dominate at the lower densities within the bulk.

The cytochrome *c* fluorescence emitted from $H_2O$/Alcohol solutions can be modeled by assuming a two-state transition between the native state and a less compact conformation in the presence of alcohol. The change in free energy, $\Delta G_{un}$, associated with a denaturing transition induced by an alcohol concentration, [A], can be represented by $\Delta G_{un} = \Delta G^{o}_{un} - m[A]$. Where $\Delta G^{o}_{un}$ is the free energy of the transition in the absence of alcohol and $m = -(d\Delta G^{o}_{un}/d[A])$. Figure 8 shows the fluorescence data fits and parameters obtained from solution and droplet denaturation experiments using MeOH and 1-PrOH solutions. The lower $\Delta G^{o}_{un}$ and *m* values for droplet fluorescence measurements may be interpreted in terms of the different environment at the droplet/air interface.

# Droplet Fluorescence Model

**Protein diffusion to droplet/air interface:**




$v_D = N\langle Z\rangle E_z\mu$

for $\mu \sim 1$ cm²/V.s

$D \sim 10^{-5}$ cm²/s

$N_{surface}/N \sim 60 - 80$ %

(~100% convection)

**Fluorescence emission during absorption:**

Adsorption at non-polar interface[1]

Energy transfer quenching observed

($R_o \sim 50$ Å)[2]

Protein surface area exposed to alcohol?

**Uncertainties:**

Alcohol dependence of NATA fluorescence

Surface composition:

$\gamma_{water}$ = 73 dynes/cm

$\gamma_{alcohol}$ = 22 dynes/cm

Droplet size

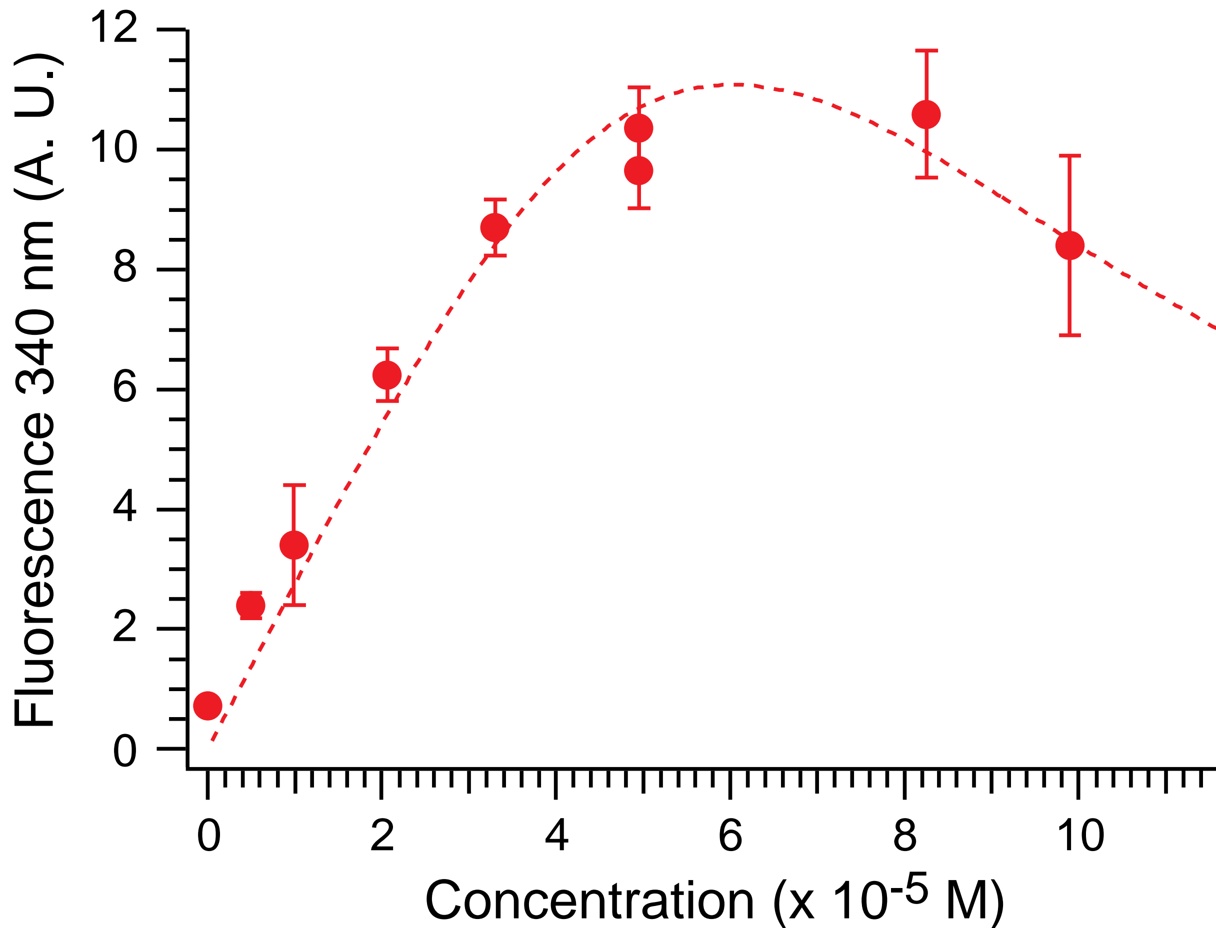
Electrostatics

References: 1. Tilton, R. D.; Robertson, C. R.; Gast, A. P. *Langmuir* **1991**, *7*, 2710-2718.

2. Forster, Th. In Biological Physics; Mielczarek, E. V.; Greenbaum, E.; Knox, R. S., Eds.; American Institute of Physics: New York, NY, 1993.

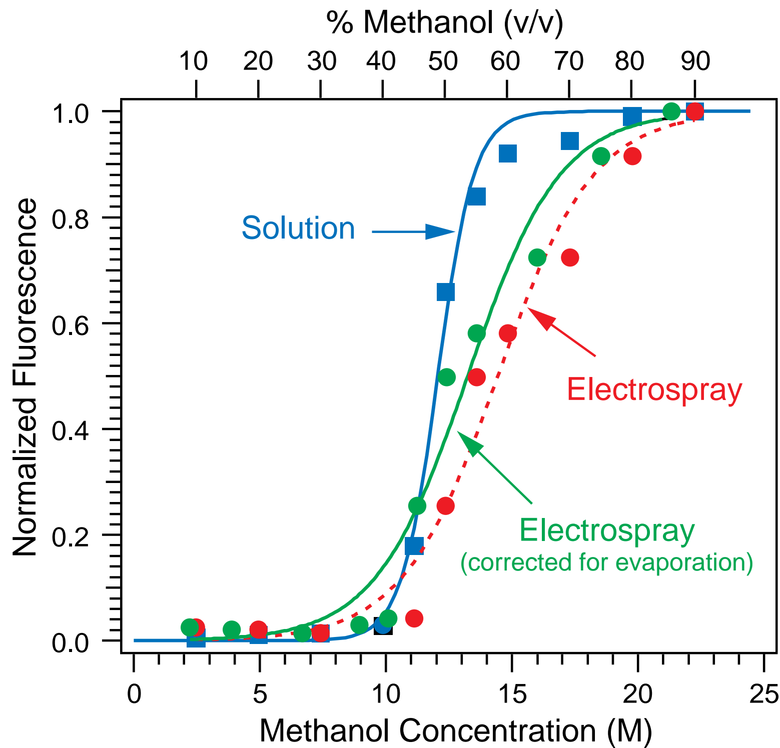
Figure 7

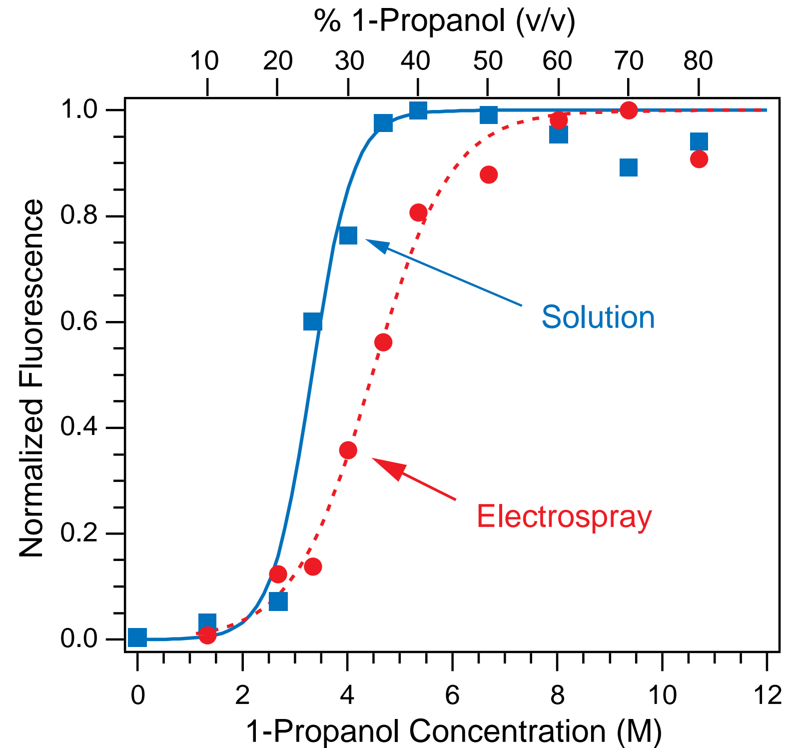
Cytochrome *c* Electrospray Fluorescence as a function of Protein Concentration



# Figure 8

## Cytochrome *c* Fluorescence Fitting Parameters




Solution
$\Delta G^{o}_{un}$ = 9-11 kcal/mol
$m$ = 0.7-0.9 kcal/mol·M
Electrospray
$\Delta G^{o}_{un}$ = 3.9-5.1 kcal/mol
$m$ = 0.3-0.4 kcal/mol·M

Solution
$\Delta G^{o}_{un}$ = 4-6 kcal/mol
$m$ = 1.2-1.8 kcal/mol·M
Electrospray
$\Delta G^{o}_{un}$ = 3.2-4.0 kcal/mol
$m$ = 0.7-0.9 kcal/mol·M

# CONCLUSIONS

- The conformation of cytochrome *c* molecules within ES droplets has been investigated using laser induced fluorescence, and results have been compared with bulk solution measurements.
- From electrospray vs solution denaturation experiments:
    - Protein adopts both folded and partially unfolded conformations in solution and within the droplets.
    - Both cytochrome *c* and NATA fluorescence measurements suggest environment in droplets is different from that of bulk solution.
    - Different protein denaturation curves for electrospray may be due to location of proteins at the droplet/air interface.

# FUTURE DIRECTIONS

We have demonstrated that the combination of fluorescence spectroscopy and electrospray ionization provides insights into the effects the electrospray process has on solution analytes. Fluorescence studies on trapped ions would significantly increase our knowledge about the structure and conformation of gas-phase biomolecules. Current efforts in our laboratory are being directed toward this goal.

# ACKNOWLEDGEMENTS

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