Interpreting Crowding Effects on FRET Signals for Protein Kinetics Analysis

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ABSTRACT

In biological studies in vitro and in vivo, techniques involving Förster resonance energy transfer (FRET) and FRET quantification use the interaction of CyPet-SUMO1 and its E2 ligase, YPet-Ubc9, to determine the dissociation constant (K_D). Dipole-dipole resonance interactions, where energy transfers from an excited donor to an acceptor chromophore, allow the detection of molecular interactions to elucidate protein interactions in many regulatory cascades spanning signal transduction, medical diagnostics, and optical imaging. This study aims to explore how protein-protein interactions are affected by the crowded environment typically found within cells using FRET signals. An in vitro assay using a 96-well plate was conducted using varying concentrations of bovine serum albumin (BSA) to simulate crowded conditions and determine their effect on K_D values. FRET measurements were conducted in a solution phase to mimic the protein interaction affinity in living cells. In contrast, other K_D measurement methods such as radio-labeled ligand binding assay, surface plasmon resonance (SPR), or isothermal titration calorimetry (ITC) require extensive preparation or orientation on solid surfaces, making them less representative for such assessments. Emission wavelengths from CyPet-SUMO1 (414 nm to 475 nm) and YPet-Ubc9 (475 nm to 530 nm) were obtained to determine fluorescence signals along with K_D. A comparison between protein interactions in crowded and uncrowded settings was made with varying K_D value results. This investigation provides insights into protein interactions and cellular crowding, with potential implications for pharmaceuticals, bioseparations processes, and drug discovery targeting protein-protein interactions.

KEYWORDS: protein-protein interactions, K_D, qFRET, FRET signals, bovine serum albumin (BSA), crowded proteins, SUMOylation

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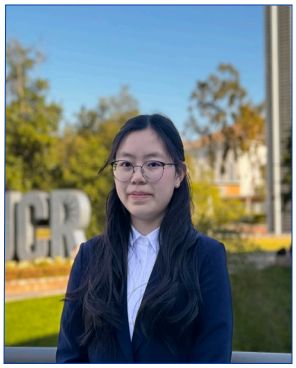

Mandy Hsieh

Mandy Hsieh is a third-year Bioengineering major with a minor in Data Science. She leads the Power Team in AIChE's ChemE Car Competition and is passionate about regenerative medicine, tissue engineering, and biophysics. A part of the B2K Group under Dr. Victor G. J. Rodgers, Mandy also contributes to the chemical synthesis/bioprinting team led by Dr. Iman Noshadi in the Innovative Biomaterials Laboratory, receiving a mini-grant award. Mandy aspires to earn a Ph.D. in chemical engineering or bioengineering.

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INTRODUCTION

Traditional approaches to mimic cellular environments for studying enzymatic processes often involve observing reactions in dilute buffers. However, the intracellular environment of living cells is densely crowded with macromolecules such as proteins, nucleic acids, ribonucleoproteins, polysaccharides, and metabolites. This combination of concentrated multicomponent solutes is known as crowding. Crowding has been found to significantly impact enzymatic activities both in vitro and in vivo, thereby challenging the validity of these representations.[1] Through the use of quantitative Förster resonance energy transfer (qFRET) imaging, an investigation is conducted to determine whether a crowded environment influences the dissociation constant (K_D) values in experimental settings. The protein, bovine serum albumin (BSA), is used as a crowding agent, and qFRET technology is utilized to interpret FRET signals and quantify binding kinetics in crowded environments. Understanding the related kinetics can provide valuable insights into protein interactions in vivo, contributing to a better understanding of cellular processes and potentially guiding drug discovery efforts targeting protein-protein interactions.

Crowding agents are employed to simulate the densely packed environment, mimicking the crowded conditions of cell interiors. This affects molecular interactions and can influence various biochemical processes, as crowded conditions are shown to alter binding affinities. In this study, BSA, whose pH closely resembles that of a cellular environment, acts as a crowding agent to replicate the crowded conditions within cells. Albumin, a protein present in BSA, contains histidine residues, and its functional group of imidazole permits effective protonation and deprotonation based on its surrounding environment. This property makes serum albumin, including BSA, an excellent buffer and a suitable candidate for maintaining protein behaviors and properties without alteration. However, other inert crowding agents like polyethylene glycol (PEG), dextran, and Ficoll can also be effective alternatives as these agents increase viscosity, reducing free space, to simulate cellular environments.[1, 2]

This work focuses on the reversible reaction (SUMOylation) between a small ubiquitin-like modifier (SUMO) and its E2 ligase, Ubc9 to elucidate how crowding affects protein-protein interactions. SUMOylation is a post-translational modification (PTM) that involves a multistep enzymatic cascade reaction that results in peptide activation and substrate conjugation.[3] Other PTMs include ubiquitin (Ub) and ubiquitin-like (Ubl) proteins that regulate protein activities and half-lives in eukaryotes.[4] Unlike some interactions, this interaction does not require an activation cascade to begin due to its inherent nonvalent affinity.[3] The engineered fluorescent proteins, CyPet-SUMO1 and YPet-Ubc9 pairs, will help determine the K_D of the SUMO1 and Ubc9 interactions. The dissociation constant in this case is given as $K_D = \frac{[A][B]}{[AB]}$, where [A] is CyPet-SUMO1, [B] is YPet-Ubc9, and [AB] is the concentration of the complex in equilibrium. FRET signal analysis and K_D measurement are made from FRET responses, allowing for the direct determination of K_D from the FRET signal. The objective of the study is to compare the binding affinity of CyPet-SUMO1 and YPet-Ubc9 in various crowding conditions, using BSA as the crowding agent. With this, K_D represents the ratio of the concentrations of free and bound proteins. A one-way ANOVA test will be performed to determine the statistical significance among the means between crowded and non-crowded environments.

Interpreting Crowding Effects on FRET Signals for Protein Kinetics Analysis

METHODOLOGY

Experimental Setup for Finding Absolute FRET (EmFRET) Signals

A Costar® black and clear-bottom 96 well-plate was utilized, and a solution containing 350 g/L of BSA yielded a measured pH reading of 7.94. The BSA solution comprised equal parts of artificial cerebrospinal fluid (aCSF) to a 350 g/L, pH 7.4 BSA solution. The artificial cerebrospinal fluid (aCSF) salt solution contained 0.214 g of dibasic sodium phosphate and 0.027 g of monobasic sodium phosphate dissolved in 500 mL of pyrogen-free sterile water. The 350 g/L BSA solution was diluted with aCSF salt solution to acquire different concentrations of FRET readings in the 96 well-plate, with 200 µL per well, repeated three times with a total of 600 µL per well and an extra 200 µL to account for pipetting errors. Fluorescent proteins, CyPet-SUMO1, with a concentration of 55.50 µM, and its E2 ligase, YPet-Ubc9, with a concentration of 61.1 µM, were obtained through protein purification. A calculation was performed using 1 µM for CyPet and YPet to determine the required µL needed to achieve the correct concentrations of BSA, ensuring that each well contains 200µL of total suspended solution. By diluting 350 g/L of BSA with aCSF salt solution, various concentrations of BSA ranging from 70-100 g/L were achieved for low-crowding, while 250-300 g/L of BSA were obtained for high-crowding. Because of the high viscosity of the solutions, the pipette tips were trimmed to ensure precise measurements for high BSA concentrations. EmFRET signals were determined using a spectrophotometer (SpectraMax M3™, Molecular Devices, San Jose, CA).

Additionally, BSA was measured separately from the fluorescent proteins due to its slight yellow coloration. BSA's signal readings were subtracted from the tagged protein emission wavelengths of CyPet-SUMO1 at 414 nm to 475 nm, YPet-Ubc9 at 475 nm to 530 nm, and the total emission wavelengths of both fluorescent proteins from 414 nm to 530 nm. This subtraction ensured accurate protein affinity measurements and eliminated potential interference from other colorations and wavelengths on the EmFRET readings.

After pipetting all necessary elements to achieve varying crowding scenarios at different BSA concentrations with 200 µL per well, the proteins were incubated at 37°C to mimic a cellular environment for about 10-15 minutes. They were centrifuged and mixed until homogeneous, and the well-plate was transferred to a plate reader where the software, SoftMaxPro (version 6.1, Molecular Devices, San Jose, CA) was employed to generate EmFRET readings, crucial for determining K_D. There, a selective comparison between the high-crowding and low-crowding scenarios was made and a decision was selected for K_D calculations. K_D measurements were conducted under crowding conditions of 0 g/L of BSA for non-crowding, 95 g/L of BSA for low-crowding, and 290 g/L of BSA for high-crowding.

K_D Determination

For K_D determination, the same setup was employed, including the use of a black and clear-bottom 96 well-plate, 350 g/L pH 7.4 BSA solution, aCSF salt solution, fluorescent proteins at consistent molar concentrations (CyPet-SUMO1 & YPet-Ubc9), the incubation step, and the software, SoftMaxPro. K_D values were calculated using Prism 5 (GraphPad Software, La Jolla, CA) for non-crowding (no BSA), low-crowding (95 g/L of BSA), and high-crowding (290 g/L of BSA) scenarios, with three replicates conducted to ensure the accuracy of protein affinity measurements. In both the setups with 95 g/L and 290 g/L of BSA, the molarity of YPet-Ubc9 varied with the volume of the aCSF solution to maintain the same BSA concentration, while CyPet-SUMO1 remained constant. From this, 14 K_D incremental steps with different substrate values

Interpreting Crowding Effects on FRET Signals for Protein Kinetics Analysis

(μM) were used, resulting in 15 total YPet-Ubc9 (μM) EmFRET signals including the initial step at 0 μM. The alpha (α) and beta (β) values were calculated once, where alpha values were measured with CyPet-SUMO1 without YPet-Ubc9 (1 starting step), and beta was calculated with only YPet-Ubc9 present, without CyPet-SUMO1 (13 incremental steps). The EmFRET signal is determined from

$$Em_{FRET} = (FL_{DA}) - \alpha(FL_{DD}) - \beta(FL_{AA}), \quad (1)$$

where FL_{DA} is the total fluorescence emission at the acceptor wavelength when excited at the donor excitation wavelength, FL_{DD} is the fluorescence emission at the donor wavelength when excited at the donor excitation wavelength, and FL_{AA} is the fluorescence emission at the acceptor wavelength when excited at the acceptor excitation wavelength.[3] These incremental steps involved altering the volume while maintaining the same concentration for three experiments: high-crowding conditions with 290 g/L of BSA, low-crowding conditions with 95 g/L of BSA, and non-crowding conditions with 0 g/L of BSA. While keeping the concentration of one reactant constant and changing its volume, an observation of how the equilibrium shifts with respect to its changes in concentration was made with its K_D value. This offered valuable insights into the strength of the interaction between the protein molecules and aided in determining K_D. After obtaining the necessary data from SoftMaxPro and Prism 5, the following equations[3] were regressed to find K_D:

$$Em_{FRET} = Em_{FRET_{max}}\left(1 - \frac{2K_D}{X-A+K_D+\sqrt{2(X-A-K_D)+4XK_D}}\right) \quad (2)$$

where A is the fixed concentration of CyPet-SUMO1, X is the different concentrations of YPet-Ubc9, and $Em_{FRETmax}$ is the maximum Em_{FRET} signal.

RESULTS AND DISCUSSION

A concentration of 290 g/L of BSA was selected to characterize high-crowded conditions for K_D determination. Conversely, 95 g/L of BSA is chosen to represent low-crowded conditions. Tables 1-3 summarize the EmFRET signals in relative fluorescence units (RFU) obtained.

BSA (g/L)	Trial 1	Trial 2	Trial 3
300	2343 ± 65	2406 ± 39	2759 ± 105
290	2503 ± 24	2524 ± 15	2657 ± 39
280	2584 ± 3	2506 ± 29	2642 ± 26
270	2795 ± 48	2584 ± 38	2655 ± 9
260	2884 ± 26	2932 ± 6	3025 ± 32
250	3007 ± 110	2575 ± 67	2634 ± 43
aCSF	6413 ± 239	7768 ± 314	6812 ± 76

Table 1. Three trials were conducted for each test to minimize inaccuracies, using BSA concentrations ranging from 250 g/L to 300 g/L to create a highly crowded environment.

BSA (g/L)	Trial 1	Trial 2	Trial 3
100	5443 ± 121	5402 ± 104	4598 ± 224
95	4473 ± 14	4433 ± 3	4413 ± 11
90	3803 ± 8	3796 ± 5	3751 ± 13
85	4108 ± 64	3958 ± 3	3788 ± 67
80	4009 ± 30	4039 ± 43	3754 ± 73
75	4088 ± 22	4205 ± 26	4133 ± 4
70	4433 ± 61	3840 ± 181	4576 ± 120
aCSF	6187 ± 95	6074 ± 49	5599 ± 145

Table 2. Three trials were conducted for each test to minimize inaccuracies, using BSA concentrations ranging from 70 g/L to 100 g/L to create a low-crowded environment.

Additionally, a control group with no crowding agent (BSA) present is collected in both high-crowded and low-crowded environments, and their values are compared. This simulates the condition of cells in a non-crowded environment, providing a basis for comparison in the study of protein-protein interactions in crowded environments.

Interpreting Crowding Effects on FRET Signals for Protein Kinetics Analysis

aCSF with CyPet/YPet	Trial 1	Trial 2	Trial 3
aCSF in Table 1	6413 ± 239	7768 ± 314	6812 ± 76
aCSF in Table 2	6187 ± 95	6074 ± 49	5599 ± 145

Table 3. A non-crowded environment is demonstrated by excluding BSA, using only aCSF salt solution from Table 1 and Table 2's experiment values with the fluorescent proteins.

In general, all EmFRET signals follow the same shape of the depicted curve in Figure 1 with CyPet-SUMO1 and YPet-Ubc9 fluorescent-tagged proteins. The fluorescent emission at the donor, CyPet, ranges from 414 nm to 475 nm, while the fluorescent emission at the acceptor, Ypet, ranges from 475 nm to 530 nm. CyPet is quenching, or losing energy, and Ypet is excited, gaining energy.[3] Once the EmFRET signals are recorded, K_D and EmFRETmax can simultaneously be determined in Equation 1, where a nonlinear regression is used for each set of experiments with different total concentrations of YPet-Ubc9 (μL). The K_D of CyPet-SUMO1 and YPet-Ubc9 were then plotted using the non-linear regression of EmFRET (RFU) vs. $[YPet\text{-}Ubc9]_{total}$ (μM), and the resulting K_D values are shown in Figure 2.

Figure 2 summarizes the K_D values obtained. The experiments revealed that binding in crowded environments is reduced due to crowding as evidenced by the respective increase in K_D values. The ANOVA test in Figure 2 yielded P values of 0.0123 for non-crowding vs. low-crowding, and 0.0340 for non-crowding vs. high-crowding, both values falling below 0.05. This suggests sufficient evidence to reject the null hypothesis and conclude significant differences exist between non-crowded and crowded environments. However, the Brown-Forsythe test comparing high-crowding and low-crowding conditions, yielded a P value of 0.3891, which exceeds the significance threshold of 0.05. This indicates that there is no significant difference between the high and low crowding conditions. In addition, altering the concentration of the crowding agent, BSA, from 95 g/L to 290 g/L does not affect the K_D results, as revealed by the comparison between the two, which shows no significance. However, a notable distinction emerges between a non-crowded environment (the absence of BSA) with the crowded environments in both low and high-crowded settings.

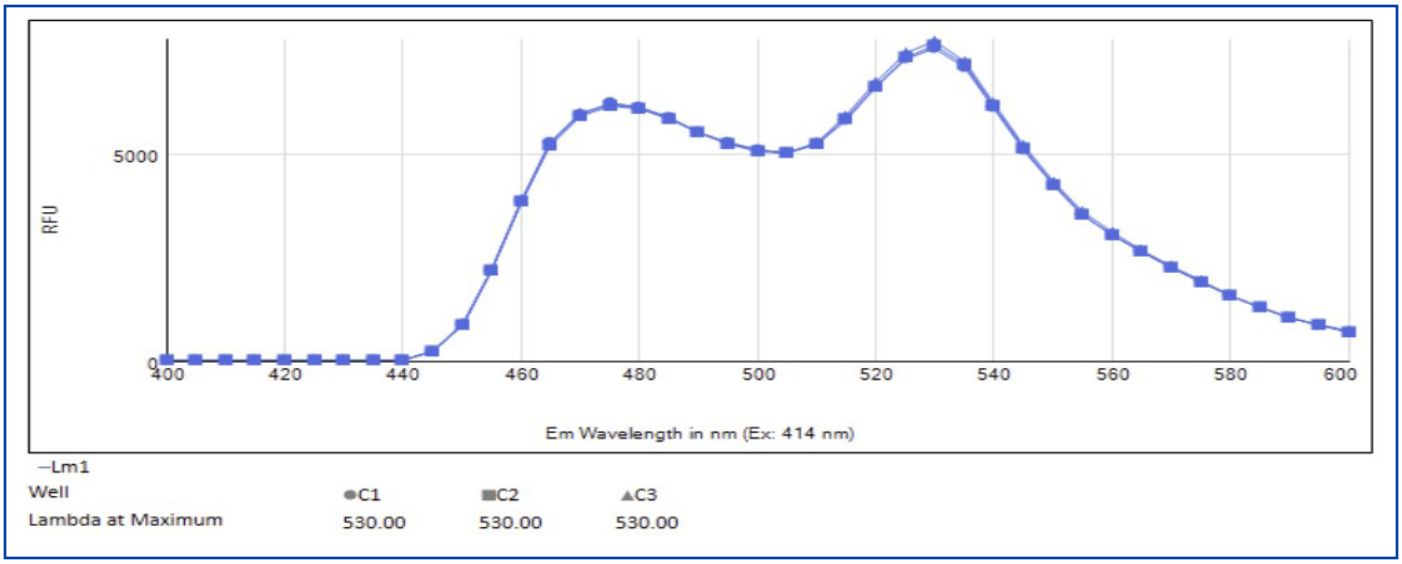

Figure 1. The general trend for FRET Emission Signals (EmFRET) in RFU Reading Peaks for 290 g/L of BSA in Table 1. The plots represent the three trials run in the well plate.

Interpreting Crowding Effects on FRET Signals for Protein Kinetics Analysis

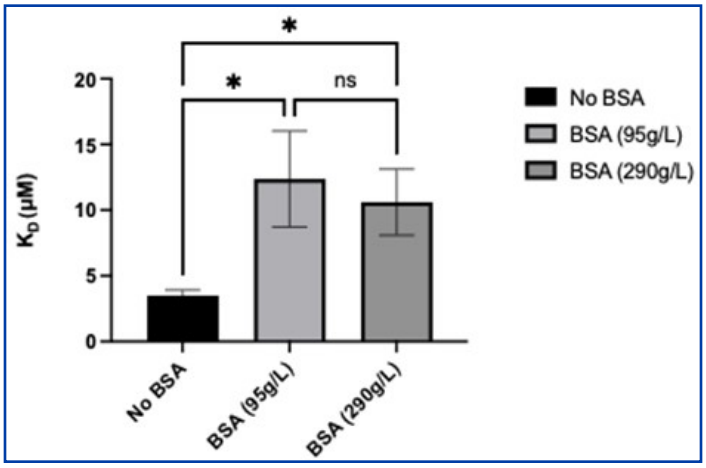

Figure 2. Resulting K_D values. The presence of the crowding agent, BSA, increases K_D by approximately a factor of three. Further increasing BSA concentration from 95 g/L to 290 g/L does not statistically change K_D. Error analysis was determined using one-way ANOVA. The * indicates $P < 0.05$. ns indicates no significant difference.

CONCLUSION

Using the qFRET assay, we determined K_D for the dissociation equilibrium constant for CyPet and YPet with SUMO1 proteins under crowded conditions.[3, 5] A higher K_D value suggests weaker binding, whereas a lower K_D value suggests stronger binding. A crowding concentration of BSA resulted in K_D values of 3.5 ± 0.5 μM, 12.4 ± 3.7 μM, and 10.6 ± 2.5 μM for 0, 95, and 290 g/L BSA, respectively. The data indicates that the crowding agent elevates K_D by approximately a factor of three, underscoring the significant impact of crowding on the dissociation constant, K_D. This indicates that in crowded conditions, the presence of other molecules or crowders reduces the affinity between proteins, leading to weaker binding compared to non-crowded environments or diluted solutions. This conflicts with traditional approaches to mimic cellular environments for studying enzymatic processes using dilute buffer solutions. Figure 2 also depicts that the EmFRET signals appear noisier at 290 g/L of BSA than in other concentrations, suggesting that crowding may interfere with the spectrometer signals.

Future investigations will focus on identifying the point at which crowded conditions are established and their subsequent impact on K_D values. Alternative crowding agents such as human serum albumin (HSA), polyethylene glycol (PEG), dextran, or Ficoll could be used in future studies to assess the significance of crowding agent compositions on K_D and better simulate environments in various cell types. This includes determining different crowding effects, for example, in cardiomyocytes or osteoclasts, particularly for reversible reactions in cells. Further reassessment of K_D values determined in dilute solutions can be conducted and compared with the K_D values in crowded conditions. Additional studies include measuring osmotic pressures in protein solutions, as previous research established that crowding agents impact osmotic pressure in cellular environments.[7-12] These studies can provide insights into how proteins behave in different environments, and because cells have high osmotic pressure, the pressure in the presence of crowding agents can be used to measure the value of K_D. This understanding could aid in designing controlled-release systems and predicting drug behavior in physiological environments, with potential implications for pharmaceuticals, bioseparations processes, and drug discovery targeting protein-protein interactions.

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Interpreting Crowding Effects on FRET Signals for Protein Kinetics Analysis

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