Application Note

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# UV Fluorescence Polarization as a Means to Investigate Protein Conformational and Mass Change

## Using intrinsic tryptophan fluorescence in conjunction with UV-capable polarizers


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## Abstract

The essential amino acid tryptophan is intrinsically fluorescent with excitation in the UV range of the spectrum. This physical characteristic can be used to investigate peptides and proteins that contain this amino acid. This application note describes the use of the Agilent BioTek Synergy H1 multimode reader in conjunction with UV-capable fluorescence polarizers to investigate conformational and mass changes of proteins in solution.

## Introduction

Fluorescence polarization (FP) is a fluorescence detection technique that is based on the observation that fluorescent molecules, when excited by plane polarized light, will emit plane polarized light. In solution, proteins are free to rotate, so the plane of polarized light emitted can change based on the fluorescence lifetime of the fluorophore in question, and the extent of rotation the molecule undergoes during that time frame. The molecule's rotational speed is influenced by solution viscosity, absolute temperature, molecular volume, and the gas constant. If one keeps viscosity and temperature constant, then the key variable for rotational speed differences is molecular volume or, to a first approximation, molecular weight.

FP measurements are made with two different polarizing filters that are parallel and perpendicular to the plane of the polarized excitation source. Polarization values for any fluorophore complex are inversely related to the speed of molecular rotation of that complex. Because the speed of rotation is related to the size of the molecule, polarization values will be high with large molecule complexes, and low with small molecules. Typically, the measured fluorescence is produced by an exogenous fluorescent compound such as fluorescein or rhodamine. These compounds are covalently linked to peptides or nucleic acids and used as tracers to assess changes in mass resulting from binding events or proteolysis. Intrinsic polarization measurements are made without an exogenous fluorescent tracer, using the inherent fluorescence of the polypeptide.

The intrinsic fluorescence of a folded protein is a mixture of the fluorescence from individual aromatic residues, with the tryptophan providing the most significant portion. Tryptophan has an indole ring structure with wavelength of maximum absorption at 280 nm and an emission peak that ranges from 300 to 350 nm, depending in the polarity of the local environment (Figure 1). Tryptophan is a relatively rare amino acid with many proteins containing only one, or a few, tryptophan residues. Therefore, tryptophan fluorescence polarization can be a sensitive measurement of the conformational state of individual tryptophan residues. The advantage compared to extrinsic probes is that the protein itself is not changed. The use of intrinsic fluorescence polarization for the study of protein conformation is, in practice, limited to cases with few (or only one) tryptophan residues, since each experiences a different local environment, which would produce an averaged response.

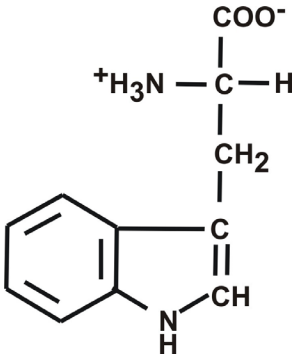


**Figure 1.** Tryptophan structure.

## Materials and methods

Protease (part number P5147), NATA (part number A6501), RNase T1 (part number R1003), glucagon (part number G2044), melittin (part number M2272), human serum albumin (part number A9511), bovine serum albumin (part number A3294), basic myelin (part number M1891), lysozyme (part number L6876), tryptophan HCl (part number T8941), and Trp-Met-Asp-Phe-$NH_2$•HCl peptide (part number T6515) were purchased from Sigma-Aldrich (St. Louis, MO). Solid black 96-well microplates (part number 3915) were from Corning (Corning, NY). All other chemicals were reagent grade.

### Ratiometric experiments

Mixtures of different molar ratios of human serum albumin (HSA) and tryptophan were made from 100 µM stock solutions. Eight replicate aliquots (100 µL) of each mixture were pipetted into wells of a black sided 96-well plate and the fluorescence polarization determined. Reactions were carried out under denaturing (8 M guanidine or 10 M urea) and native conditions.

### Protein digestion

HSA protein (100 µM final concentration) was digested with various concentrations of protease enzyme in the presence of 10 mM phosphate (pH 7.5) buffer at 37 °C for four hours. The fluorescence polarization was then measured. For kinetic experiments, reactions were initiated by the addition of HSA and fluorescence polarization monitored every 10 minutes.

### Protein denaturation

HSA protein (100 µM final concentration) was incubated with various concentrations of guanidine HCl in the presence of 10 mM phosphate (pH 7.5) in a total volume of 100 µL per well buffer at room temperature for two hours. After incubation the fluorescence polarization was determined.

### Melittin polymerization

2,000 Cells Melittin protein (100 µM final) was incubated with various concentrations of sodium chloride in the presence of 10 mM phosphate (pH 7.5) buffer at room temperature for 30 minutes. After incubation, the fluorescence polarization was then measured.

### Polarization measurements

Fluorescence polarization was measured in a Synergy H1 multimode reader configured with UV-compatible polarizers. For all measurements, a 284/10 excitation and a 340/30 emission filters were used in conjunction with a 320 nm cutoff dichroic mirror. PMT gain setting was set to 50 with 10 reads per data point.

## Results and discussion

The fluorescence polarization values for mixtures of large and small molecules are determined by their relative ratio. An excess of large molecules relative to small will result in a large polarization value, while an excess of small molecules will result in low polarization. This was demonstrated by mixing tryptophan amino acid with human serum albumin (HSA). HSA is a 67kD protein that only contains one tryptophan amino acid. However, due to its size, fluorescence polarization values obtained using intrinsic fluorescence will be high. Conversely, the amino acid tryptophan is small, and would be expected to have a low polarization value in solution. Figure 2 demonstrates the ability of the Synergy H1 with UV-capable polarizers to distinguish different molar ratios of tryptophan and HSA. Mixtures that are predominately HSA have polarization values of approximately 175 mP, while those that are predominately tryptophan have values approximately 40 mP. In addition, these data are a compilation of multiple experiments, indicating the repeatability of the polarization measurements.

Polarization measurements using the intrinsic fluorescence of proteins are based on the ability of tryptophan to rotate in solution. Tryptophan in proteins is normally located within the hydrophobic center of most proteins as part of a tertiary structure, drastically reducing its ability to rotate independently of the entire peptide. If the tertiary structure is completely denatured, tryptophan moieties within polypeptide have some rotational freedom within the polymer that the rigid native peptide would not necessarily have. This is demonstrated in Figure 3, where the fluorescence polarization of tryptophan-HSA mixtures was determined in their native and denatured state.

Under denaturing conditions, the maximal FP values are significantly reduced (40%) as compared to that observed under native nondenaturing conditions. Samples that are substantially tryptophan are not affected to any extent.

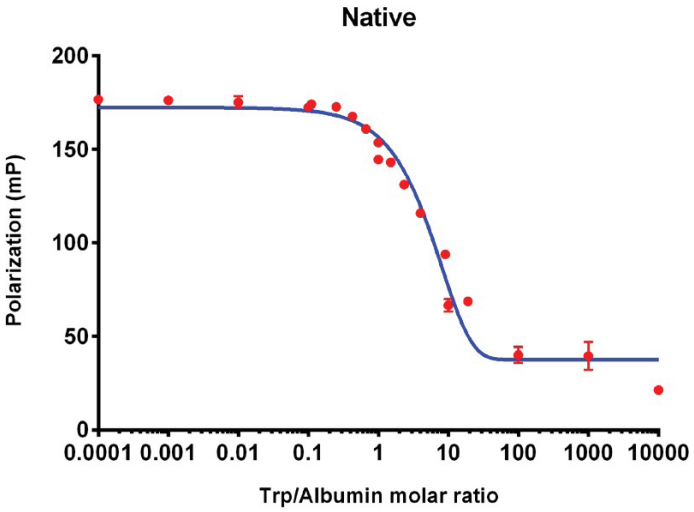


**Figure 2.** Polarization values of mixtures of human serum albumin and tryptophan in nondenaturing conditions. Data represent the mean and standard deviation of at least eight separate determinations from multiple experiments.

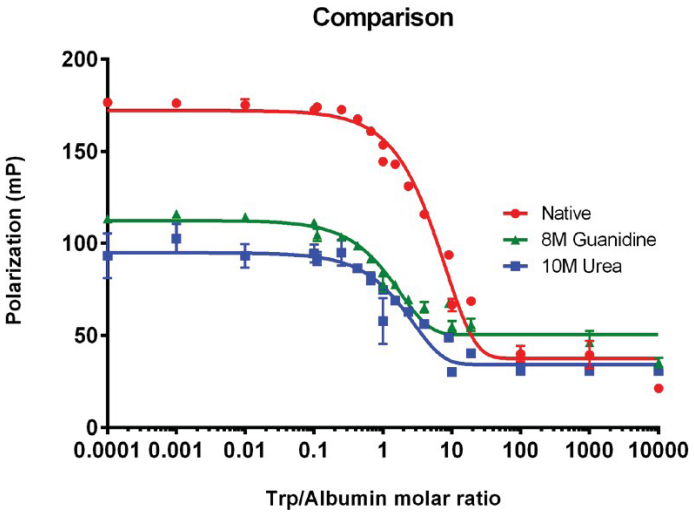


**Figure 3.** Comparison of native and denatured polarization. Mixtures of HSA and tryptophan were made under native or denaturing (8 M guanidine or 10 M urea) conditions and the fluorescence polarization determined. Data represent the mean and standard deviation of at least eight replicates from multiple experiments.

The ability of tertiary structure to influence fluorescence polarization is demonstrated in Figure 4. When human serum albumin (HSA), which only contains a single tryptophan moiety, is subjected to increasing concentrations of the denaturing compound guanidine, the fluorescence polarization first increases approximately 10% before significantly decreasing. This increase is due to the preliminary unfolding of the protein, which increases its molecular volume, while the decrease is attributable to unfolding, which releases the tryptophan residue from the hydrophobic core of the protein allowing the indole ring to rotate independently of the protein. Because the tryptophan is still part of the protein backbone, the polarization value is still significantly greater than tryptophan in solution when the protein is completely denatured.

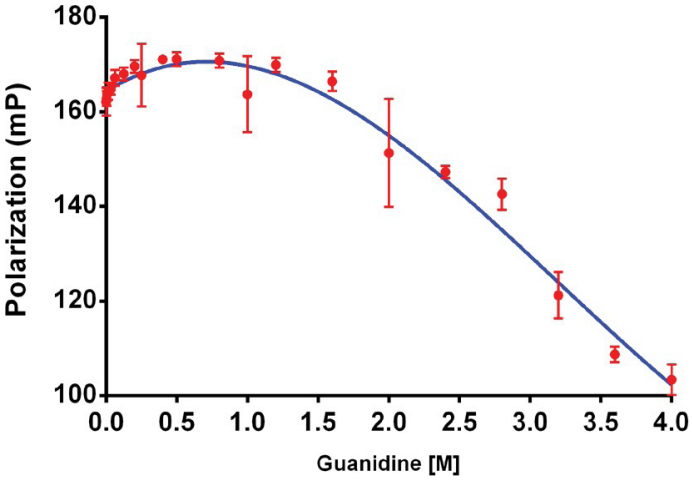


**Figure 4.** Polarization of HSA with increasing guanidine concentrations. Human serum albumin (HSA) was treated with different amounts of guanidine-HCl. After a 30-minute incubation, the fluorescence polarization with UV excitation was determined. Data represent the mean of at least eight separate determinations from multiple experiments.

The essence of fluorescence polarization is the ability to distinguish differences in mass. Digestion of human serum albumin with general protease enzymes results in the protein being reduced into smaller and smaller peptides. Because of the single tryptophan moiety in the HSA protein, only peptide fragments containing the amino acid are fluorescent. Therefore, with the continual digestion of HSA with protease the fluorescent fragments would be expected to become smaller over time. As demonstrated in Figure 5, samples containing either 100 µg/mL or 500 µg/mL protease enzyme have decreasing polarization values over time, while samples that lack protease have a constant polarization.

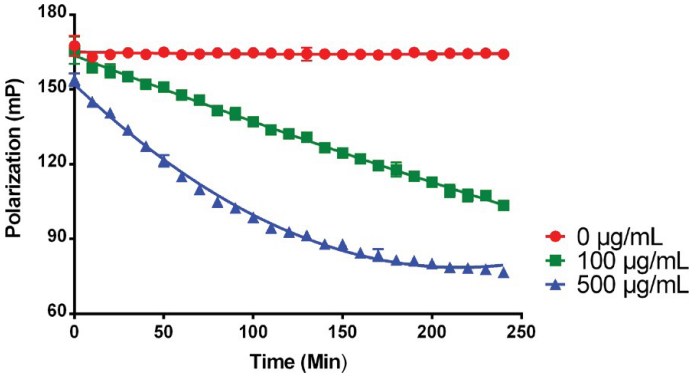


**Figure 5.** Change in polarization over time of HSA with digestion with protease. Samples in replicates of eight were treated with 0, 100, or 500 µg/mL of protease enzyme at 37 °C and the fluorescence polarization determined kinetically. Data represent the mean and standard deviation of eight determinations.

Using a series of protease concentrations in conjunction with a fixed digestion time one can plot changes in polarization as a function of enzyme concentration. As demonstrated in Figure 6, with increasing protease concentrations the resultant polarization value decreases after a four-hour digestion. HSA, which consists of 350 amino acids with only one tryptophan moiety, is cleaved into smaller fragments over time. Because only fragments containing tryptophan are excited with UV light, fluorescence polarization measurements reflect the relative length of only the fragments with that amino acid.

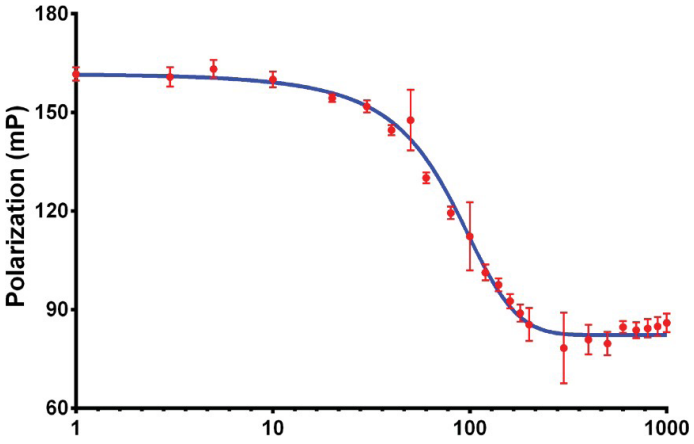


**Figure 6.** Fluorescence polarization of HSA with protease enzyme titration. HSA was treated with various concentrations of protease enzyme for four hours at 37 °C. Data points represent the means and standard deviation of at least eight replicates from multiple separate experiments.

Figure 5 shows that fragment length decreases with increasing amounts of time, while Figure 6 shows that increasing amounts of enzyme also causes fragment length to decrease. Because the digestion does not proceed to the point of individual amino acids, the polarization values at maximal enzyme concentrations are greater than that observed with tryptophan.

UV-fluorescence polarization can be used to discern peptides with different molecular masses. As demonstrated in Figure 7, the differences in mass of native proteins can be discerned using fluorescence polarization. While short peptides with molecular weights less than 1,000 behave in a linear fashion, larger peptides have less significant increases in polarization with increasing mass, suggesting that secondary and tertiary structure plays a role in the ability of tryptophan to freely rotate in solution. Many of these proteins (e.g. HSA, melittin, basic myelin, glucagon, RNaseT1, and Trp-Met-Asp-Phe peptide) possess only one tryptophan per polypeptide chain, and polarization values from these would reflect the rotation of this single amino acid. BSA and lysozyme contain two and four tryptophans, respectively. As such, the FP value returned would reflect an average.

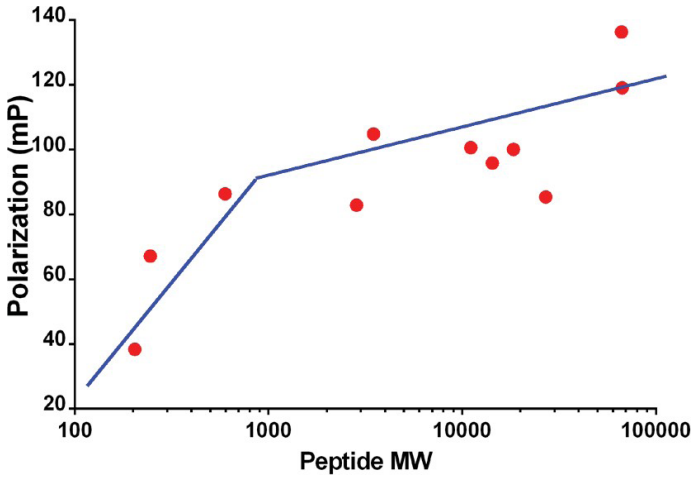


**Figure 7.** Comparison of polarization and peptide mass. The fluorescence polarization of several different peptides (Protease, NATA, RNase T1, glucagon, melittin, human serum albumin, bovine serum albumin, basic myelin protein, lysozyme, Trp-Met-Asp-Phe-$NH_2$•HCl peptide, and tryptophan) with different molecular weights was determined at pH 7.5 under native nonreduced conditions. Data represent the mean of eight determinations.

Melittin, a 26-amino acid peptide from Apis mellifera bee venom, has been used as a model to study protein folding.[1] Melittin converts from a monomeric random coil to an α-helical tetramer with increasing ionic strength.[1] With dimer and tetramer formation the rotational mass increases substantially. As shown in Figure 8, this change in mass can be monitored with fluorescence polarization.

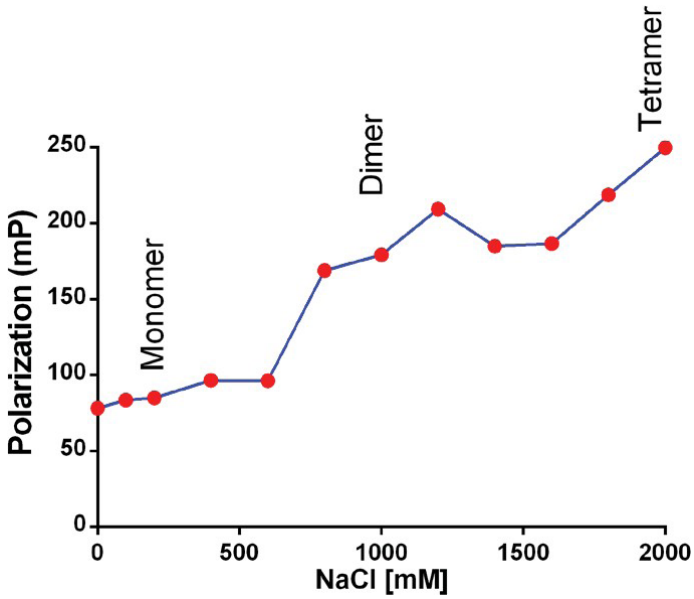


**Figure 8.** Melittin multimer formation. Melittin protein was incubated for 30 minutes with increasing concentrations of sodium chloride at pH 6.5 and the fluorescence polarization determined. Data represent the mean of eight determinations.

While the measurement of intrinsic protein fluorescence in microplates has been used extensively[1], the determination of fluorescence polarization from the same signal has not received appreciable use. These data indicate that appropriately configured Agilent BioTek Synergy multimode readers can be used to make fluorescence polarization determinations from intrinsic protein fluorescence.

Fluorescence polarization is, in essence, an assay that measures changes in size. Binding events where a small labeled ligand attaches to something larger result in a larger rotational mass. The fluorescence polarization measurements of these reactions are effectively the average of a mixture of labeled ligand (small) and labeled bound ligand (large) molecules, with the unlabeled large molecule being invisible. Tryptophan and HSA, which only have one tryptophan, are functional equivalents of a labeled ligand and a ligand bound to a receptor, respectively. Mixtures of the two molecules should present with different polarizations values based on their molar ratios.

The digestion of a polypeptide will result in fragments of smaller size than the original molecule. Because intrinsic fluorescence is generated primarily from tryptophan emission, polarization measurements of those peptides effectively mimic the size of peptides that contain tryptophan, while the other fragments are invisible. Proteins with more than one tryptophan will result in a multitude of fluorescent fragments with the resultant polarization value being the average polarization of all the fragments.

Denaturation studies suggest that the tryptophan moiety in HSA is located in a region of tertiary structure.[3] Fluorescence polarization in completely denatured conditions is markedly less than that observed under native conditions (Figure 3). In experiments with various amounts of denaturing reagent, the FP values increase slightly with low amounts of denaturant before decreasing (Figure 4). Fluorescence polarization with UV excitation of HSA is essentially a measurement of the ability of a single tryptophan to rotate freely. The native protein of course has a tertiary structure that allows the protein to rotate as a unit. With low concentrations of guanidine denaturation, the protein begins to unravel, effectively increasing in rotational size while still keeping the tryptophan amino acid locked in its local tertiary structure, resulting in a higher polarization value. With further denaturation, the protein is completely relaxed, allowing the tryptophan amino acid to rotate freely within the polypeptide amide linkage of the protein that is measured as a decrease in the polarization value.

## Conclusion

Fluorescence polarization measurements using visible light excitation wavelength in microplates have been performed as a matter of routine. These experiments used a covalently linked fluorescent tracer attached to the ligand of interest. The assumption of these assays is that the tracer does not interfere with the measurement. Polarization measurements of individual peptides and proteins with small numbers of tryptophan moieties can be advantageous in that there is no requirement for modification of the protein. The intrinsic physical property of the polypeptide is used as the fluorescence source. The caveat is that the reader hardware is capable of making measurements in the UV portion of the light spectrum. These data are an example of the capability of the Agilent BioTek Synergy H1 multimode reader when configured with UV-capable polarizing filters.

## References


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3. Kumar, S.; Swaminathan, R. Employing the Fluorescence Anisotropy and Quenching Kinetics of Tryptophan to Hunt for Residual Structures in Denatured Proteins, *J. Chem. Sci.* **2007**, *119*, 141–145.


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