Spectroscopy and Light Scattering

ABSTRACT

Light can interact with matter in different ways. In this laboratory module, you explore how light absorption, emission and scattering provide information on the system being probed. You then monitor the time-dependent optical density of a solution of collagen to investigate the kinetics of collagen fibril formation.

I. OBJECTIVES

- Learn the physics principles behind absorption and fluorescence of visible light
- Learn the physics principles behind FRET and how the technique is applied to biological questions
- Learn the physics principles behind light scattering
- Determine the concentration of an unknown solution of dye molecules
- Determine the how light scattering depends on wavelength
- Determine the kinetic stages of self-assembly of collagen proteins into fibrils

II. BACKGROUND: LIGHT-MATTER INTERACTIONS

When light interacts with a sample, its intensity is attenuated due to two factors: absorption by the sample and scattering by particles within the sample. The former is described by its absorbance density, α, and the latter by its turbidity, τ. The attenuation of intensity as a function of distance x through a sample is given by

$$-dI_\lambda(x) = (\alpha_\lambda + \tau_\lambda)\, I_\lambda(x)\, dx\,. \tag{1}$$

Notice how each process, absorbance and turbidity, makes a separate contribution to the loss of light. If multiple species are present in the solution, with different concentrations c_j, the absorbance density and turbidity are a sum of the contributions of each:

$$\alpha_\lambda = \sum_j c_j \varepsilon_{j,\lambda}\,, \qquad\qquad \tau_\lambda = \sum_j c_j \sigma_{j,\lambda}\,. \tag{2}$$

The extinction coefficient ε_λ is a function of the electronic structure of the species and relates to the strength of electronic transitions as a function of wavelength (see below). The scattering cross-section σ_λ depends on particle geometry, size and refractive index, and is

described in the following section. Integrating Eq. (1) shows that the intensity of light at a distance x through the sample reduces exponentially with absorbance density and turbidity:

$$I_\lambda(x) = I_{\lambda,0}(x)\, e^{-(\alpha_\lambda+\tau_\lambda)\, x}\,. \tag{3}$$

The attenuation of light intensity by a sample can be measured using an instrument known as a spectrophotometer. Figure 1 shows a schematic of this instrument. The illuminating light passes through the sample and the amount of light reaching the detector is recorded. The instrument records the *transmittance* of light, T, through the sample, relative to a reference sample (e.g., a blank cuvette):

$$T(\lambda) \equiv \frac{I(\lambda)}{I_0(\lambda)}\,. \tag{4}$$

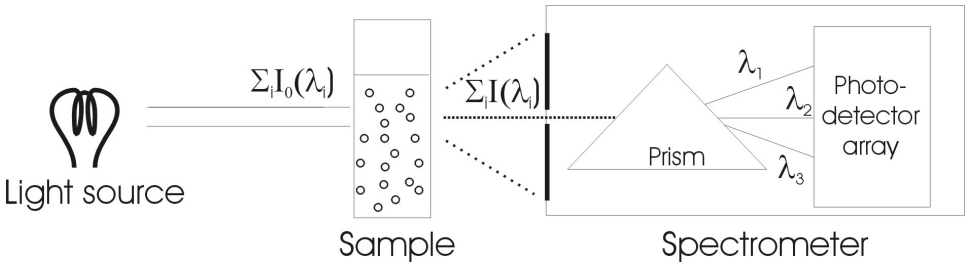

FIG. 1. Light path through a spectrophotometer. Absorption or scattering of light by species in the sample decreases the light intensity reaching the spectrometer. In the spectrometer, a prism disperses different colours onto an array of photodetectors. The spectrum of transmitted light is given by the intensity of light falling onto the different pixels in this array.

Here, $I(\lambda)$ is the intensity of light of wavelength λ transmitted through the sample when light of intensity $I_0(\lambda)$ is incident on it.

A more common measure of the attenuation of the light by the sample is given by the *optical density* (OD) of the solution, which is a function of wavelength λ:

$$\mathrm{OD}(\lambda) = -\log_{10}\left[\frac{I(\lambda)}{I_0(\lambda)}\right] = \log_{10}\left[\frac{I_0(\lambda)}{I(\lambda)}\right]\,. \tag{5}$$

Note that OD is occasionally defined using a natural log (ln) instead of base 10.

Pre-lab question 1: If a sample attenuates the incoming light to 10% of the incident value, what is the observed OD of the sample?

Note that in spectroscopy, the optical density is defined as the base-10 logarithm of the ratio of intensities, while the exponential relationship of Eq. (3) would more naturally suggest the use of natural logarithm. Extinction coefficients and scattering cross-sections

are likewise quantified using the spectroscopist's convention of base-10 logarithms, which we follow here.

Comparing with Eq. (3), we see that the optical density through a sample of thickness d is given by

$$\mathrm{OD}(\lambda) = (\alpha_\lambda + \tau_\lambda)\, d\,. \tag{6}$$

The optical density is often referred to as the *absorbance* of a sample, though this nomenclature can be confusing since the attenuation of light in a sample is due to both absorbance and scattering.

A. Scattering

Light scattering depends on the wavelength of the illuminating light, on particle size, shape and refractive index, and on the concentration of the particles in solution. For spherical particles much smaller than the wavelength of light,[1] scattering can be described analytically as Rayleigh scattering. In this case, the scattering cross-section for a spherical particle of radius R is given by

$$\sigma_\lambda = f \frac{R^6}{\lambda^4} \left(\frac{n^2 - 1}{n^2 + 2} \right)^2 , \tag{7}$$

where $n \equiv n_{\mathrm{sphere}}/n_{\mathrm{medium}}$ is its index of refraction relative to the surrounding medium and f is a constant prefactor.

For a single type of scattering species in dilute solution, Eqs. (2), (5), and (7) give

$$\sigma_\lambda \propto \frac{cR^6}{\lambda^4} \left(\frac{n^2 - 1}{n^2 + 2} \right)^2 , \tag{8}$$

i.e., the optical density depends linearly on concentration and is inversely proportional to λ^4. This relationship can be used to determine the relative concentration of scattering particles in solution and can be made absolute if the relation between concentration and OD is known.

Pre-lab question 2: Would you expect to detect more transmitted light for 300 nm or 500 nm illumination? What ratio of attenuated light intensity would you predict for these two conditions, assuming Rayleigh scattering?

[1] Rayleigh scattering is conventionally assumed to hold if the particle radius $R < \lambda/20$.

Pre-lab question 3: If you wanted to determine the power-law relationship between optical density and wavelength (i.e., determine or verify the exponent of λ in Eq. (8), what measurements would you perform and how would you plot and analyse your data?

B. Absorption

Molecules that absorb visible light absorb a photon and promote an electron to a higher-lying molecular orbital. These electronic excitations occurring in the visible range of the spectrum (750–400 nm) correspond to electronic states separated by energies of 1.6–3.1 eV. Electronic transitions can also occur to states of higher energy; some molecules are colourless and their lowest accessible electronically excited states lie in the ultraviolet range of the spectrum. By contrast, absorption of infrared light is associated with much lower energy vibrational transitions, and rotational energy transitions are yet lower in energy, in the microwave region of the spectrum. Selection rules of quantum mechanics determine which transitions are allowed based on the symmetries and spins of the participating states.

Figure 2 shows an example of a Jablonski diagram, illustrating the types of transitions that can occur between different electronic states of a molecule. Here, the notations S_0, S_1, and S_2 refer to electronic states of singlet spin, where S_0 is the ground state of the molecule. Transitions from a singlet state to a triplet state, such as the lowest-energy T_1 triplet state, are not quantum mechanically allowed, and so these transitions happen rarely. If a triplet state is populated, it is generally long lived, since transitions out of this to the ground electronic state, which is usually a singlet state, are also disallowed.

Each molecule possesses a unique spectroscopic fingerprint, by which it can be identified under specified experimental conditions. For example, DNA absorbs strongly at 260 nm, where an electron in a π-bonding orbital can be excited to a π^*-antibonding orbital. (The notation π refers to electronic wavefunctions possessing a nodal plane.) By contrast, proteins absorb most strongly around 280 nm, which is due mostly to electronic excitations from π-bonding to π^*-antibonding orbitals on the aromatic amino acids tryptophan and tyrosine. (π to π^* excitation of electrons in the peptide bonds gives rise to a large absorbance peak further into the UV, at 200 nm.) Thus, the purity of a DNA sample relative to contaminating proteins can be determined from the ratio of its absorbance at 260 nm to 280 nm.

For a solution containing a single type of absorbing species, Eqs. (2) and (6) give

$$\mathrm{Abs}(\lambda) = \mathrm{OD}(\lambda) = \varepsilon_\lambda c d\,. \tag{9}$$

This is the well-known Beer-Lambert law of spectroscopy, which describes the linear relationship between the solution concentration and the measured absorbance. Here, ε_λ represents the wavelength-dependent extinction coefficient of the absorbing molecule, c its concentration and d the optical pathlength through the sample cuvette (generally 1 cm).

The quantity ε_λ is specified in units of $\mathrm{M}^{-1}\mathrm{cm}^{-1}$ and is usually given for the wavelength corresponding to the maximum absorbance. The extinction coefficient can vary with solution conditions, so may depend on the solution's ionic strength, pH, and temperature, among

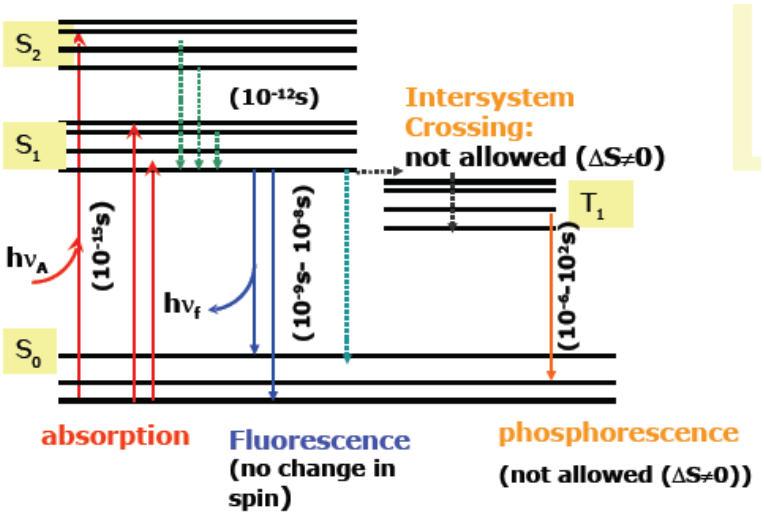

FIG. 2. Jablonski diagram showing possible relaxation pathways and their timescales following absorption of a photon and excitation of an electron. Lines represent energy levels of specific quantum mechanical states, where for illustration purposes a few vibrational states are included within each electronic manifold. Figure courtesy of Marcia Levitus, Arizona State University.

other possibilities. The linear relationship of Eq. (9) holds over a range of concentrations that is spectrometer dependent; a good rule of thumb is that it holds from $0.1 < \mathrm{OD} < 1.0$, and often to an order of magnitude lower OD.

By measuring the absorbance spectrum of a solution containing a molecule of known extinction coefficient under the solution conditions used, one can determine its concentration in solution. Alternatively, by making a dilution series of a molecule in solution, one can determine its extinction coefficient from a plot of A vs. c. Spectrophotometers generally have built-in software that reports absorbances $A(\lambda)$ as output, though it is important to keep in mind, as stated above, that this measured attenuation of light can result not only from absorption of light by a sample but also from scattering.

C. Fluorescence

A fluorescent molecule, or *fluorophore*, absorbs light of energy corresponding to allowed transitions of electrons in the molecule (see above), and then emits light at longer wavelengths when the electron that was excited by the absorbed light relaxes to a low-lying state. The possible types of transitions and associated timescales are shown in Fig. 2, the Jablonski diagram familiar to spectroscopists. Because of rapid relaxation to low-lying vibrational states within each electronic state, the downward transitions (e.g. fluorescence) are of lower energy than the excitations (absorbance). Thus, a molecules fluorescence/emission spectrum is said to be *red shifted* (shifted to longer wavelengths / lower energies—i.e., towards the red region of the spectrum) compared to its absorbance spectrum.

Figure 3 shows the absorbance and fluorescence emission spectra for the dye molecule

Rhodamine 6G, which will be used in this laboratory module. Illumination with the common laser wavelength 532 nm excites rhodamine near the peak of its absorbance spectrum. Its fluorescence emission can be detected at wavelengths longer than this excitation wavelength. For this reason, Rhodamine 6G is also used in the module on fluorescence correlation spectroscopy (FCS).

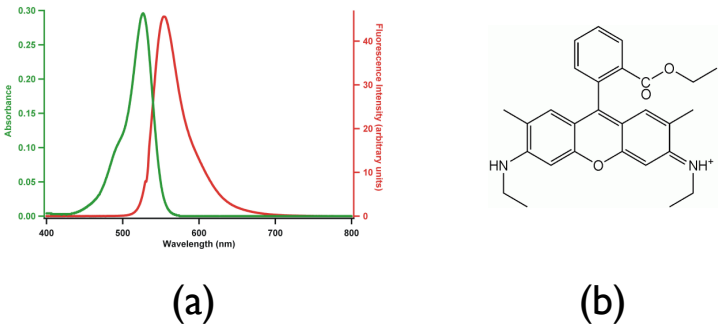

FIG. 3. Rhodamine dye molecule: (a) Absorbance (green) and emission (red) spectra. (b) Chemical structure.

Generally, we measure fluorescent light at 90° with respect to the excitation source. Since, on the slow timescale of these measurements, emission of photons by the fluorophore is isotropic, any detection angle should give rise to an equal fluorescence signal. On the other hand, the forward direction contains the highest intensity of the excitation source. The signal-to-background ratio is thus better if we measure fluorescence by detecting at 90°, rather than in the forward direction.

D. Förster Resonance Energy Transfer (FRET)

Förster Resonance Energy Transfer (FRET)[2] is an extremely popular technique in current biophysics research, employed to provide information about the spatial separation between two parts of a molecule or different molecules. It relies on the spectral overlap between the emission of a donor fluorophore and the absorbance of an acceptor fluorophore. If these coincide, the two species are spatially close, and other conditions are met, as described below, one fluorophore (donor) can absorb light and a second (acceptor) emit fluorescence. Measuring the emitted light intensity at wavelengths corresponding to donor fluorescence and comparing with the emitted light intensity at wavelengths corresponding to acceptor fluorescence provides information about whether the two fluorophores are separated by short distances (acceptor emission observed) or larger distances (donor fluorescence observed).

FRET occurs due to coupling of the transition dipoles for the two transitions involved (de-excitation of the donor and excitation of the acceptor); see Fig. 4. This dipole-dipole interaction depends on the spatial separation between the two dipoles, r, as well as the

[2] The technique is commonly but incorrectly called *fluorescence resonance energy transfer*. It is not fluorescence that is transferred; the form of energy transfer was first described by Förster as dipole-dipole coupling.

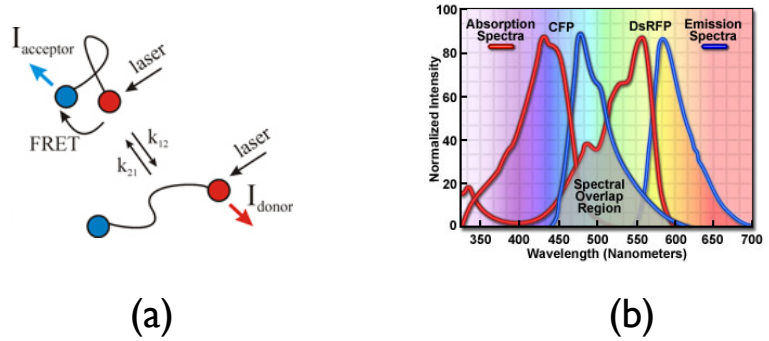

FIG. 4. FRET. (a) Schematic illustrating the spatial dependence to emission of light intensity by a donor or acceptor species, in response to excitation of the donor. Figure from Marcia Levitus. (b) Absorption and emission spectra of a possible FRET pair, cyan fluorescent protein (CFP) and red fluorescent protein (DsRFP). The emission spectrum of CFP shows extensive spectral overlap with the absorption spectrum of DsRFP, meaning CFP could act as a donor and DsRFP could act as an acceptor. A common small-molecule FRET pair is Cy3-Cy5, two cyanine-based dye molecules. Figure from Olympus.

relative orientation of the dipoles. The separation is usually defined relative to the critical Förster distance R_0, the separation at which 50% of absorbed intensity is transferred to the acceptor. The efficiency of energy transfer is given by

$$E(r) = \frac{1}{1 + \left(\frac{r}{R_0}\right)^6} \,. \tag{10}$$

where the Förster distance is given by

$$R_0^6 \sim Q_D \kappa^2 n^4 J \,, \tag{11}$$

Here, Q_D is the fluorescence quantum yield of the isolated donor, κ^2 is a geometric factor that depends on the relative angular orientation of the two dipoles, n is the refractive index of the medium, and J is the integrated spectral overlap of the donor and the acceptor, as illustrated schematically in Fig. 4b.

For most FRET pairs, R_0 lies in the range of 2–6 nm, which is conveniently similar to the size of proteins and on the scale of many biological processes. Examples of studies employing FRET include protein and nucleic acid folding, protein-protein binding interactions (*in vitro* and *in vivo*), molecular motor stepping, and vesicle fusion. Many of these studies can be done in "bulk" (using a cuvette and sample in solution); however, much richer information emerges when FRET is studied at the single-molecule level and the kinetics of transitions, e.g., between folded and unfolded states of a system, can be monitored in real time. You will not study FRET in this module, but please keep it in mind for your independent project.

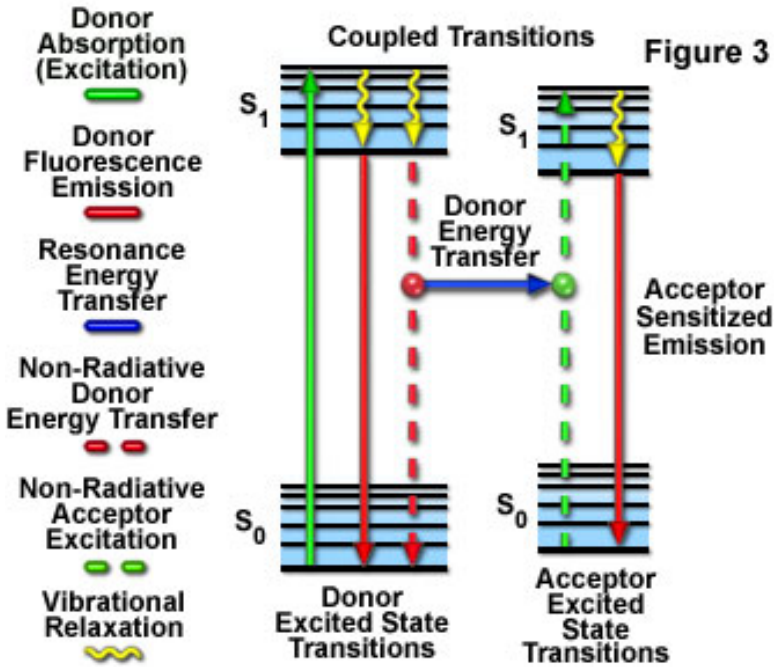

FIG. 5. Jablonski diagram illustrating the resonant energy transfer between a donor and an acceptor, where this transfer can occur if (a) both transitions are the same energy; (b) symmetries of the states; and (c) spatial orientation and separation of the two species make the transfer allowed. Figure from Olympus.

III. BACKGROUND: COLLAGEN FIBRIL FORMATION

Collagens are a class of proteins with a unique triple helical structure. They are the predominant proteins in vertebrates, comprising greater than 25% of our total protein content. They form the basis of the majority of our connective tissues (including tendons, cartilage, bone and skin) and of the extracellular matrix, the network of supports within which our cells grow. The mechanics of collagen are critical to physiological function and can exert profound influences over cell fate, as seen in fetal development, in cancer progression, and in stem cell differentiation in engineered devices that utilize collagen as a biomaterial.

Most collagens are fibrillar, meaning that they assemble into well-ordered, higher-order structures called "fibrils." Fibrillar collagens are triple helical proteins of about 300 nm (1000 amino acid residues, 100 kDa per chain) in length and about 1.5 nm in diameter. The fibrils formed by these collagens are extremely well ordered structures, as seen by the distinct dark-light "D-banding" pattern observed in electron microscopy, resulting from staggered overlap and gap regions of collagen proteins in the fibrils (Fig. 6). Fibrils are typically a few hundred nanometres in diameter and can be tens of micrometers in length. These long, rope-like structures in turn can undergo further assembly (sometimes alone and often with other proteins or minerals) into fibres, networks or other structures that form our connective tissues. The process of assembly from collagen proteins into fibrils is so well specified by the chemical sequence of collagen that this process can occur *in vitro*, i.e., in a tube in the

lab, with no chaperones or other assisting proteins normally found in the cellular milieu necessary.

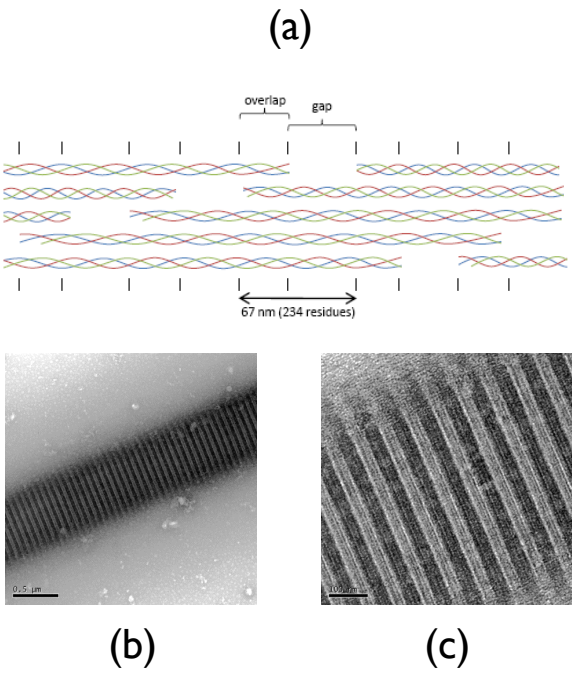

FIG. 6. The structure of a collagen fibril. (a) Collagen triple helices pack laterally, offset from one another, giving rise to bands of protein overlap and gaps (fewer overlapping regions) transverse to the fibril axis. (b) A collagen fibril can extend for tens of micrometres, exhibiting high degrees of order along its axis. The gap regions are preferentially bound by heavy-atom stains used in electron microscopy, giving rise to dark bands when negatively stained and imaged using transmission electron microscopy (TEM). Scale bar = 500 nm. (c) A close-up view of the collagen fibril in part b reveals substructure in the overlap and gap regions. Scale bar = 100 nm. TEM images of type II collagen fibrils were recorded at SFU's *Nanoimaging Facility*. Images courtesy of Clara Chan, former SFU Biological Physics honours undergraduate student.

Collagen proteins are generally stored in an acidic solution, as they are highly positively charged at low pH, leading to electrostatic repulsion between proteins. To initiate fibril formation, the solution is neutralized, usually by diluting the collagen into a solution buffered around pH 7, and salts are added. Under these conditions, collagens can associate and start to pack into fibrils.

Pre-lab question 4: Why might increasing the pH to 7 allow collagen molecules to associate? Why might adding salt to the solution allow collagen molecules to associate?

Collagen fibril formation, during which collagen changes from being isolated as a triple helical protein in solution into being tightly packed in a fibril, is entropically driven. At first

glance, this entropic mechanism might seem impossible: as collagen proteins are bound into fibrils, they *lose* their configurational entropy and are constrained to lie relatively straight the well-ordered structure of a fibril. However, the necessity of packing three amino acid chains into a triple helix means that the side-chains (so-called R-groups) must protrude into solution, rather than being shielded inside the folded protein core, as is the case for globular proteins. To keep these side-chains solvated requires structured water molecules, which form water bridges and contribute to salt bridges. As the collagen proteins associate laterally in their preferred staggered configuration, the structured water molecules are able to return to the bulk water, where they gain significant entropy, more than offsetting the configurational entropy loss of the collagen proteins.

The kinetics of fibril formation are easily monitored via light scattering measurements. Initially, the collagen proteins are isolated in solution and are too small to scatter significant amounts of visible light. As they start to encounter each other in solution, small aggregates can nucleate (nucleation phase of fibril formation). These are proposed to have a well-defined structure consisting laterally of five or six collagen molecules in a so-called microfibril. These structures are also too small to scatter significant amounts of visible light, but as they assemble into fibrils, the optical density of the solution rapidly increases as the fibrils grow sufficiently large to scatter light. This is the growth phase of fibril formation, and the slope of scattered light intensity versus time in this region gives the rate at which fibrils grow. Finally, the solution becomes depleted of free collagen molecules, as the majority has been incorporated into fibrils, and so the amount of scattered light no longer continues to increase (the plateau region). You will observe and characterize the kinetics of collagen fibril formation in this lab.

IV. INVESTIGATIONS

1. Measure the absorbance spectrum of Rhodamine 6G dye at a range of known concentrations in solution, to determine its extinction coefficient at 532 nm. Can you find a literature value to compare to?

2. Measure the fluorescence spectrum of Rhodamine 6G dye in response to excitation at 532 nm, and determine the fraction of the emitted intensity that falls above 550 nm, the cutoff wavelength of the filter used in the Fluorescence Correlation Spectroscopy module.

3. Determine whether 30 nm diameter polystyrene spheres scatter light as expected for Rayleigh scattering, choosing an appropriate range of wavelengths for your analysis.

4. Measure the absorbance and fluorescence spectra of a solution of 36 nm diameter "fluorospheres" over the wavelength range 250–900 nm. (Decide what part of the spectrum is useful.) Determine the scattering, absorption, and fluorescence properties of the spheres.

5. Measure the kinetics of collagen fibril formation by taking advantage of the enhanced light scattering of fibrils compared with isolated proteins in solution.

V. MATERIALS AND EQUIPMENT

- Spectrophotometer equipped for absorbance and fluorescence measurements.
- Disposable cuvettes.
- Stock solution of Rhodamine 6G (10 μM in water) **IMPORTANT: Rhodamine is a health hazard. Dispose in chemical waste; wear gloves and eye protection.**
- Concentrated solution of 31-nm-diameter polystyrene spheres (8% w/v).
- Concentrated solution of 36-nm-diameter fluorescently labelled polystyrene spheres (1% w/v).
- Concentrated solution of type I collagen, extracted from rat tail tendons, in acetic acid (2.5 mg/ml).
- Concentrated PBS buffer solution, pH 6.9 with excess phosphates, for fibril formation (5X working concentration).[3]

VI. SUGGESTED TIMELINE

Day 1: Measure the absorbance and fluorescence spectra for each of the Rhodamine 6G and nanosphere solutions. To determine the extinction coefficient of Rhodamine 6G, you will need to measure its absorbance spectrum at a range of concentrations. To measure its fluorescence spectrum, you will need to determine the appropriate concentration, as well as integration time on the spectrometer, in order to see a curve of good signal-to-noise ratio. To measure the spectra of the microspheres, dilute them so the solution is not too optically dense. Try a 100-fold dilution.

Day 2: Measure the kinetics of collagen fibril formation. Come to lab having analyzed the spectra from Day 1 experiments and answered the following pre-lab questions.

Pre-lab question 5: What features in the spectra from Day 1 arise from light scattering?

Pre-lab question 6: What type of measurement (e.g., absorbance or fluorescence mode) are you going to perform to determine the amount of light scattering during collagen fibril formation? Justify your choice.

[3] Working (1X) stock concentration of the buffer is 273.8 mM NaCl, 5.4 mM KCl, 42.2 mM dibasic sodium phosphate (Na_2HPO_4) and 8.8 mM monobasic potassium phosphate (KH_2PO_4).

You will need to measure the intensity of scattered light as a function of time. *Protocol—Using the Spectrophotometer* explains how to set up the instrument to record a time series of measurements. Start your measurements rapidly after initiating fibril formation, so that you have enough time to observe the plateau phase of fibril assembly and growth. To initiate fibril formation, mix—**on ice**—the 5X buffer solution, pure water, and concentrated collagen solution to obtain working concentrations of

1X buffer and ≈ 0.3 mg/ml collagen.

Start recording your spectra immediately after mixing and putting in the cuvette (i.e., ensure the spectrophotometer is properly zeroed and ready to go *before* mixing your sample). The measured kinetics of collagen fibril formation depend very strongly on initial conditions (see Wood and Keech,1960). For one experimental run, as you will perform here, these are less critical, but it is important to keep materials cold so that fibril nucleation does not initiate prior to the start of your measurements.

Pre-lab question 7: What amounts of buffer, collagen and water will you mix to create a final sample 1 ml in volume?

- 5X buffer: _____ μl
- ddH_2O: _____ μl
- collagen (2.5 mg/ml stock): _____ μl

VII. ITEMS TO INCLUDE IN LAB WRITE-UP

- Answer all pre-lab questions.
- Plot the Rhodamine 6G absorbance spectrum at a given concentration (and state the concentration).
- Determine the extinction coefficient of Rhodamine 6G at 532 nm from a plot of absorbance versus concentration.
- Plot Rhodamine 6G-fluorescence spectrum at a given concentration (and state the concentration).
- Determine the percentage of emitted light intensity at wavelengths longer than 550 nm from Rhodamine 6G excited by a laser at 532 nm.
- Plot the relationship between OD and wavelength of 31 nm diameter polystyrene spheres, and determine whether this follows Rayleigh scattering.

- Plot absorbance and fluorescence spectra of 36 nm diameter polystyrene "fluorospheres," labelling contributions from scattering, absorption and fluorescence
- Plot light scattering versus time throughout the course of collagen fibril formation, labelling nucleation, growth and plateau phases.
- Determine growth rate of fibrils from the kinetics measurements.
- Discuss the above results, as well as errors and experimental difficulties encountered, if any.
- Discuss at least two (Phys 433) or all (Phys 833) of the points from the following section.

VIII. POINTS TO PONDER

- How would the fluorescence spectrum of Rhodamine 6G change if the solution were excited at 500 nm? At 550 nm?
- Describe and sketch the expected OD(λ) curve for a solution containing (a) 30 nm diameter polystyrene spheres; (b) 100 nm diameter polystyrene spheres; and (c) equal numbers of the two sizes of spheres.
- Find an example in the literature of using FRET to study some aspect of a biological system. Citing the reference, summarize in one or two paragraphs a key example of how the experiment was conducted and what was learned.
- How would the kinetics of fibril formation change if the collagen concentration were decreased? Describe any changes expected in each of the three kinetic phases.
- How would the kinetics of fibril formation change if the temperature were increased? Describe any changes expected in each of the three kinetic phases.

IX. ADDITIONAL READING

- R.N Zare, B.H. Spencer, D.S. Springer and M.P. Jacobson, "Light Scattering from Disordered Systems" in *LASER Experiments for Beginners*. University Science Books (1995).
- C. Garland, J. Nibler and D. Shoemaker, "Spectroscopy," in *Experiments in Physical Chemistry*. McGraw-Hill (2008).
- C. Joo, H. Balci, Y. Ishitsuka, C. Buranachai and T.J. Ha, "Advances in Single-Molecule Fluorescence Methods for Molecular Biology." *Annual Review of Biochemistry* **77**, 51–76 (2008).
- An introduction to FRET from Olympus (the microscope company).
- C.G. Wood and M.K. Keech, "Formation of fibrils from collagen solutions 1. Effect of experimental conditions: kinetic and electron-microscope studies," *Biochemical Journal* **75**, 588–598 (1960).
- C.G. Wood, "Formation of fibrils from collagen solutions 2. Mechanism of collagen-fibril formation," *Biochemical Journal* **75**, 598–605 (1960).
- B.R. Williams, R.A. Gelman, D.C. Poppke and K.A. Piez, "Collagen fibril formation. Optimal in vitro conditions and preliminary kinetic results," *Journal of Biological Chemistry* **253**, 6578–6585 (1978).

Some more detailed articles about FRET:

- "Recent advances in FRET: distance determination in protein-DNA complexes." A. Hillisch, M. Lorenz and S. Diekmann. *Current Opinion in Structural Biology* **11**, 201–207 (2001).
- "Fanciful FRET." S.S. Vogel, C. Thaler and S.V. Koushik. *Science STKE* 2006 (331).

