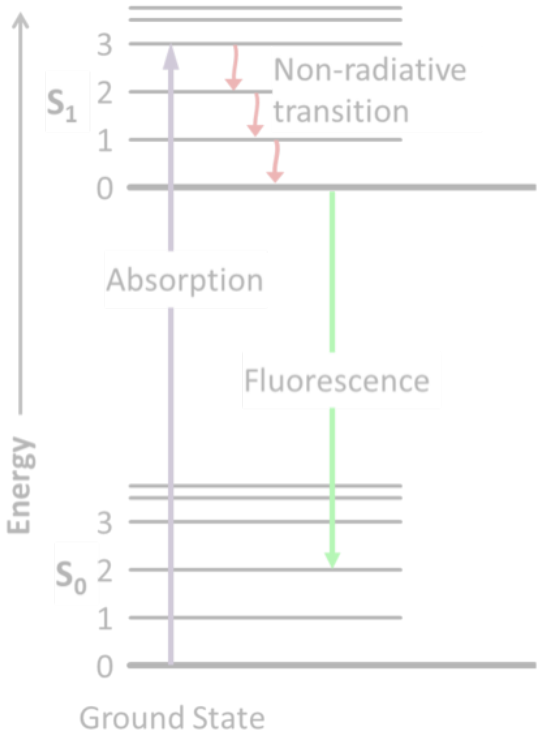
EMISSION SPECTROSCPY

The emission phenomenon
The emission lifetime
Fluorescence spectroscopy
Analytical applications
Fluorescence for amino acids
Fluorescence of proteins
Fluorescence for nucleic acid



Master course 2018

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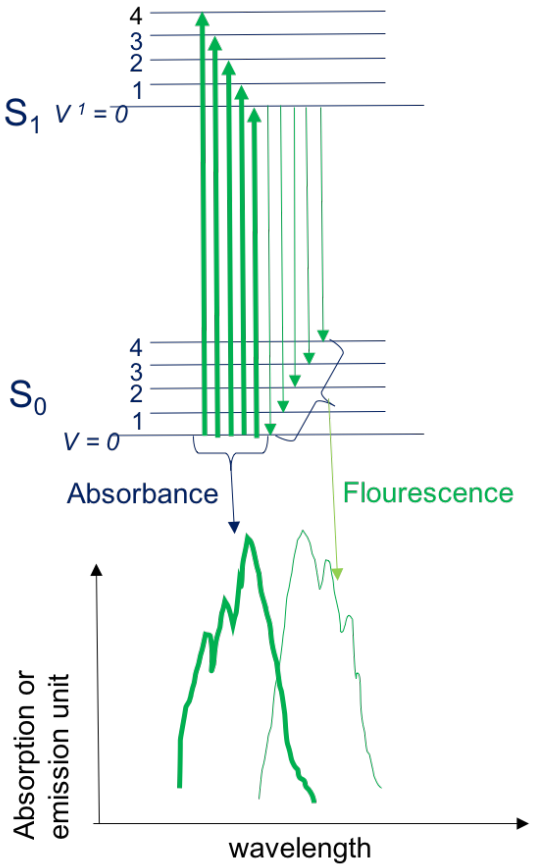
Emission spectroscopy

When a molecule absorbs photons of light, it will be excited from the ground state to a higher energy level. In many cases the liberated energy transfers to heat to the surroundings. However, in some cases the light emitted by the molecule could be liberated as fluorescence or phosphorescence. We will discuss general concepts about emission and leave any exception behind.

1.1. The emission phenomenon

If the molecule absorbed light and excited to a higher energy level (S_1), but did not do a chemical reaction (collision, dissociation, chemical rearrangement), then the molecule will liberate this energy as photons *hv* and go back to the ground state (S_0), See Figure 1.
The **emission spectrum** of a molecule or a chemical compound is the frequented spectrum of the electromagnetic radiation that emitted due to electronic transitions from an excited energy level to a lower energy level. This phenomenon is also called **fluorescence**. At first glance, the fluorescence process may appear to be the reverse of the absorption steps. However, if we compare the spectra for each, we will notice that the spectra for absorption is not superimposed on that for the emission. The two spectra form a mirror image of each other, except that the emission spectrum is displaced toward the longer wavelength.
The emitted photon has an energy equal to the energy difference between the two levels. Each atom has many possible electron transitions, and each transition has a specific energy difference. These different transitions collectively will lead to different radiated wavelengths, which show as an emission spectrum.
Each element has a unique emission spectrum. Therefore, fluorescence spectroscopy frequently used to identify the elements in an unknown mixture. Similarly, the emission spectra of molecules can be used in chemical analysis of substances.



1.2. The emission lifetime

The time required to induce a vibration is much less than the decay time or the lifetime of the fluorescent state (time of vibration=10^{-14} – 10^{-13} s < the decay time= 10^{-9}-10^{-5} s). Therefore, the excess of vibrational energy is dispatched as a heat to the surroundings and the decay is now occurring from the ground vibrational level (S_1, S_2,..) to the ground state (S_0). Emission is then occurring when the photon relaxing from the lowest vibrational energy level (V_1) of S_1 to (most often) a higher vibrational level of S_0, see Figure 2.
The emission lifetime is the time that the electron spends between the vibrational levels and stages before decay back to the ground state.

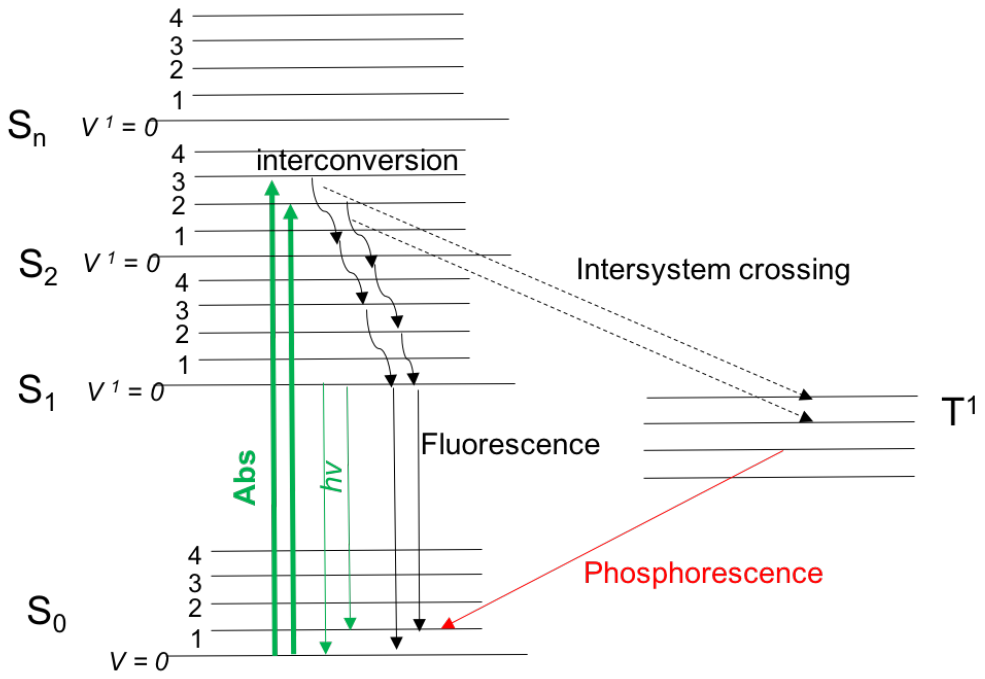


Figure 2. The fluorescence lifetime. S_0, S_1, S_2 and S_n are the singlet ground, first, 2nd and unlimited electronic energy levels, respectively. These energy levels have different vibrational energy levels (1,2,3 and 4). When photons of light are absorbed to a higher energy level, it will either undergo internal conversions (interconversion) to a lowest vibrational level of S_1 before returning to S_0 and emit the light (fluorescence), or it will inter-crosses the system to another lower energy level (T) to liberate the light as phosphorescence and return to the ground state S_0.

1.3. Fluorescence spectroscopy

Understanding the process from the ground vibrational level is the key to understand how the fluorescence spectroscopy works and how to use it for different applications.
Fluorimeters are designed for general-purpose to measure the fluorescence spectrum, the polarization and/or the lifetime. A typical fluorimeter includes a light source such as xenon arc lamps, a specimen chamber with integrated optical components, and detectors (Figure 3).

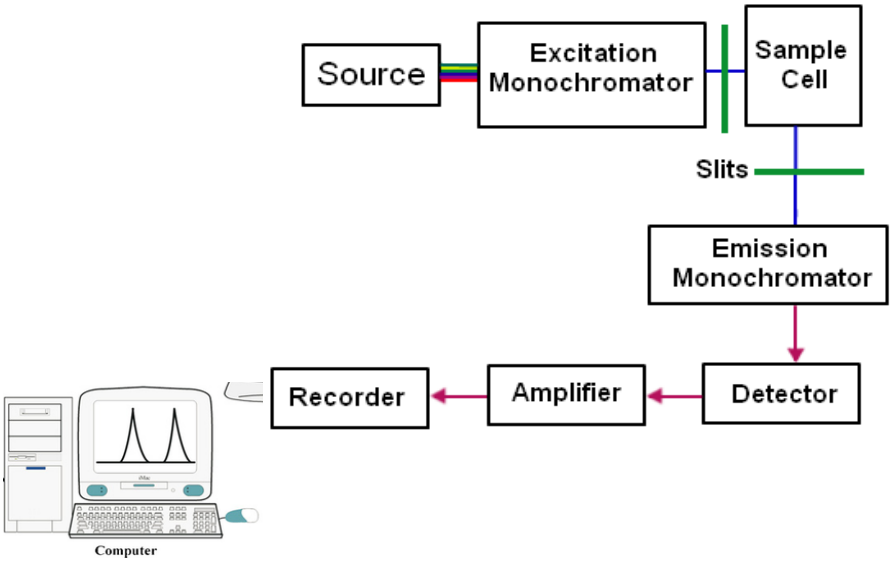


Figure 3. simple diagram of the fluorescence instrument

The lamp (Xe) provides a relatively uniform intensity over a broad range (UV to the near IR). The optical paths of the excitation and the detection light paths are along the orthogonal axis. The orthogonal arrangement ensures minimal leakage of excitation light into the detection side. High sensitivity photodetectors such as photomultipliers or charge coupled device cameras are commonly used.
The measurement of fluorescence intensity, lifetime and detect the spectrum is a sensitive method of monitoring the biochemical environment of a fluorophore.
Measurement of the fluorescence intensity determine the presence of fluorophores and their concentrations in the certain molecule or compound. The fluorescence intensity measurement is important in the biochemical assays because it is a good indication about the location of the fluorophore within the whole structure of the molecule.
Fluorescence technique uses light to excite the sample. The light should pass through a monochromatic subject to choose the wavelength of excitation that is suitable to the sample. In addition, another monochromator is needed after the sample to scan the different wavelengths of the emitted light, (Figure 3). Herein, the second monochromator should have a wavelength setting different than that of the first monochromator to detect the emitted light which usually appears in longer wavelength as showed in Figure 1 and Figure 4.

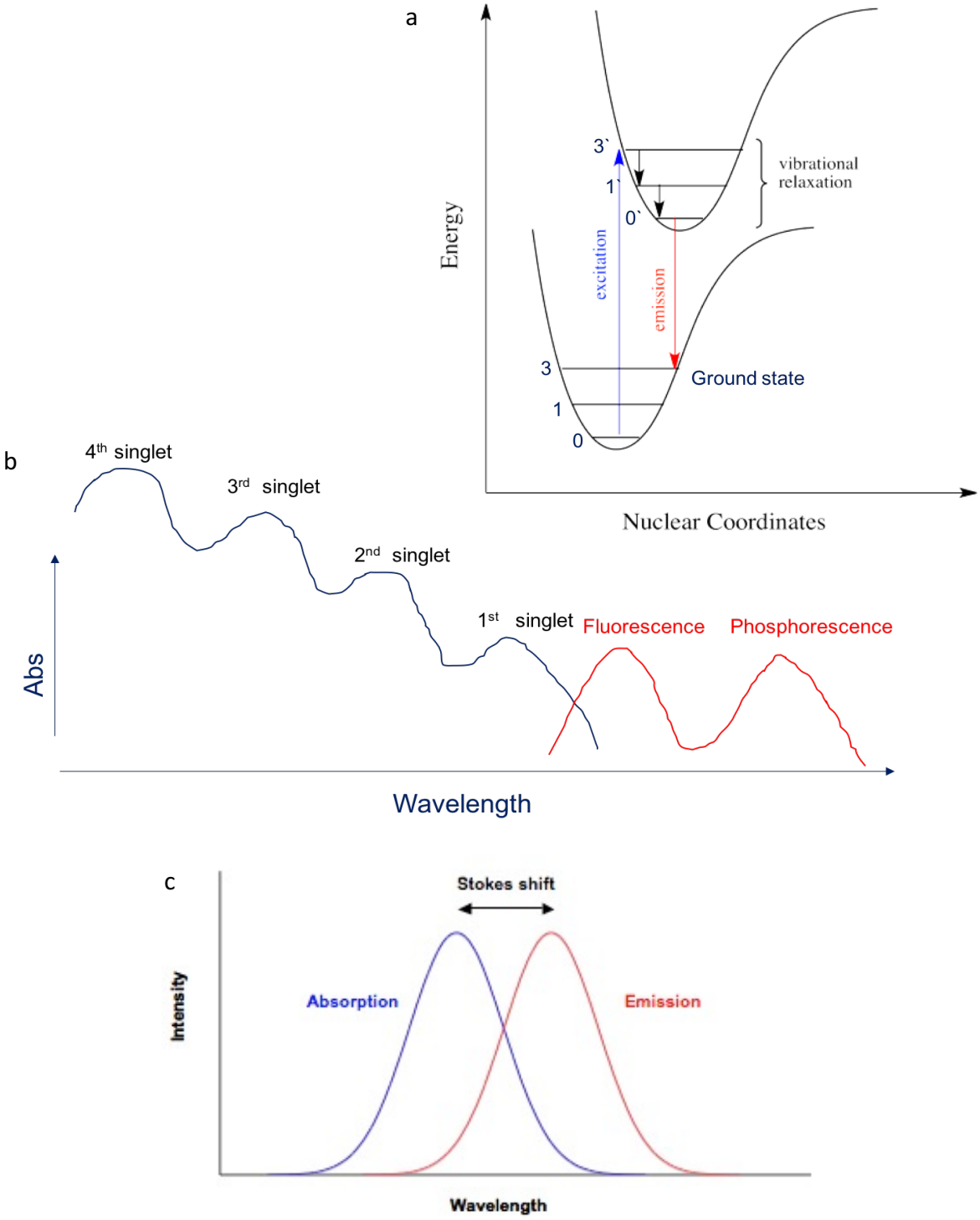


Figure 4. a) The ground and the first singlet electronic states, b) the absorption and emission of a hypothetical molecule, c) the Stokes shift. The typical absorption and emission of a fluorophore where the photons absorbed at higher energy and short wavelength but emitted at lower energy and longer wavelength.

The absorption from the ground state (quantum number =0) up to any vibrational level of the excited state for a hypothetical molecule is shown in Figure 4a.
The lower ground vibrational level will require less energy than the 2nd or 3rd vibrational levels. Therefore, excitation to a higher vibrational level require more energy and it appears in lower wavelength as shown in Figure 4b; each absorption wavelength has its the fluorescence spectrum. The difference between maximum absorption and maximum emission is called Stokes Shift, Figure 4c.
For spectral measurement, monochrometers are placed in the excitation and emission light paths (before and after the sample) to select a specific wavelength spectral band.
The excitation spectrum is the fluorescent intensity measured as a function of excitation wavelength at a constant emission wavelength.
The emission spectrum is the fluorescent intensity measured as a function of emission wavelength at a constant excitation wavelength.

Fluorimeters have also been designed to measure the fluorescence lifetime. Accurate lifetime measurement requires photodetectors and signal processing electronics. High-precision fluorimeters have been designed in both the time and frequency domains.
For polarization measurement, polarizers are inserted into the excitation and emission light paths. With the excitation polarizer, the emission polarizer can be rotated to measure the parallel ($I_{\prod}$) and the perpendicular ($I_{\perp}$) components of the fluorescence emission.

1.4. Applications of fluorescence to study biological molecules

1.4.1. Amino acids

The fluorimeter has been widely used for studying the proteins and amino acids with aromatic side chains. They found to have an intrinsic fluorescent when excited using UV light.
There are also special proteins such as Green Fluorescent. The three amino acids that absorb by UV and show a fluorescence are: Tryptophan (Trp), Tyrosin (Tyr) and Phenylalanine (Phe), Figure 5.

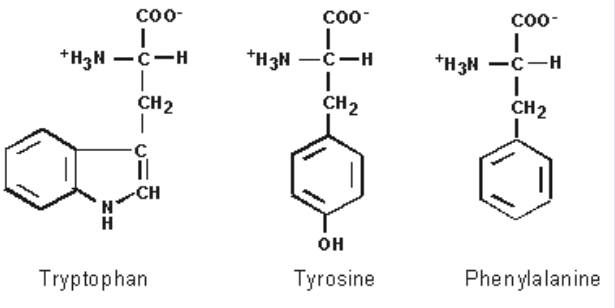


Figure 5. the chemical structure of aromatic amino acids.

These residues have their distinct absorption and emission wavelengths (Table 1). However, Trp is the dominant intrinsic fluorophore in proteins, where the protein fluorescence is highly sensitive to the local environment of Trp. The polarity of the solvent has an effect on the absorbance of these amino acids. If the polarity of the solvent decreases, the Trp would tend to buried inside the hydrophobic region of the folded protein. This phenomenon has been

utilized to study the denaturing protein. Tyrosine and Trp can be excited at similar wavelengths, but emits at a different wavelength. Although Tyr is less fluorescent than Trp, it provides a significant signal because it presents in many proteins. Figure 6 shows the spectra of these amino acids with a control baseline.

Table 1. The absorption and emission wavelength of the aromatic amino acids residues. Trp has the strongest fluorescence while Phe has the weakest.

Residues	**Absorption λ/nm**	**Emission λ/nm**
Phe	257	282
Tyr	274	303
Trp	280	348

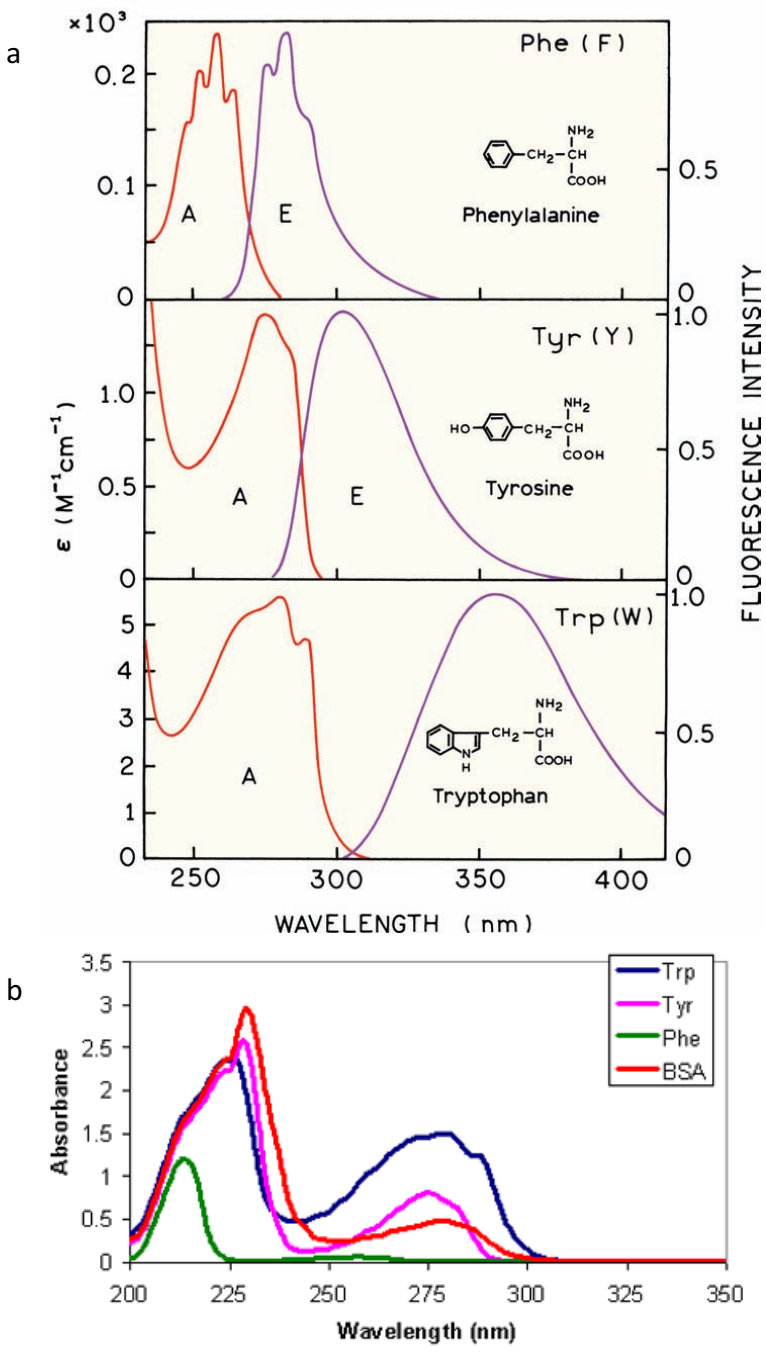


Figure 6. Fluorescence emission of the aromatic amino acid residues. a) the absorption and emission spectra of the three amino acids. Tryptophan has the highest emission intensity. b) the emission spectra of all aromatic amino acids in one graph.

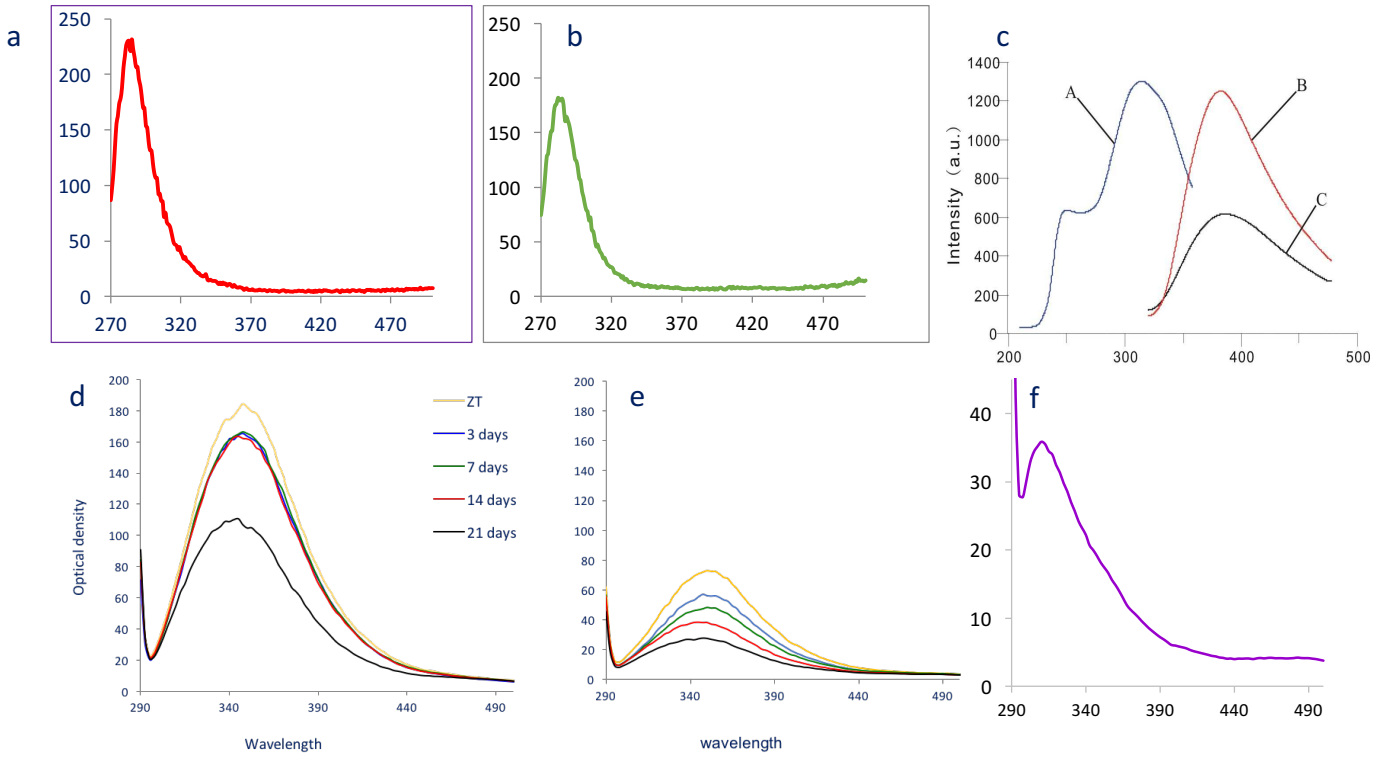


Figure 7. Fluorescence emission of Phe within mature fibrils of the peptide KFFEAAAKRFFE (a), and within a silica nanowire of the same peptide (b). Figure (c) is the excitation fluorescence (blue) and emission fluorescence (red) of silver nanoparticles, d) the decay time of the emission spectra of Tyr within a peptide (Ac-IHIHIYI-$CONH_2$) grown with Zn^{+2} for 21 days, e) the decay time of the emission spectra of Tyr within the same peptide in (d) but, grown without Zn^{+2}, f) emission spectra of Tyr within mature fibrils of the peptide IHIHIYI.

Figure 6 shows the absorption and emission spectra of the three amino acids. The emission spectra of Trp is affected by the surrounding environment, which affect the indole group specifically. These affected factors could be related to conformational transition, substrate binding, or denaturation. Also, Trp and Tyr can easily be quenched by added quenchers or by other nearby groups within the sequence of peptide or protein.

Figure 7 is showing the fluorescence emission spectra of some aromatic amino acids within mature fibrils. Figure (7a) and (b) is a comparison between the spectra of Phe within a peptide and a nanowire structure templated by the same peptide in Figure 7a. The intensity of the emission spectra became less in (b) than (a), which perhaps occurred due to quenching of Phe by the precursor used to prepare the nanoparticle (tetraethoxy orthosilicate). Alternatively, it could due to the polymerization of silica around the surface of the fibrils which made Phe that is buried inside the fibrils be less sensitive to the absorbed light and therefore emitted less. The emission spectra of Tyr also could also affect by the surrounding ligand or metal ions, which either enhance or quench the emission, as shown in Figure (7d) and (7e) where the signal of Tyr emission enhanced in the presence of zinc ion.

The appearance of a high intensity signal at zero time indicated the position of Tyr within the peptide architecture, which was buried at the hydrophobic core, therefore showed a high intensity emission. Over time, the peptide started to precipitate and Tyr became more exposed to the solvent, therefore, lower emission spectra at the specific wavelength (290- 380 nm),

1.4.2. Proteins

Proteins contain aromatic amino acids residues that when excited by appropriate wavelength of light, they will emit a light at another wavelength. The emission appears at longer wavelength because it is at lower energy. (Figure 4).
Fluorescence of protein is frequently used to detect the folding/ misfolding of the protein. Protein folding occur when it adopts the 3D native fold which is the functional form of the protein. Simply, folding passes through many stages starts by the formation of the secondary structure which then folds into tertiary 3D, and sometimes further into the quaternary structure 4D, Figure 8.

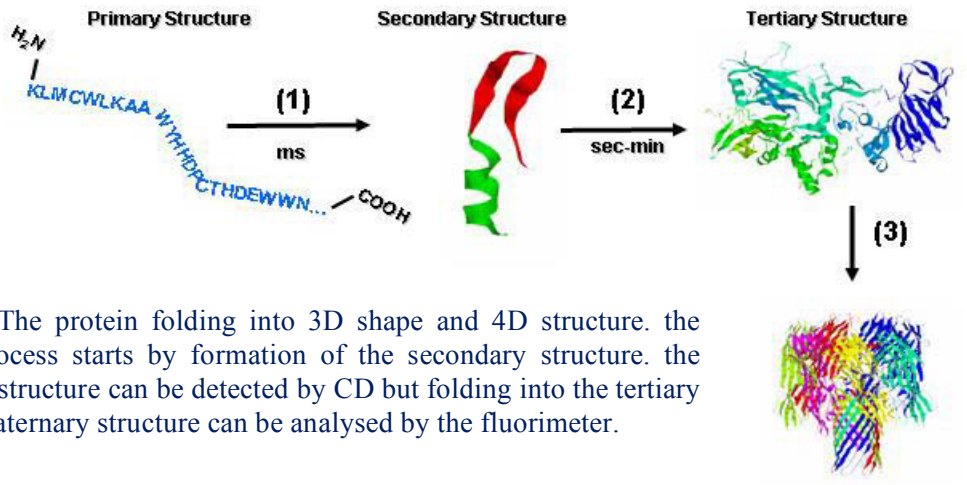


Figure 8. The protein folding into 3D shape and 4D structure. the folding process starts by formation of the secondary structure. the secondary structure can be detected by CD but folding into the tertiary and the quaternary structure can be analysed by the fluorimeter.

Protein fluorescence is useful to illustrate the change in quantum yield (emitted photons/excited photons), the effect of energy transfer, the change in polarization with time and study the stoichiometry of complex formation. All this information is useful to monitor the shape of the protein molecule. Whilst, lipids, saccharides, membranes are non-fluorescent and the fluorescence of DNA is very week.

The three amino acids Tyr, Trp and Phe have a fluorescence in protein. However, Trp is the predominant fluorophore in proteins, which presents at ~ 1mole % due to metabolic expense of its synthesis. On the other hand, it is difficult to interpret the fluorescence of a protein has multi amino acid fluorophore because the spectral properties of each residue are different than other. Also, Trp emission affects by the polarity of solvent.

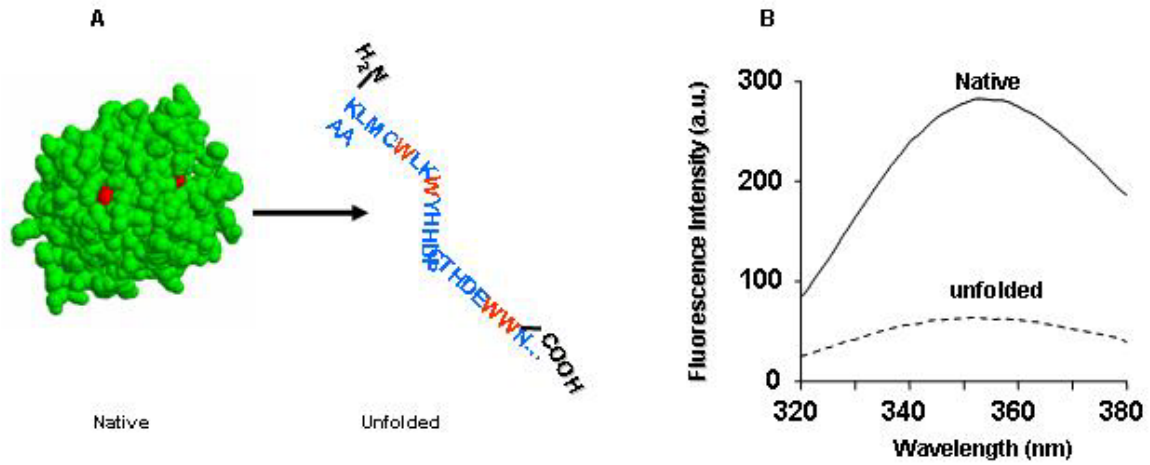


Figure 9. The fluorescence emission of the native and misfold protein.

The fluorescence of Trp and Tyr is useful to follow the protein folding because they are sensitive to the change in the environment, especially Trp. Figure (9) shows the native form of a protein where Trp (red) is buried inside the hydrophobic core (A). Therefore, the native protein has a higher fluorescence of Trp than the misfolded form in (B), where Trp became more exposure to the solvent so the fluorescence decreased.

Figure (10) shows the fluorescence emission spectra of native and denature IgG protein. The fluorescence emission was sensitive to the change in the protein structure when exposed to a high temperature up to100 C, which caused aggregation and denaturation. The upper large image is indicated the meditation of the spectra to demonstrate the obvious shift of the denature band toward long wavelength.

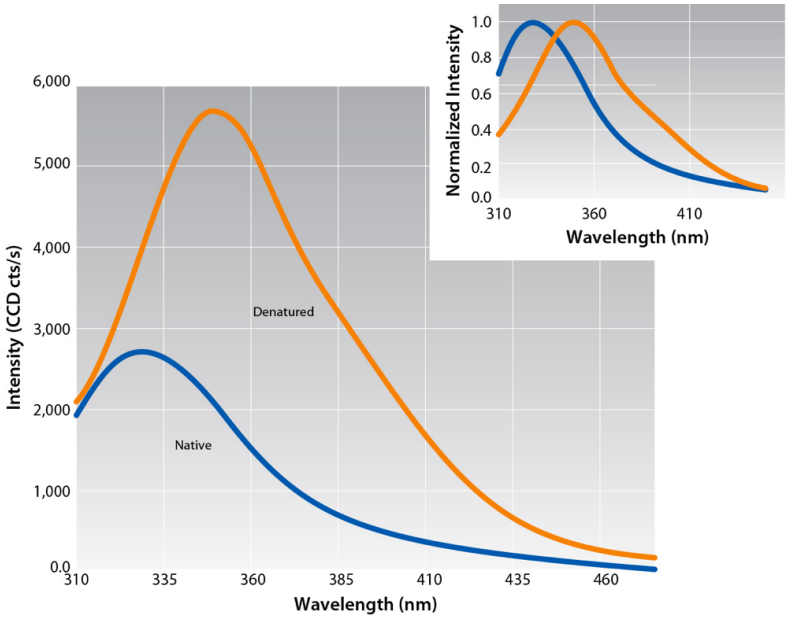
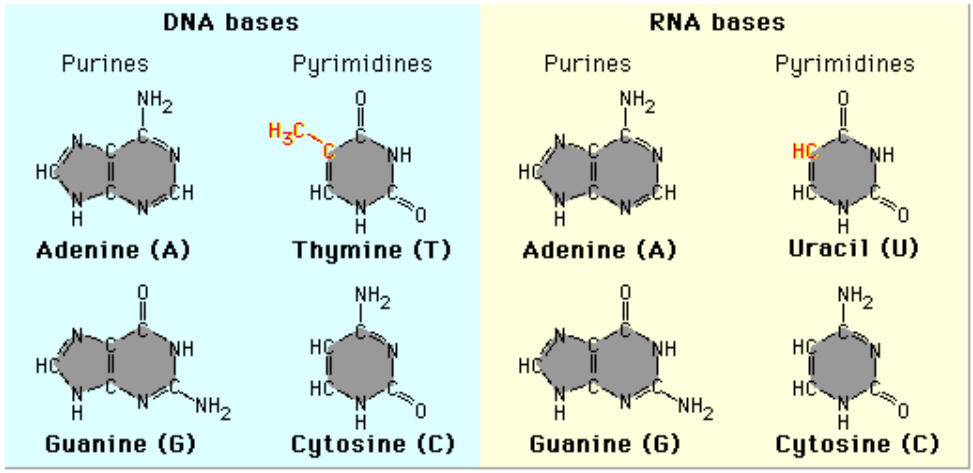


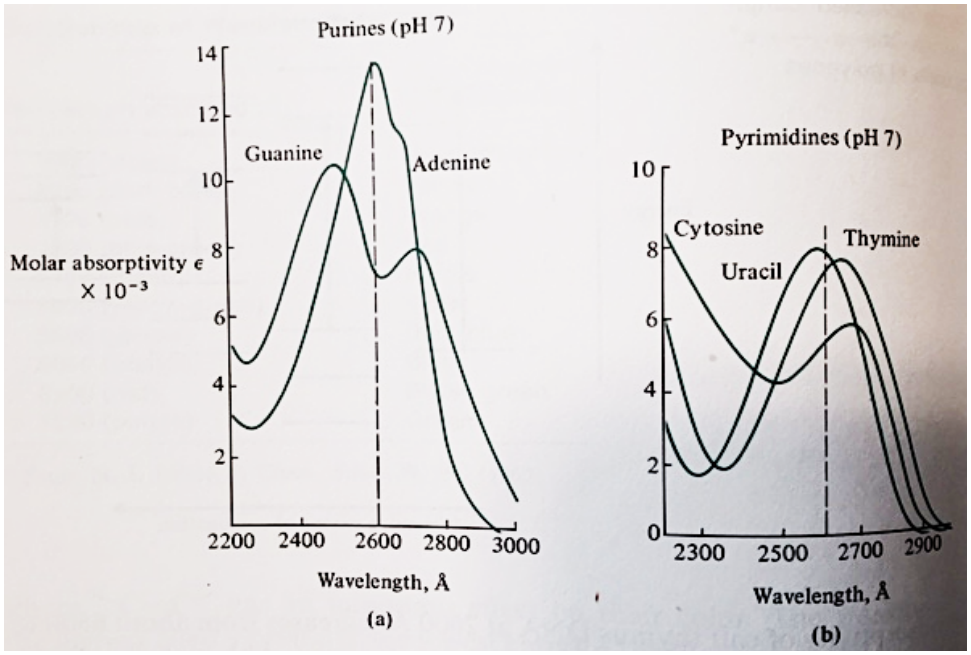
Figure 9. The fluorescence emission of the native and denature IgG protein after

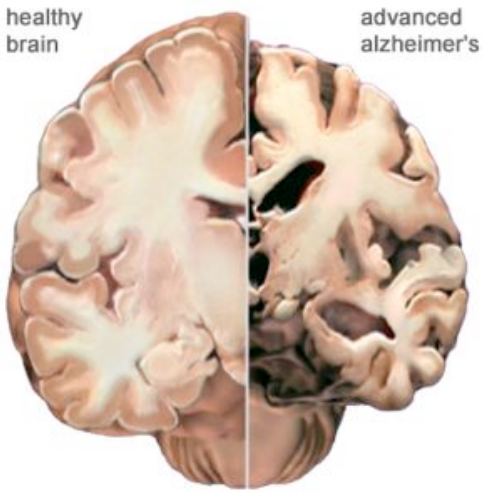
1.4.3. Nucleic acids

Many enzymatic cofactors, such as FMN (Flavin mononucleotide), FAD (Flavin adenine dinucleotide) and NAD (Nicotinamide adenine dinucleotide) have intrinsically fluorescents, add to the protein fluorescence. All these moieties have a common structure of aromatic ring which absorbs UV light for excitation.

The DNA bases have very little emitted light (fluorescence), therefore, to study the fluorescence of nucleic acids, they usually bind to a fluorophore or substitute to a fluorescence analog.



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