Molecular Spectroscopy

UV-Vis Spectroscopy

Absorption Characteristics of Some Common Chromophores

Chromophore	Example	Solvent	λ_{max}, nm	ε_{max}	Transition Type
Alkene	$C_6H_{13}CH{=}CH_2$	*n*-Heptane	177	13,000	$\pi \rightarrow \pi^*$
Alkyne	$C_5H_{11}C{\equiv}C{-}CH_3$	*n*-Heptane	178	10,000	$\pi \rightarrow \pi^*$
			196	2000	—
			225	160	—
Carbonyl	$CH_3\overset{\overset{O}{\|}}{C}CH_3$	*n*-Hexane	186	1000	$n \rightarrow \sigma^*$
			280	16	$n \rightarrow \pi^*$
	$CH_3\overset{\overset{O}{\|}}{C}H$	*n*-Hexane	180	large	$n \rightarrow \sigma^*$
			293	12	$n \rightarrow \pi^*$
Carboxyl	CH_3COOH	Ethanol	204	41	$n \rightarrow \pi^*$
Amido	$CH_3\overset{\overset{O}{\|}}{C}NH_2$	Water	214	60	$n \rightarrow \pi^*$
Azo	$CH_3N{=}NCH_3$	Ethanol	339	5	$n \rightarrow \pi^*$
Nitro	CH_3NO_2	Isooctane	280	22	$n \rightarrow \pi^*$
Nitroso	C_4H_9NO	Ethyl ether	300	100	—
			665	20	$n \rightarrow \pi^*$
Nitrate	$C_2H_5ONO_2$	Dioxane	270	12	$n \rightarrow \pi^*$

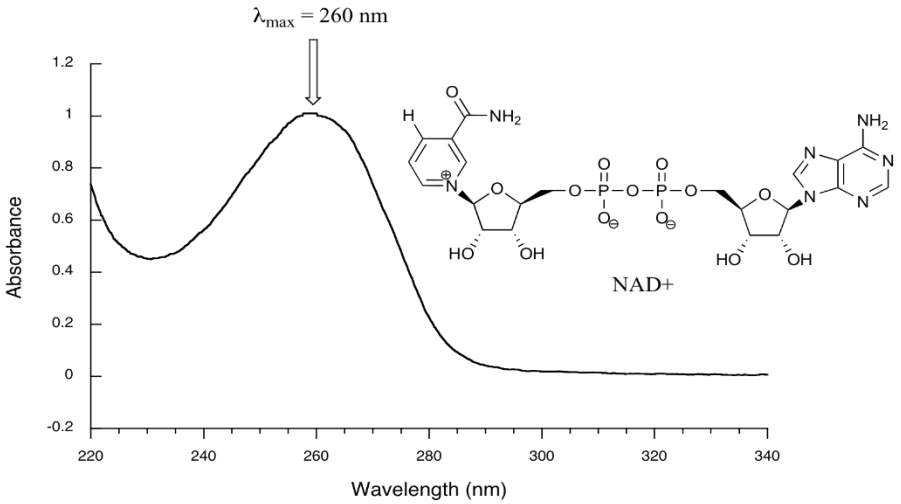
UV-Vis Spectroscopy

Absorption Characteristics of Aromatic Compounds

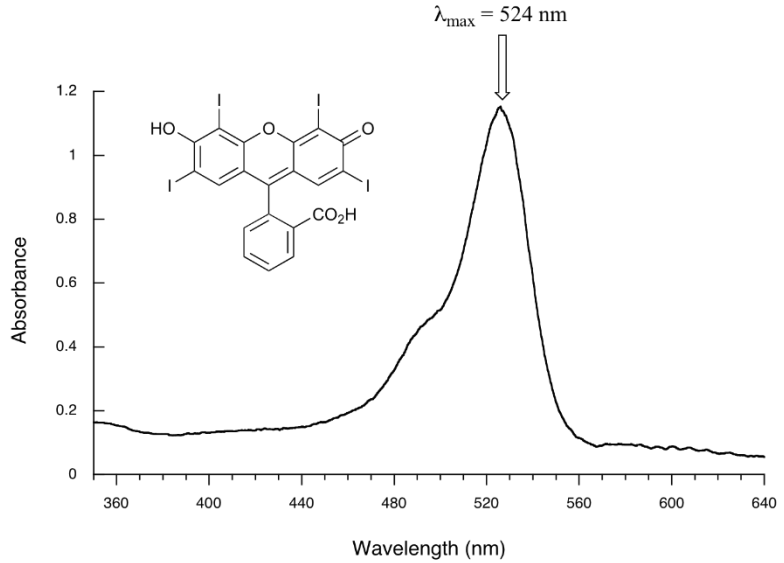
Compound		E_2 Band		B Band	
		λ_{max}, nm	ε_{max}	λ_{max}, nm	ε_{max}
Benzene	C_6H_6	204	7900	256	200
Toluene	$C_6H_5CH_3$	207	7000	261	300
m-Xylene	$C_6H_4(CH_3)_2$	—	—	263	300
Chlorobenzene	C_6H_5Cl	210	7600	265	240
Phenol	C_6H_5OH	211	6200	270	1450
Phenolate ion	$C_6H_5O^-$	235	9400	287	2600
Aniline	$C_6H_5NH_2$	230	8600	280	1430
Anilinium ion	$C_6H_5NH_3^+$	203	7500	254	160
Thiophenol	C_6H_5SH	236	10,000	269	700
Naphthalene	$C_{10}H_8$	286	9300	312	289
Styrene	$C_6H_5CH{=}CH_2$	244	12,000	282	450

UV-Vis Spectroscopy

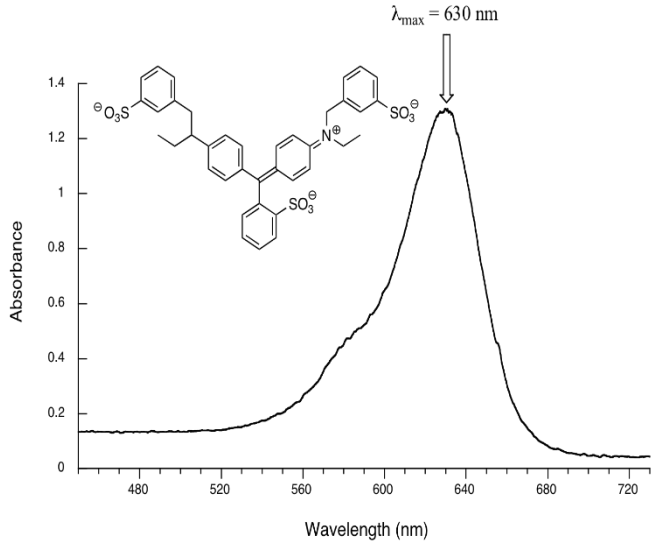
Effect of extended system of conjugated π-bonds



Nicotinamide adenine dinucleotide (NAD^+)



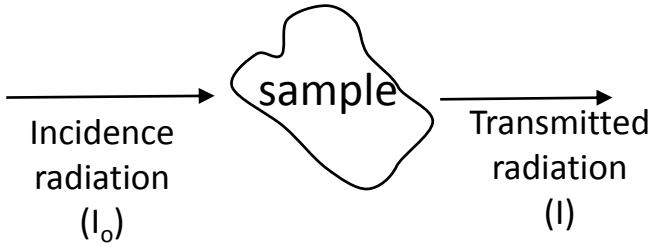
Erythrosine



Brilliant Blue FCF

UV-Vis Spectroscopy – Applications

1. QUANTIFICATION



Beer - Lambert Law:

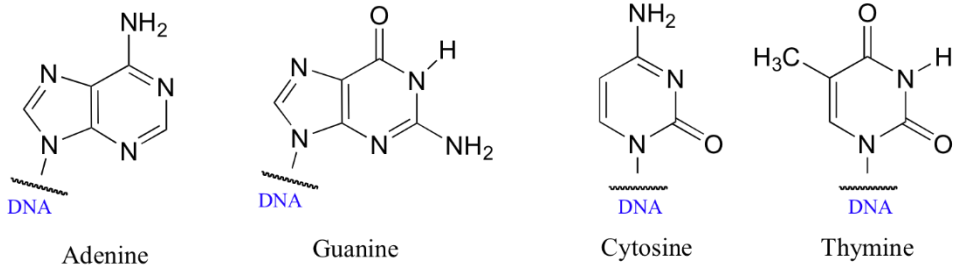
$$A = -logT = -log\frac{I}{I_0} = \varepsilon bc$$

A = absorbance
T = transmittance
c = concentration (molar)
ε = absorptivity ($molar^{-1}cm^{-1}$)
b = pathlength (cm)

Case Study: DNA or RNA

λ_{max} = 260 nm
ε (double-stranded DNA) = 0.020 $ng^{-1}\times mL$

Concentration determination

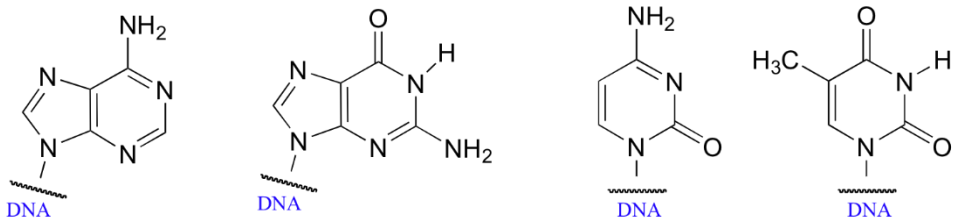


UV-Vis Spectroscopy – Applications

2. PURITY DETERMINATION

λ_{max} = 260 nm

ε (double-stranded DNA) = 0.020 $ng^{-1} \times mL$

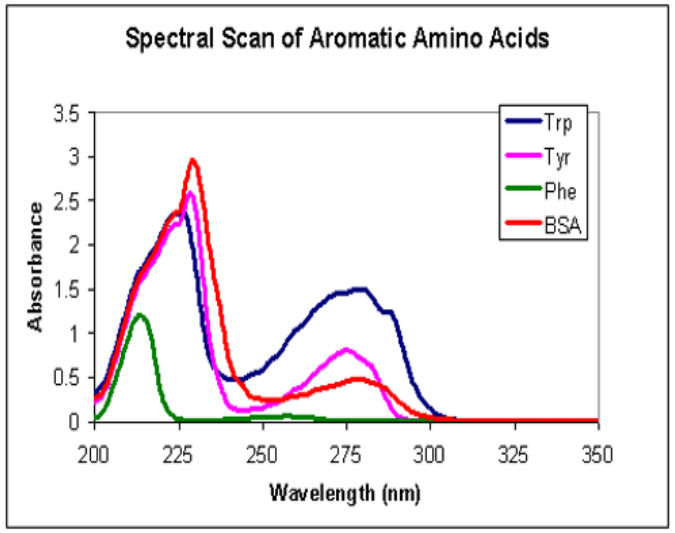


Adenine Guanine Cytosine Thymine

Common impurity in DNA samples is proteins.

Proteins typically absorb light at 280 nm

The ratio of absorptions at 260 nm vs 280 nm is commonly used to assess DNA contamination with proteins

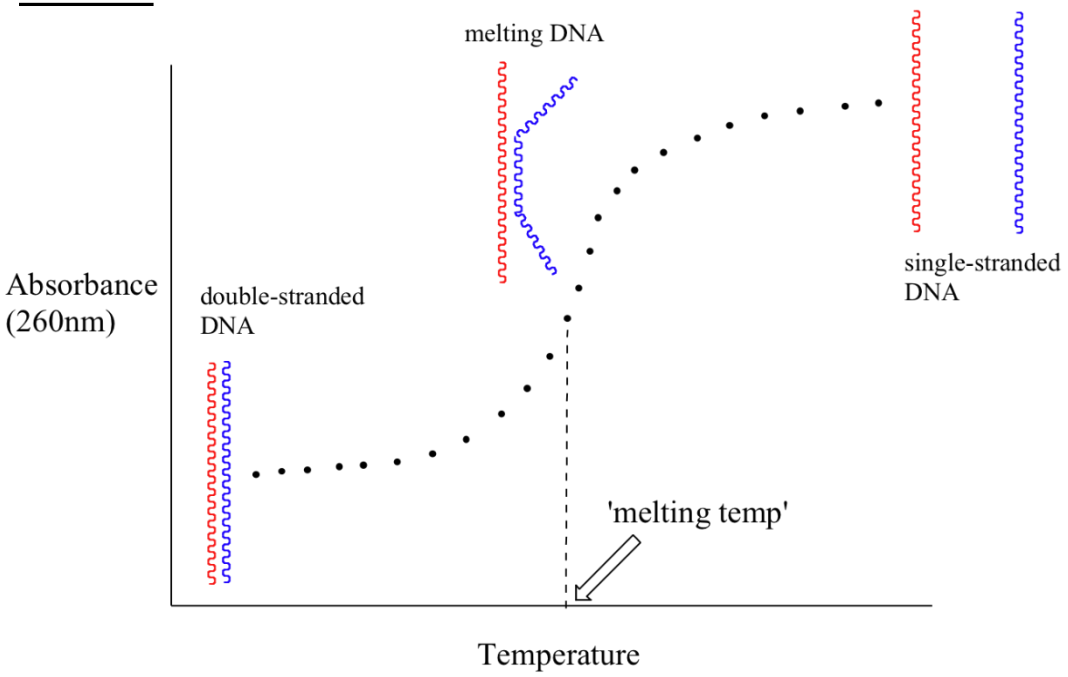


Likewise, proteins can be quantified by using these chromophores at 280 nm

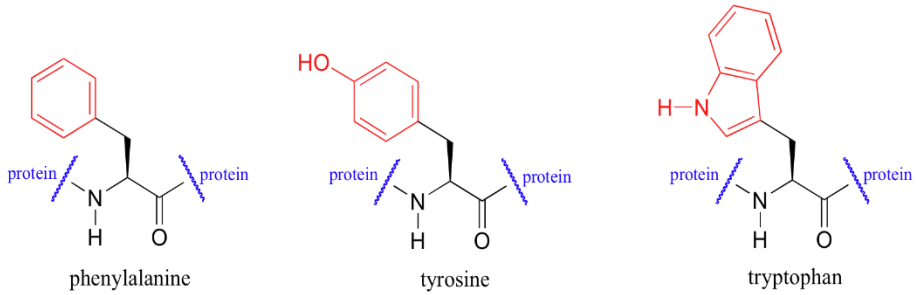
UV-Vis Spectroscopy – Applications

3. CONFORMATIONAL CHAGES

a. DNA



b. Proteins

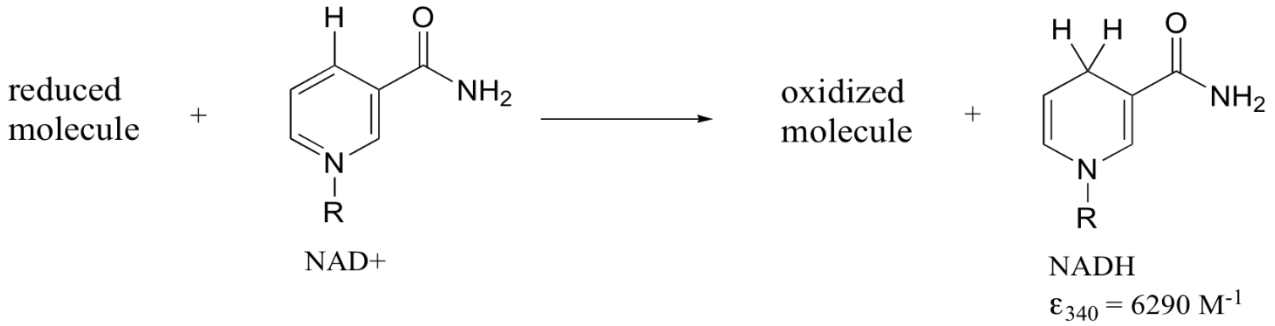


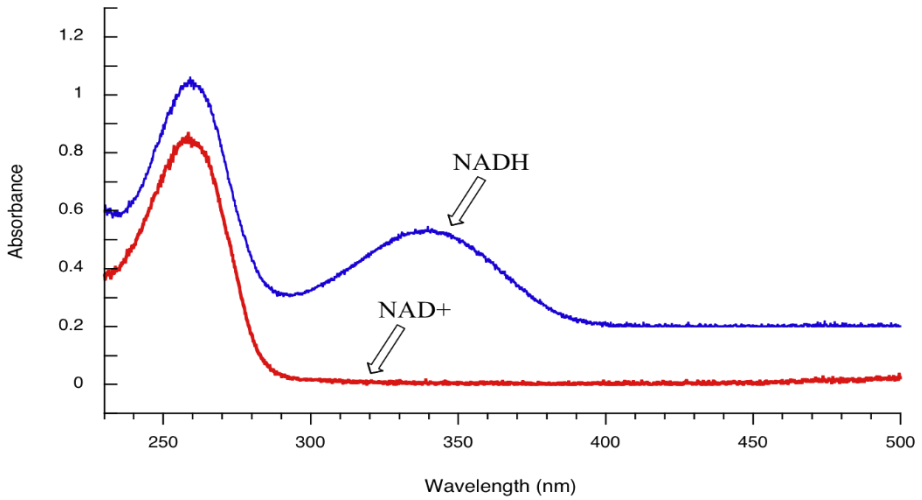
The mid-point of the curve, called the melting temperature, provides a good indication of how tightly the two strands of DNA are able to bind to each other.

$$T_m = -\frac{\Delta G^\circ}{R \ln \frac{[AB]_{initial}}{2}}$$

UV-Vis Spectroscopy – Applications

4. REACTION MONITORING





By monitoring the absorbance of a reaction mixture at 340 nm, we can 'watch' NADH being formed as the reaction proceeds, and calculate the rate of the reaction

Molecular Luminescence Spectroscopy

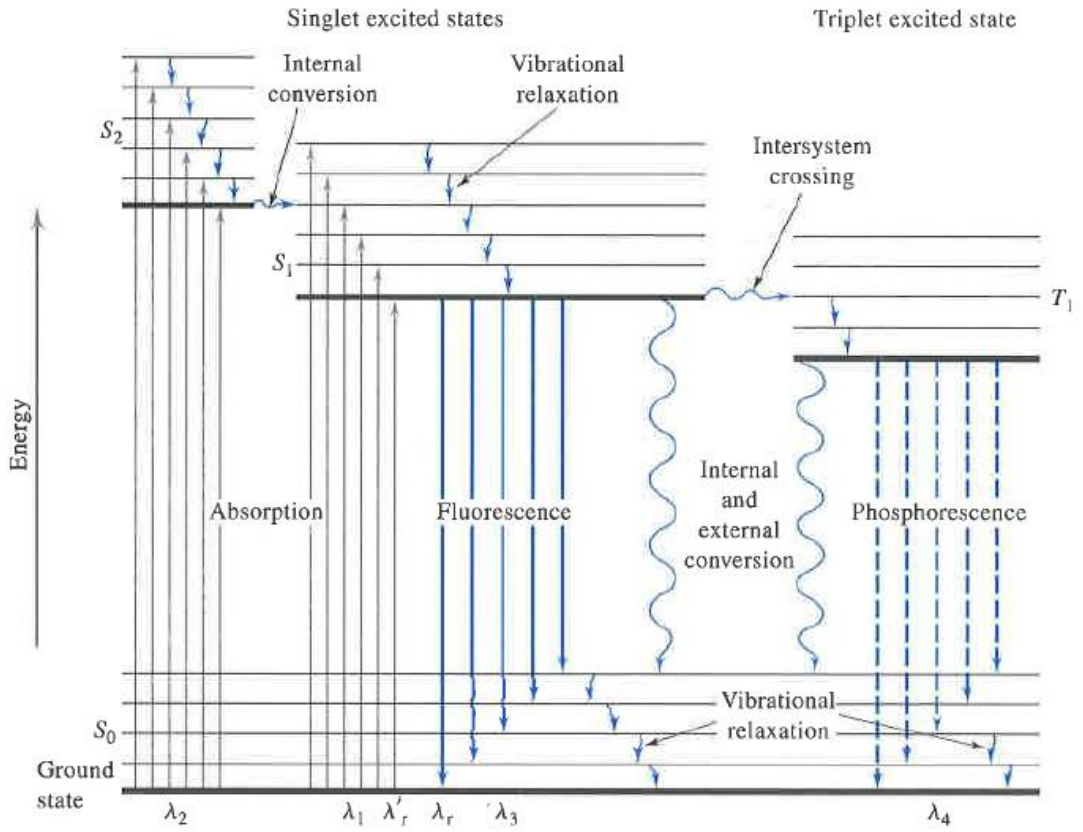
1. Fluorescence (mostly used)
2. Phosphorescence
3. Chemiluminescence

Advantages

- Highly sensitive (up to single molecules)
- Both organic and inorganic species
- Large linear concentration range

Challenges

- Only few molecules luminescence
- Quantitative luminescence methods are subject to serious interference



Joblonski Diagram

Deactivation Processes for the excited State

1. Vibrational relaxation
2. Internal conversion
3. External Conversion (involves interaction between other excited species or solvent molecules)
4. Intersystem crossing
5. Phosphorescence

Fluorescence and Phosphorescence

Factors Affecting Fluorescence and Phosphorescence

Mainly determined by chemical structure and environment; these two conditions also determine the intensity of the emitted light.

1. Quantum Yield

$$\text{Number of molecules that luminescence}$$

$$\text{Total number of excited molecules}$$

$$\phi = \frac{k_f}{k_f + k_i + k_{ec} + k_{ic} + k_{pd} + k_d}$$

Structure → k_f, k_{pd} and k_d

Environment → k_i, k_{ec}, and k_{ic}

Deactivation processes:

fluorescence (k_f), intersystem crossing (k_i), external conversion (k_{ec}), internal conversion (k_{ic}), predissociation (k_{pd}), and dissociation (k_d).

Fluorescence and Phosphorescence

2. Transition Type

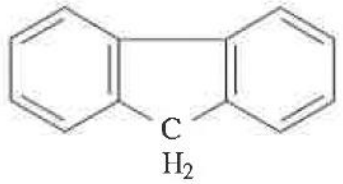
- Fluorescence can only occur for π → π* and *n* → π* transitions because σ → σ* require radiation of <250 nm; the corresponding energy (140 kcal/mol) can easily dissociate many bonds in organic compounds

3. Quantum Efficiency and Transition Type

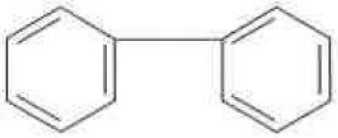
- Fluorescence is common for π → π* type (i.e., greater quantum efficiency) than n → π*
- Inherent lifetime for π → π* transition is much shorter (10^{-7} to 10^{-9}) compared with 10^{-5} to 10^{-7} for n → π* transitions, and so not susceptible to many deactivating processes

4. Structure: conjugation and rigidity

- π → π* transitions; conjugation increases molar absorptivity resulting in short lifetime of excited states
- Incorporation of heteroatoms (e.g., nitrogen) into the aromaticity tends to prevent fluorescence
- Rigid structures fluorescence more → less internal conversion



fluorene


biphenyl

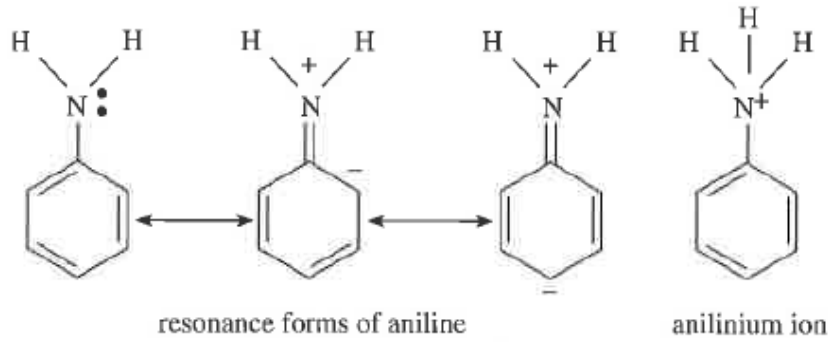
Fluorescence and Phosphorescence

5. Temperature and Solvent Effects

- Quantum efficiency decreases with increasing temperature because of increased frequency of collisions at elevated temperatures
- The likelihood of external conversions increases with solvent viscosity

6. pH

- Aromatic compounds with acid/base substituents are affected by pH



6. Concentration and Nonlinearity

- *Secondary absorption* is common at high concentrations, and when emission wavelength overlaps with absorption, causing the emitted light to be re-absorbed

Fluorescence and Phosphorescence

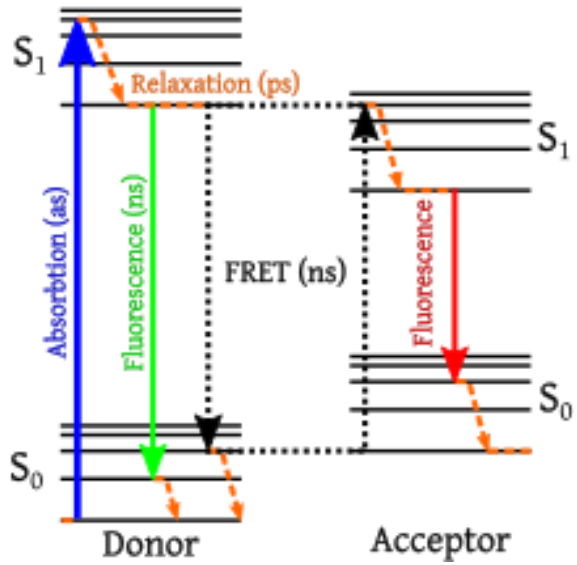
7. Dynamic Quenching

Specifically refers to collisional quenching, and requires physical contact between excited species and quenching agent. It is controlled by diffusion. Rate of quenching is proportional to quencher concentration

Fluorescence and Phosphorescence - Applications

Mostly applied in biology and medicine based
Information is obtained from the:

(1) fluorescence spectrum,
(2) fluorescence lifetime, and
(3) fluorescence polarization

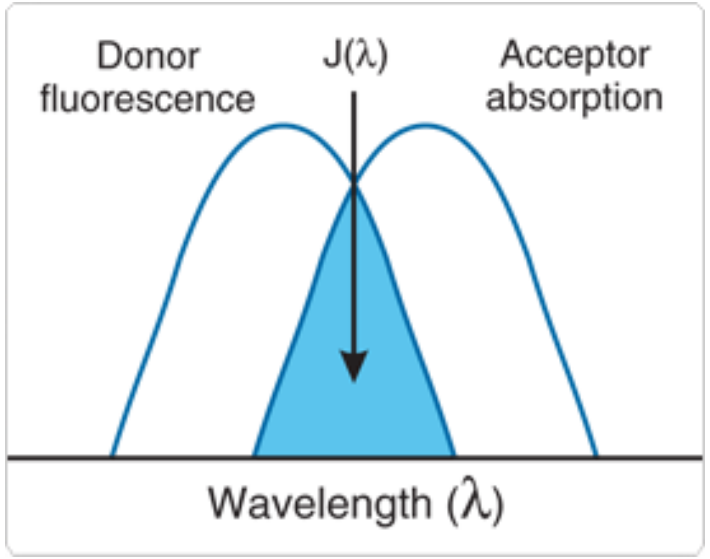


1. <u>The fluorescence spectrum</u>

- Highly sensitive to the biochemical environment of the fluorophore
  - ✓ Spectra can change as a function of the concentration of metabolites
  - ✓ spectral changes yield information about protein structure and folding

<u>2. Fluorescence resonance energy transfer (FRET)</u>

- Protein domain structure and motion on the sub-nanometer scale can be monitored
- FRET is a non-radiative process in which the energy is transferred between two fluorophores. FRET requires that the emission spectrum of one fluorophore (the donor) overlaps the absorption spectrum of a second fluorophore (the acceptor)
- The efficiency of this process is a <u>strong function of the molecules' relative distance, r</u>
- Protein conformational changes can be monitored by labelling the relevant structures with a FRET pair



Fluorescence and Phosphorescence - Applications

3. The Fluorescence lifetime

- Fluorescence lifetime provides complementary information to spectral measurement
- Many fluorophores may respond to environmental changes with lifetime variations
- E.g., oxygen concentration measurement based on the dynamic quenching of long-lifetime fluorophores

- Lifetime measurements are also used to distinguish dynamic and static quenching mechanisms

- Lifetime-resolved FRET measurement allows the determination of distance distribution of a population of FRET pairs

4. Fluorescence polarization

- Fluorescence polarization measures the rotational diffusion rate of macromolecules
- Rotational diffusion contains information related to the shape of the proteins

- E.g., monitoring of protein–ligand binding
- The smaller ligand molecules are labelled by a fluorophore. The binding of the small ligand to a larger protein results in a significant increase in the hydrodynamic radius of the composite particle and a slower rotational diffusion rate. The change in rotational diffusion rate can be measured using fluorescence polarization assay.
- The fraction of bound molecules can be estimated by quantifying the optical signal contributions from the fast and slow diffusers. The association constant of this protein–ligand interaction can also be measured by quantifying the fractions of bound and free proteins at different protein–ligand mixing ratios

Fluorescence and Phosphorescence - Applications

5. Fluorescence Imaging

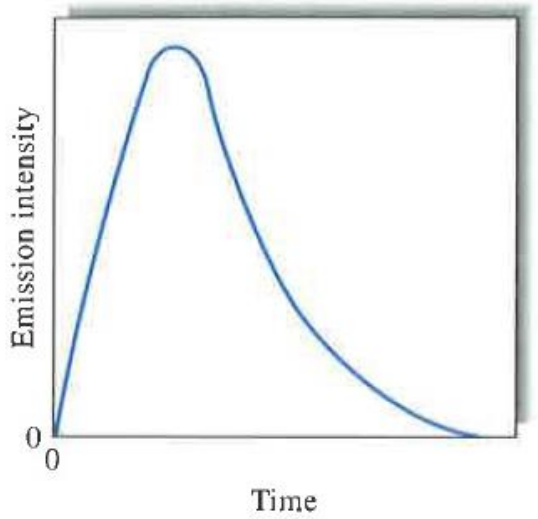
- Native fluorescence biomolecules
- Fluorescence indicators; e.g., ion probe for the detection of the presence of Na^+ or Ca^{2+}
- Fluorescence lifetime imaging – combines fluorescence microscope with fluorescence lifetime

Analytical Applications of Chemiluminescence

Principle

$$A + B \rightarrow C^* + D$$

$$C^* \rightarrow C + h\nu$$



Instrumentation is quite simple:
Reaction vessel and photo-multiplier tube

Advantages:

1. Since signal is generated through a chemical reaction, the sensitivity of the analysis can often be enhanced by changing reaction conditions
2. Analysis is selective since the excitation is selective (i.e., only species involved in the chemical reaction have the opportunity to become excited
3. Instrumentation is simple because of the possibility of selective excitation
4. The method has zero-background. That is, since no excitation light source is used, there is no background light signal to interfere with the measurement

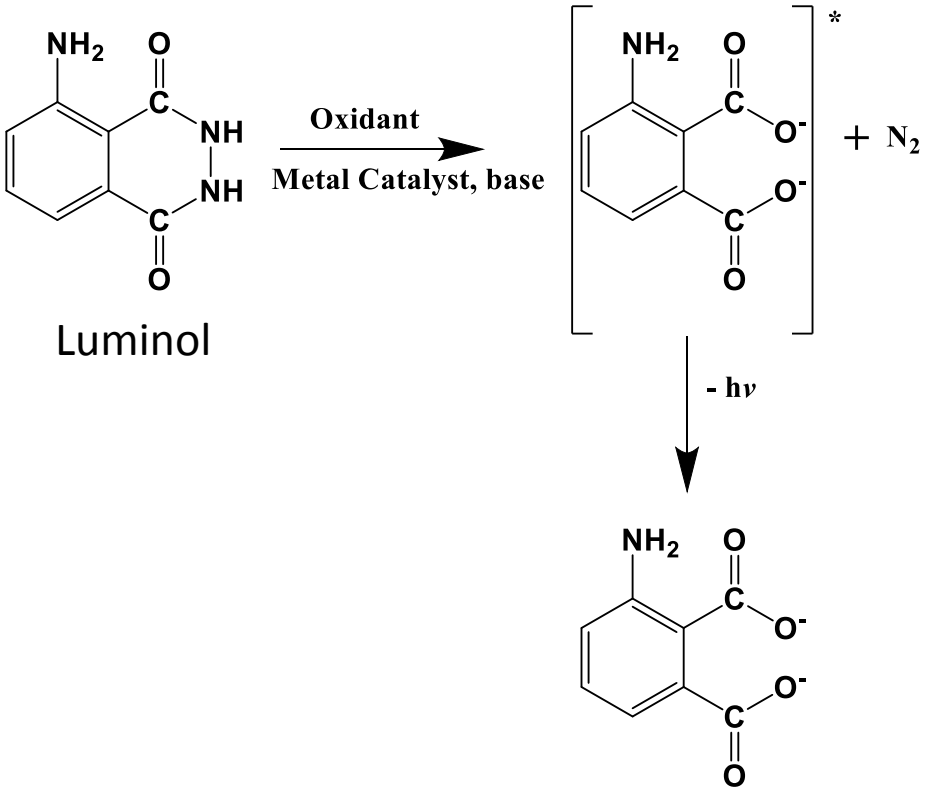
Analytical Applications of Chemiluminescence

1. Gas Analysis

$$NO + O_3 \rightarrow NO_2^* + O_2$$
$$NO_2^* \rightarrow NO_2 + h\nu (\lambda = 600 \text{ to } 2800 \text{ nm})$$

Ozone is drawn continuously into the reaction vessel, and the luminescence radiation is monitored by a photomultiplier

2. Inorganic species (H_2O_2, metals)



Luminol

3. Biosensor

$$\text{sucrose} + H_2O \xrightarrow{\text{invertase}} \alpha\text{-D-glucose} + \text{fructose}$$

$$\alpha\text{-D-glucose} \underset{}{\overset{\text{mutatrotase}}{\rightleftharpoons}} \beta\text{-D-glucose}$$

$$\beta\text{-D-glucose} + O_2 \xrightarrow{\text{glucose oxidase}} \text{gluconic acid} + H_2O_2$$

Luminol is used to determine the concentration of H_2O_2