Biomolecules

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Spectroscopy (of Biomolecules)

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What Will You Learn Today?

Spectroscopy: *working principle and application to biomolecules*

- Light absorption
- Light emission (different luminescent processes)
- Spectroscopy in the THz region (10¹²Hz)

Spectroscopy: Relevance for Biomolecules

SPECTROSCOPY

how electrons interact with light

- spectral lines and/or energy of a sample
- to study sample absorbance and/or transmittance and the structure, molecular composition and arrangement

radiation	radiowaves n	nicrowaves		IR VIS UV	X-ray
frequency	MHz (m)	GHz (mm)	THz (μm)	PHz (nm)	EHz (pm)
spectroscopic observables	nuclear spins	rotatio	n v	vibration electronic transition	electronic diffraction
relevance to biomolecules	FT-methods, imaging			microscopy	crystal structure

(Optical) Excitation & Jablonski Diagram

A molecule can be excited to its electronic excited state



non-radiative decay -

Internal Conversion (IC): *radiation-less transition between states with the same spin state* • timescale: 10⁻¹⁴-10⁻¹¹s

Intersystem Crossing (ISC): *radiation-less transition between states with different spin state* • timescale: 10⁻⁸-10⁻³s Absoprtion: e⁻ is excited from low energy level to a higher energy level (energy transfer from a photon to the electron)
allowed λ = energy difference between the levels (E>E_G)
timescale : 10⁻¹⁵s



radiative decay -

Fluorescence: *electron in excited states (singlet) decays to a lower energy state* • timescale: 10⁻⁹-10⁻⁷s

Phosphorescence: *electron in excited states (triplet) decays to a lower energy state*

• timescale: 10⁻⁴-10⁻¹s

Molecule Absorption (and UV-Vis)



Why is Absorption Important?

- Absorbance/transmittance of a species
- Identify compounds (through the absorption maximum)
- Color determination
- Impurity concentration
- Reaction dynamics

How to Measure Absorption



Measuring Absorption...

... as function of molecule concentration = R_=OCH. Curcumi = OCH,, R = H Demethoxycurcumin = R_=H Bisdemethoxycurcum Absorption Intensity ton (upimi) 0 Cur N1-1 µg Cur N1- 2.5µg Cur N1- 5 µg Cur N1- 10 µg Cur N1- 20 µg 0.0 440 480 520 320 360 400 560 600 Wavelength (nm)

Absorption spectra of curcumin-ethanol solutions for different curcumin concentration. Curcumin solution shows absorbance @ λ =425nm and linear increasing absorption with increasing concentration [SpringerPlus **5**, 1147 (2016)]



UV-Vis absorption of different acenebased molecules (anthracene, tetracene, pentacene) [Chem. Comm. **47**, 10112 (2011)]

... as function of molecules form

solution vs. film vs. crystal



UV-Vis spectra of P3HT in different forms: crystal, solution, thin film on glass. Differences arise from *aggregation* (solution), *molecular packing* (thin film) and *crystal structure* [RCS Adv. **4**, 11121 (2014)]

Absorption of Nucleotides



Photosynthesis: Absorption in Nature



Chlorophylls is based on a porphyrin backbone and represents one of the key pigments for photosynthesis in plants, algae and photosynthetic bacteria.

Electronics excitation energy is used for charge separation,

to be used to drive all subsequent biochemical reactions

Chlorophyll *a* and *b* are the most common and they exhibit two dominant bands in the blue (400-500nm, Soret band) and red (600-700nm, Q_y band)



Monitoring Photosynthesis

Upon optical excitation, chlorophyll also exhibits (high) fluorescence (~700nm), which can be used to measure plant parameters







when exposed to different illumination conditions, plants regulate the amount of excitation energy

Fluorescent Molecules





adding fluorescent molecules





biomolecules can be identified

Fluorescent molecules:

- narrow absorption spectrum
- high extinction coefficient
- high quantum yield ($\Phi = \frac{number of photons emitted}{number of photons absorbed}$)
- narrow fluorescence spectrum
- soluble in aqueous environment
- chemical active site
- photostability



rhodamine green

tetramethylrhodamine (TMR)

Rhodamine





Rhodamine enables visualization of large number of structural details (*i.e.* adhesion filipodia in both HDF and HE cells, the multinuclear nature of SCC4 cancer cells, and the formation of motoneuronal axon on RMN cells) [*PLOS ONE* **5**(5): e10459 (2010)]

Fluorescein



Absorption and fluorescence in the VIS range are separated and do not overlap with absorption and fluorescence of proteins and DNA



Fluorescein angiography picture of ischemic branch retinal vein occlusion in 30-year-old woman.

High-magnification fluorescein angiogram showing a branch retinal vein occlusion.

Fluorescein-based Imaging for the Eye



Moisten the fluorescein strip with 1 drop of saline or topical anesthetic. Depress the lower lid and gently place a wetted strip onto the inside of the patient's lower lid so that only the smallest amount is instilled.





Positive Seidel test. Fluorescein seen streaming down the cornea indicates an open-globe injury. (From Krachmer JH, Mannis M, Holland E, eds. Cornea. 3rd ed. St. Louis; Mosby; 2010.)



Large corneal abrasion seen with the naked eye. Smaller abrasions or corneal injuries produced by keratitis or a welder's arc flash require slit lamp evaluation to identify minor corneal defects.



Corneal abrasion as seen via a slit lamp. A moderate-sized abrasion (arrow) is revealed by fluorescein staining and blue light. (From Friedman NJ, Raiser PK, Pineda R. Massachusetts Ear & Eye Infirmary Illustrated Manual of Ophthalmology. 3rd ed. Philadelphia Saunders; 2009.)



Examine the eye with a Wood lamp or a slit The Eidolon Bluminator ophthalmic corneal and conjunctival surfaces. Because the naked eyes may not be able to appreciate small defects, magnification should be used.



lamp with a cobalt blue filter (shown). Check illuminator provides an intense blue LED for areas of bright green fluorescence on the light with 7× magnification. (Courtesy of Michael W. Ohlson, OD, FAAO, and Victor J. Doherty, Eidolon Optical, LLC.)



Vertical linear abrasions. These types of abrasions are typically caused by a foreign body trapped under the upper eyelid. (From Kliegman R, Stanton B, Behrman R, et al, eds. Nelson Textbook of Pediatrics. 19th ed. Philadelphia: Saunders; 2011.)



Superficial punctate keratitis. These diffuse, shallow corneal irregularities are caused by chemical irritation, viral illnesses, exposure to bright light, and many other conditions.



Herpes simplex keratitis. A classic herpetic epithelial dendritic lesion is seen on this fluorescein examination. (From Palay DA, Krachmer JH, eds. Primary Care Ophthalmology: Concepts and Clinical Practice. 2nd ed. St. Louis: Mosby; 2005.)

Fluorescent Proteins

Green Fluorescent Protein (GFP) can be found in jellyfishes and it shows an absorption maximum at ~ 400nm and a fluorescence maximum ~510nm





GFP forms from the residues of the serine, tyrosine and glycine, which are in close proximity in the primary sequence of the protein (autocatalytic reaction). GFP can be used as a marker.





Diversity of proteins and mutations can cover the VIS range. Multicolor labeling, living HeLa cells (TagBFP-H2B, TagGFP2-actin, phiYFP-mito, TagRFP-golgi, mKate2-zyxin). [*Physiol. Rev.* **90**, 1103 (2010)]

GFP is an excellent "reporter gene," able to attach itself to another gene without interfering with its behavior.



Chemiluminescence & Bioluminescence

light emission derived from a *chemical reaction* in which, chemically excited molecules decay to the electronic ground state and emit photons







Chemiluminescence & Bioluminescence



THz Region [10¹²Hz]



- not perceptible by the human eye
- not ionizing
- can cross many non-conducting common materials (*i.e.* paper, fabrics, wood, plastic, organic tissues)

vibration frequencies of (bio)molecules in THz region are related to *collective vibrations*, *distorted vibrations* and structural deformation

THz radiation allows *contactless* and *non-destructive* analysis of the materials under investigation with resolution higher than micro- and millimeter waves

THz Time-Domain Spectroscopy (THz-TDS)

THz-TDS directly measures the *transient electric field* (rather than its intensity)



THz-TDS can work in *transmission* and *reflectance* mode



THz-TDS of Nucleotide & DNA

THz spectrum of nucleic acids reflect intermolecular collective vibrations, the lattice vibrations and the configuration characteristics of nucleic acid molecules



Four nucleotides (adenine, cytosine, guanine, thymine) show characteristics bands near 3 THz [*Spectrochim. Acta A: Mol. and Biomol. Spec.* **179**, 255 (2017)]



Frequency dependence of THz signal for hybridized, denaturated and without DNA. THz fingerprint enables differentiation between singleand double-stranded DNA [Phys. Med. Biol. **47**, 3815 (2002)].

THz Spectroscopy for Tumor Detection

THz-TDS is capable of distinguishing between healthy and malignant tissues, being sensitive to:

- morphology
- water content (dielectric permittivity, refractive index, absorption coefficient, dielectric relaxation)



Refractive index and absorption coefficient of water molecules in the THz region, as functions of frequency and temperature.



THz absorption coefficient of two control samples of DNA (kidney cell M293T and 293T) and five types of cancer DNA: prostate cancer (PC3), skin cancer (A431), lung cancer (A549), breast cancer (MCF-7) and gastric cancer (SNU-1), originated from methylation measured at -20°C [IEEE J. of Sel. Topics in Quantum Elect. **23**(4), 8600109 (2017)].

THz Imaging for Tumor Detection



THz images generated using the maximum of the reflected pulse (E_{max}) and the ratio between maximum/minimum of the reflected pulse (E_{max}/E_{min}) , along with histology report [Opt. Exp. **17**(15), 12444 (2009)]



contactless, non-invasive

Tumor discrimination in mice using (**a**) MRI, (**b**) white light, (**c**) GFP fluorescence, (**d**) hematoxylin and eosin (H&E) stained images, (**e**) optical coherence tomography (OCT), (**f**) TRI and (**g**) 5-ALA-induced ppIX fluorescence [Sci. Rep. **6**, 36040 (2016)].

Complementary Techniques



Summary of Today

Spectroscopy: *study structure and molecular arrangement through spectral response*

- excitation and light emission (*i.e.* absorption, fluorescence)
- THz spectroscopy, with biomolecules showing a characteristics fingerprint



If you are interested in THz Spectroscopy ELEC-E4760 Terahertz Techniques (period V)