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Topic: Analytical Approaches and Instrumentation
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Four nucleotide bases can combine in millions of combinations to make DNA molecules. These are interpreted to make proteins with primary, secondary, tertiary, and quaternary structure.

Spectroscopy is based on the interaction of light with matter. The part of the proteins that is excited by light depends on the energy, or frequency, of the light.

Type of Radiation	Wavelength Range	Type of Transition
Gamma-rays	< 1 pm	Nuclear
X-rays	1 nm – 1 pm	Inner electron
UV	400 nm – 1 nm	Outer electron
Visible	750 nm – 400 nm	Outer electron
Near-IR	2.5 um – 750 nm	Outer electron, molecular vibrations
IR	25 um – 2.5 um	Molecular vibrations
Microwaves	1 mm – 25 um	Molecular rotations, electron spin flips
Radio waves, NMR	> 1 mm	Nuclear spin flips

Spectroscopic methods currently exist for smaller molecules: by studying the difference in energy states of electrons in the molecule, structural information can be derived.

Light Absorption Methods

UV Spectroscopy

Absorption of UV can be used to measure the concentration of a solution as follows: for an incident beam intensity I0 and a transmitted beam intensity I, the absorbance A is

$$A = -\log\left(\frac{I}{I_0}\right)$$

and can be equated to the product of the extinction coefficient ε ($M^{-1}cm^{-1}$), path length of light, and concentration C (M):

$$A = \varepsilon l C$$

For all proteins, a peak in UV absorption occurs at a wavelength of 280nm.

Circular Dischroism

Proteins have chirality about the alpha-carbon. Biological proteins are left-handed. The overall chirality of molecules is able to change the polarity of light. The far-UV region (190 to 260 nm) provides information about the peptide bonds, and quantitative information about secondary

structures. The near-UV region (250 to 300 nm) provides information about aromatic amino acids and disulfide bonds, and qualitative information about tertiary structures.

Secondary structures are quantified by comparing a spectrum to a spectrum of a fully characterized molecule: for example, one that is rich in alpha helices, beta sheets, or random coils. An algorithm in computer software such as CONTIN or SELCON can quantify the percentage alpha-helix, beta-sheet, beta-turn, and random coil in the sample.

Infrared Spectroscopy

IR spectroscopy reports on transitions between vibrational modes of molecular bonds, such as C=O stretch, C-H and N-H stretch, bending modes, etc. In proteins, the stretching modes for N-H and C=0 are very sensitive to secondary structure. Secondary structures lead to IR peaks at the following frequencies:

beta-turn	1670-1660 cm^{-1}
alpha-helix	1660-1650 cm^{-1}
random coil	1650-1640 cm^{-1}
beta-sheet	1640-1620 cm^{-1}

An IR spectrum often contains overlapping peaks, which are difficult to deconvolute into individual vibrational modes. This method is useful for comparing DNA (e.g., normal vs. cancerous cells), characterizing solids (e.g., biominerals), studying protein aggregation diseases (e.g., Alzheimer's, prion diseases, amyloid), and analyzing other protein and DNA conformations.

Light Emission Methods

Raman Spectroscopy

Raman spectroscopy is (according to the speaker) relative expensive for the amount of information that it provides, and is therefore less popular than other spectroscopic methods. It provides complementary information to IR spectroscopy.

The information obtained, as with IR, can reveal quantitative information about the secondary structure of proteins, and be used to detect conformational changes of proteins and DNA. In controlled Raman excitation, a 206.5 nm laser can be used to excite an amide bond, or a 230 nm laser can be used to study aromatic groups. The secondary structure is determined based on the frequency response:

Amide I	
beta-turn	1680-1665 cm^{-1}
random coil	1665-1655 cm^{-1}
alpha-helix	1655-1650 cm^{-1}

Amide III	
alpha-helix	1280 & 1305 cm^{-1}
random coil	1250 cm^{-1}
beta-strand	1240 cm^{-1}

(from Miura and Thomas 1995, Nerjot et al. 1987)

Fluorescent Spectroscopy

Fluorescent probes occur naturally in biological proteins (e.g., Trp, Tyr, and Phe residues), and can be incorporated artificially as an attached dye.

In steady-state fluorescence spectroscopy, a sample is constantly illuminated to excite flourophores, and emission is measured during the denaturation of the sample. *Denaturation is always associated with a red shift in the emission spectrum.*

In time-resolved fluorescence spectroscopy, the sample is illuminated with extremely short (picosecond) bursts of light, and the fluorescence decay is measured. This gives information about the local microenvironment, such as the amino acids neighboring the fluorophore.

Fluorescence spectroscopy is particularly useful for studying:

tertiary structure of proteins,
receptor/ligand interactions,
immunoassays,
single molecule interactions,
real-time PCR assays, and
DNA sequences.

Forster Resonance Energy Transfer: In this version of fluorescence spectroscopy, a an energy donor and acceptor are placed in close proximity (within 8 nm or each other), and decay of fluorescence on the acceptor can be used to study macromolecular structure and dynamics.

Light microscopy

Light microscopy

Cells are approximately five times smaller than the smallest thing that the naked eye can see. Light microscopy can magnify up to 2,000 times, using a lens than can transmit natural color. the method is cheap and easy to perform, and speciments can remain alive while being observed. Staining cells using dyes or more sophisticated markers such as antibodies with fluorescent dies or quantum dots allows for “live-action” shots of biological processes.

GFP in light microscopy

Cells can be labeled with fluorescent markers that can be cell-specific. The markers can be made to change color depending on the environment of the cell. E.g., GFP movie of a virus invading a cell.

Confocal microscopy

This advance in light microscopy and fluorescence microscopy allows for 3-D-like imaging of portions of cells stained with fluorescent markers. E.g., fluorescent staining of Golgi apparatus and different portions of the cytoskeleton during mitosis.

Light microscopy

Here, rather than sunlight or a light bulb, the illuminating source is a wire that emits electrons.

Transition electron microscopy

This method gives a 2-D representation of a 3-D specimen. Its incredible magnification can be as high as 500,000x, and its theoretical maximum resolution is 0.005 nm, smaller than a single atom! (This has not been achieved.) Effectively, the resolution of a TEM exceeds that of a human eye by 2,000,000 times.

TEM requires staining of a sample, and does not retain color information or 3-D information. Staining often damages a sample.

TEM-tomography

Based on the principles of TEM, a camera takes TEM micrographs of a molecule from a variety of angle and reconstructs the 3-dimensional structure by computer.

Cryo-TEM

This eliminates the need for dying the sample. The sample is cooled so rapidly that ice crystals do not have time to form or for water to evaporate. Thus, the sample can be studied without the shrinking or damaging effects of a stain. Again, computer processing of hundreds of images can produce a 3-D model.

E.g., cryo-TEM was used to analyze the structure of chaperonin, which could not be easily crystallized for diffraction methods.

Scanning Electron Microscopy

Electrons are reflected off the surface of a sample, which, if non-conducting, must be coated with a thin layer of gold. The electrons are focused through a detector and depicted as 3-D images with a high depth of field and a resolving power of about 10 nm (less than that of TEM).

Atomic Force Microscopy

A cantilever drags or taps across a surface with a constant force, and maps the topography of the surface. This method can compare the stiffness of surfaces in addition to plotting an “elevation map” of the surface.

Conclusions on microscopy:

Microscopy allows for detailed visualization of biological surfaces, and permits some types of structural determination of macromolecules.

Light scattering

Dynamic Light Scattering

Some wavelengths of light interact with matter, but no absorption takes place. This fast, easy, non-destructive method gives reproducible data about particle size distributions based on light scattering off different particle sizes. However, the data can be tricky to analyze: the hydrodynamic size must be determined by the Stokes-Einstein Equation, and gives a quantity different from the true physical size given my NMR spectroscopy. (The hydrodynamic radius includes both the molecule and its hydration shell: this radius is more relevant, for example, for determining diffusion coefficients.) The intensity of light scattering is proportional to the radius to the sixth power!

Calorimetry

Differential scanning calorimetry

This method is used to measure thermodynamic parameters, such as stability ranges, folding and unfolding processes, thermodynamic potential energies, and heat capacities.