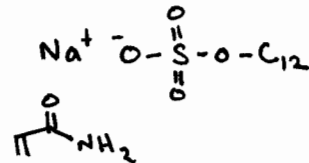


TD7: Gel Electrophoresis
 Photoaffinity probes

GEL ELECTROPHORESIS

- Summary:** migration of charged molecules through a gel under the influence of an electric field
 Can be used on: protein, DNA, RNA, peptides (just not small molecules)
 Can resolve molecules based on: size, charge, shape

- Most common gel for proteins: **SDS-PAGE gels** (aka "denaturing gel")
 SDS= sodium dodecyl sulfate, a detergent



PAGE= polyacrylamide gel electrophoresis

Purpose: resolve proteins by size and no other physical feature (ie charge or shape)

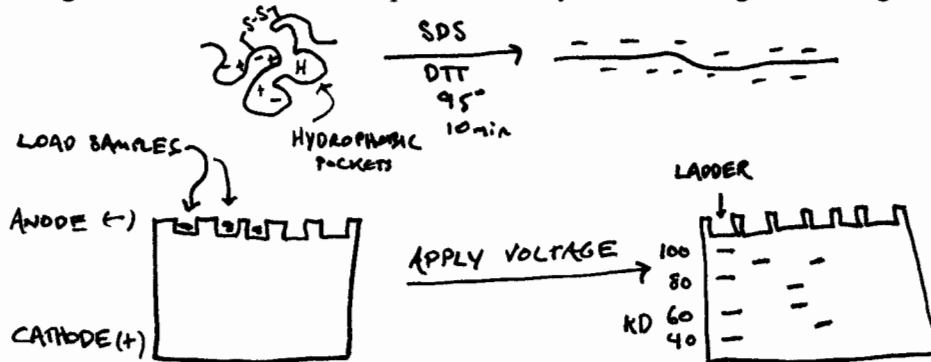
Gels: made from polymerized acrylamide, typically 6-20% (higher %= more dense gel, same protein runs slower)

Running buffer: has .1% SDS detergent

Sample: pre-treated with SDS (& DTT a reducing agent)

*SDS unfolds the protein and covers it with negative charge.

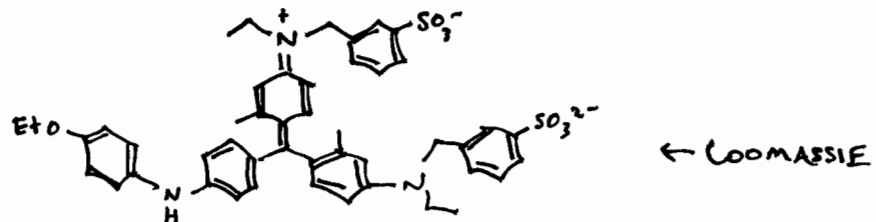
Globular protein with hydrophobic pockets, electrostatic interactions etc-> SDS, DTT 95deg for 10 min-> linearized protein evenly coated in negative charge.



Cathode attracts SDS-covered proteins, anode at top, cathode at bottom, larger proteins travel slower

Visualization of proteins in gels

- autoradiography or phosphorimaging (if the protein is radiolabeled)
- Coomassie stain (most common, cheap) binds to Arg, Trp, Tyr, His, Phe gives blue bands
- Silver Stain (more sensitive than Coomassie) proteins reduce Ag^+ to Ag metal
- Immunoblot (transfer proteins from gel to a membrane, detect using labeled protein specific antibodies)



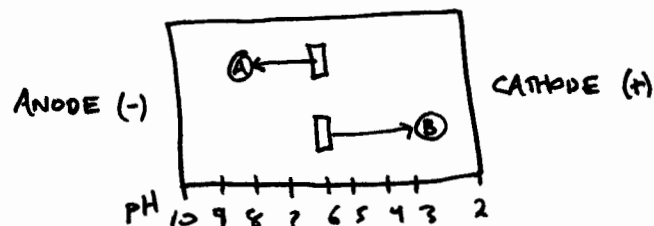
3. Other protein gel techniques:

-Native PAGE: leave out SDS

- protein migrates based on charge & shape in addition to size
- very pH sensitive (influences proteins net charge)
- can resolve folded and unfolded proteins, monomers & dimers, protein complexes

-IEF gel: Isoelectric focusing gel

- protein separation based on charge
- protein migrates to pH region where their net charge is zero- "isoelectric point"
- in the drawing, A is positively charged at pH 6, neutral at pH 8.5, B is negatively charged at pH 6, neutral at pH 2



-Peptide gel : for resolving small proteins and peptides

- use high % acrylamide and glycerol to make the gel very dense- otherwise they will run right off the gel!

4. Most common DNA gel: agarose

Agarose: linear galactam hydrocolloid

Mix with water, boil, cool-> forms colloidal suspension like jello

Negatively charged DNA runs to the cathode (cathode at bottom, anode at top)

Note- mobility is proportional to size, because charge is proportional to size (unlike with proteins)

For very high resolution, DNA can be analyzed by denaturing PAGE (urea is used to denature DNA instead of SDS)

-gives single nucleotide resolution (can distinguish 500nt from 501nt like in the Noller paper we studied)

-can be used for DNA sequencing

5. RNA is usually analyzed with denaturing PAGE gel (+urea) as described above, because RNA has too much secondary structure that can complicate results in native conditions (much more secondary structure than DNA)

Summary:

Proteins: SDS-PAGE (denaturing, size)

Native (non-denaturing, size, charge and shape)

IEF (non-denaturing, charge)

Peptides (higher % acrylamide)

DNA: agarose (size)

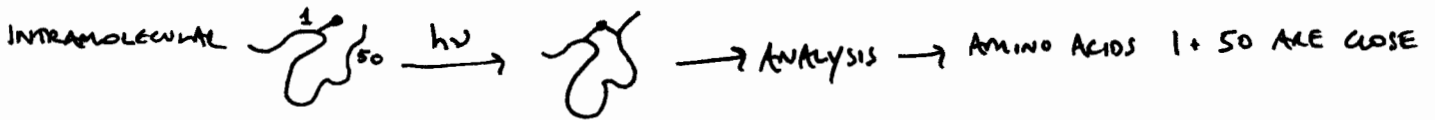
PAGE-urea (denaturing, size, high resolution)

RNA: PAGE-urea (denaturing, size, high resolution)

PHOTOAFFINITY LABELS

1. **Summary:** small probes are attached site-specifically to RNA or protein.

Irradiation w/ near-UV light activates probe, allowing rapid crosslinking to nearby C-H bonds. Useful as a way to determine protein/RNA structure or interaction partners. Crosslinking can be intramolecular or intermolecular.



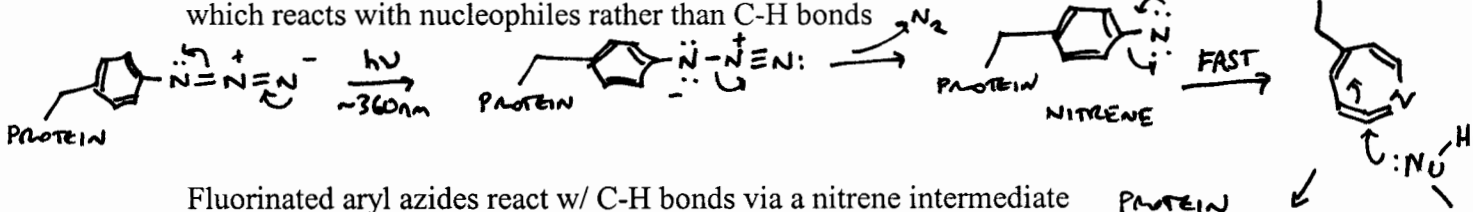
2. **Types of photoaffinity labels**

Acrylides

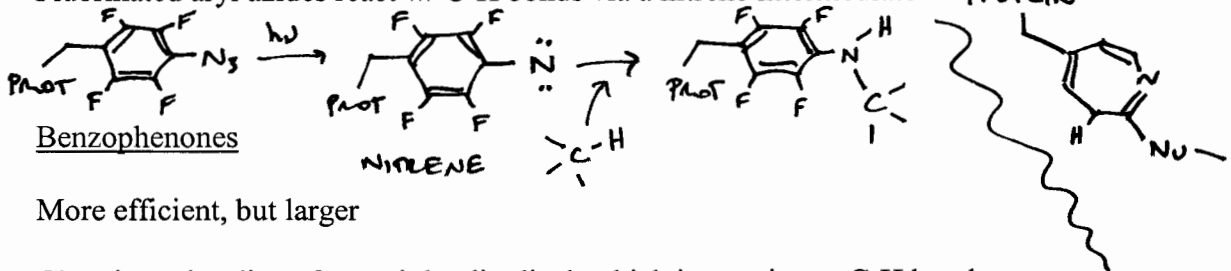


Less efficient but smaller, less invasive

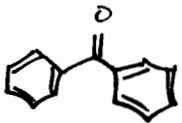
Aryl azides: irradiate, lose N₂, rapid formation of dehydroazepine intermediate, which reacts with nucleophiles rather than C-H bonds



Fluorinated aryl azides react w/ C-H bonds via a nitrene intermediate

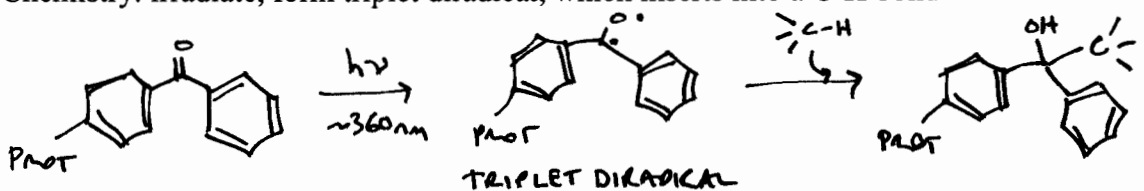


Benzophenones



More efficient, but larger

Chemistry: irradiate, form triplet diradical, which inserts into a C-H bond



Crosslinking efficiency: benzophenone > fluorinated aryl azide > aryl azide

3. Conjugation chemistries

Nucleotide labeling:

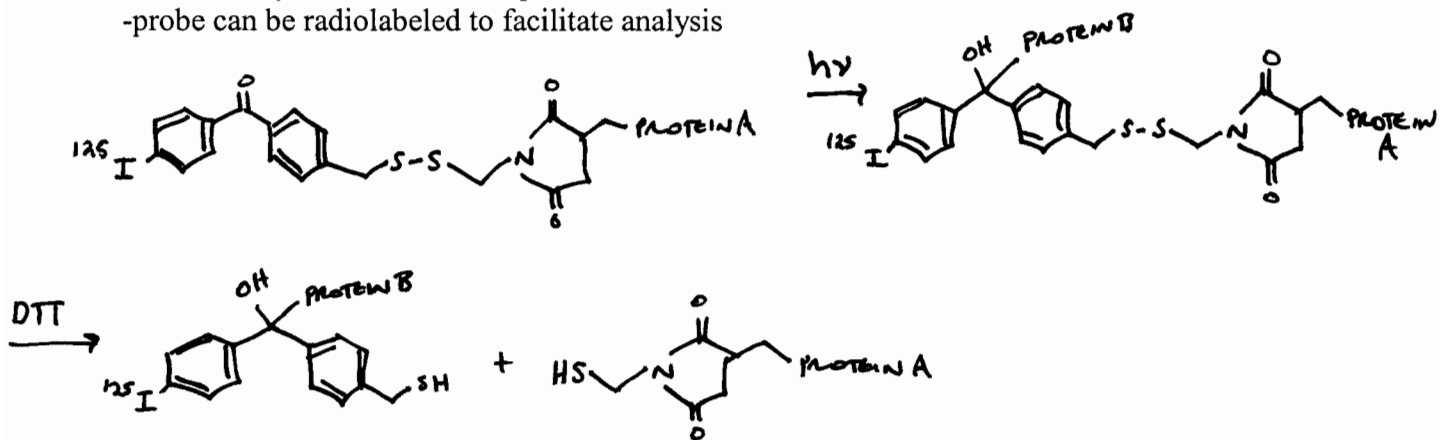
-can make DNA or RNA with site specific thiophosphate, commercially available
thioreactive probes include iodoacetamide, maleimide (as discussed in lecture)

Protein labeling:

-can label unique Cys (remove all other cys by site directed mutagenesis) with
thioreactive probes
(iodoacetamides, maleimides, dithiopyridyl)

4. Other tricks

- a disulfide bond in the probe allows one to separate crosslinked proteins after experiment
- makes analysis of crosslinked products easier
- probe can be radiolabeled to facilitate analysis



5. An example: Photoaffinity crosslinking of tRNA to P&E sites of 70S ribosome

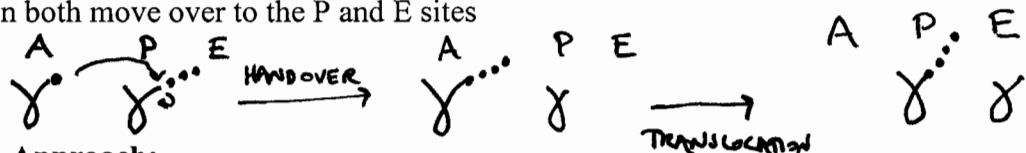
FEBS letters (2002) 514, 60-66

A. Goal: map tRNA ribosome contacts during protein synthesis
(dynamic information, different from static info that x-ray gives)

B. Background

tRNA starts in A(aminoacyl) site, moves to P(peptidyl) site then E(exit) site

growing chain on tRNA in the P site is transferred to the tRNA in the A site,
then both move over to the P and E sites



C. Approach:

Label the tRNA with photoaffinity tag (azide), specifically at 3' end

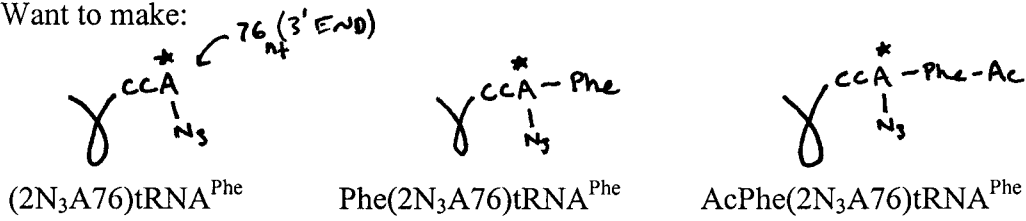
Add to 70S ribosome and mRNA

350-400nm light to crosslink

Use analytical techniques to ID protein/ RNA crosslinked to tRNA

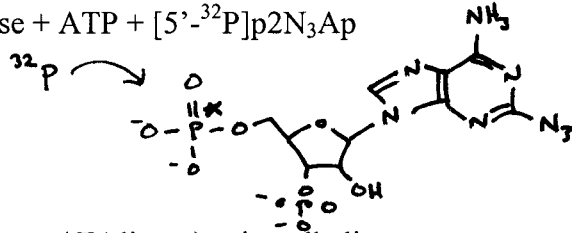
D. Step 1: preparation of azide labeled tRNA

Want to make:

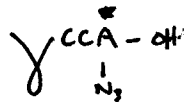


1) prepare tRNA^{Phe} (-A) by in vitro transcription $5' \gamma CC 3'$

2) combine tRNA^{Phe} (-A) + T4 RNA ligase + ATP + [5'-³²P]p2N₃Ap

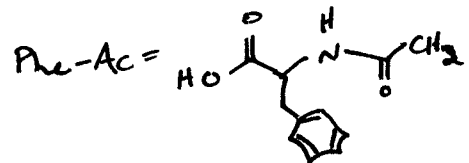
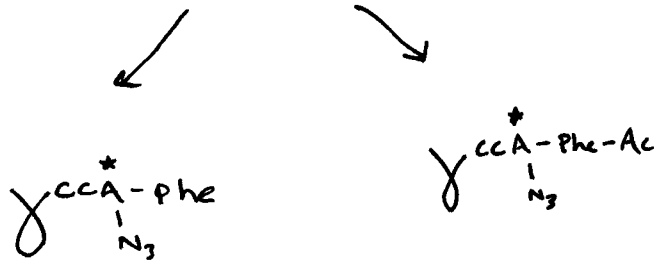


3) remove 3' phosphate (needed for ligation w/ T4 ligase) using alkaline phosphatase(AP)



4) to part of the sample add Phe amino acid + RS^{Phe} (tRNA synthetase)

5) to another part add Phe-Ac + RS^{Phe} (blocked cannot join to other amino acids)



E. Step 2: The experiment

1. load tRNAs into P site of 70S w/ polyU mRNA (tRNAs are known to prefer P site when coloaded w/ polyU)
2. 350-400nm hv, 2min, 0 deg
3. sucrose gradient centrifugation: Is 50S or 30S radiolabeled? (only 50S)
4. isolate labeled subunit

protein analysis

- treat w/ RNAase
- boil
- SDS-PAGE gel
- What proteins have ^{32}P label?

RNA analysis

- protease
 - boil
 - PAGE-urea gel
 - What RNAs have ^{32}P label?
 - Do further analysis via primer extension to find site of labeling (like Noller)
5. repeat under E site loading conditions combine tRNAs with 70S + saturating cold AcPhe-tRNA^{Phe} (binds only A+P sites leaving E site open)

F. Findings

P site loading conditions

$(2\text{N}_3\text{A76})\text{tRNA}^{\text{Phe}}$	crosslinks with L27 protein of 50S and L33 to some degree, also 23S rRNA at U2506, U2585, C2422
$\text{Phe}(2\text{N}_3\text{A76})\text{tRNA}^{\text{Phe}}$	crosslinks with L27 protein & 23S rRNA at U2506, U2585
$\text{AcPhe}(2\text{N}_3\text{A76})\text{tRNA}^{\text{Phe}}$	crosslinks with L27 protein and 23SrRNA at U2506, U2585, G2069

E site loading conditions

$(2\text{N}_3\text{A76})\text{tRNA}^{\text{Phe}}$	crosslinks with L33 & L1 proteins and 23S rRNA at C2422
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