Chemistry 5.08 (Spring 2016)
Recitation #5 (March 3-4, 2016)
Overview of cross-linking, including photo-reactive cross-linking methods (no outside reading assignment, see Discussion Questions for optional reading/resources).

I. Background

Cross-linking is the process of chemically joining two or more molecules by forming covalent bonds between the molecules. It is commonly employed in studies of protein structure and in the identification of interaction sites of a protein and a binding partner. For studies of protein three-dimensional structure and protein-protein interactions, the proximity of residues involved in a cross-link can be deduced because the cross-linking reagent imposes a distance constraint for the location of the cross-linked side-chains. Thus, cross-linking can be used to map protein tertiary and quaternary structure, and it is also useful for identifying new protein-protein interactions. Although this material focuses on protein-protein interactions, cross-linking can also be applied to study other types of interactions (e.g. protein-RNA). Moreover, cross-linking can be performed on a variety of types of samples that include purified proteins, cell lysates, and live cells.

II. Applications of cross-linking

1. Study protein tertiary or quaternary structure – the structure can be stabilized by the cross-link and subsequently analyzed (think: what methods of analysis?)
2. Capture and identify as-yet unidentified protein-protein, protein-RNA, etc. interactions
3. Investigate known protein-protein interactions to learn more about the interaction surface, residues, etc.
4. Conjugation chemistry – e.g. attachment of an enzyme/protein/tag to a purified protein or antibody
5. Immobilization – e.g. attachment of antibodies or proteins to a solid support for affinity purification or interaction studies

III. Overview of types of cross-linking reagents – terminology

There are many different approaches and different reagents! We will consider:

1. **Homobifunctional** versus **heterobifunctional** cross-linkers
2. **Non-specific** versus **specific** cross-linkers

Here, ***non-specific versus specific refers to whether a cross-linking reagent is site-specifically attached to a protein of interest.*** These terms do not refer to the functional groups or type of chemistry that occurs to make the cross-link.

Homobifunctional cross-linkers are **non-specific**.

Heterobifunctional cross-linkers may be used **non-specifically** or **specifically**.

→ *How do you select the appropriate cross-linker to employ in a given study?*

IV. Amine-reactive reagents

Most proteins contain Lys residues, and many homobifunctional and heterobifunctional cross-linkers have amine-reactive moieties. These groups react with the ε-amino group of Lys residues and the N-terminal α-amino group of polypeptides.

Examples of amine-reactive reagents:

- **Succinimidyl esters** (NHS ester)
- Isothiocyanates
- Sulfonyl chlorides
- Aldehydes

What factors affect amine reactivity?

- Type of amine (e.g. **aliphatic**, aromatic)
- **Basicity (pK_a)**
  - Lys ε-NH_2 pK_a ≈ 9
  - N-terminal α-NH_2 pK_a ≈ 7

Practical considerations for experiments:

There are many considerations to take into account when using an amine-reactive reagent in cross-linking or other applications. These considerations include:

1. Buffer pH – the kinetics of N-acylation are strongly pH dependent
2. The buffer itself – some commonly used buffers like Tris and glycine contain free amines. These amines will react with the amine-reactive cross-linker. Moreover, the buffer concentration is most likely in great excess over the biomolecule(s) of interest (consider typical millimolar concentrations of the buffer, e.g. 10 or 75 mM Tris compared to nanomolar or micromolar concentrations of a protein)
3. Decomposition of the cross-linking reagents – it is important to know the properties of your reagent. Is it sensitive to pH? Light?
4. Purity of the cross-linking reagent – have healthy skepticism about reagents obtained from commercial suppliers, even if the reagents are expensive and claim high purity.
5. Cross-reactivity – do undesired reactions occur?

V. Thiol-reactive reagents (also terms mercaptans, sulfhydryls)

Thiol-reactive moieties are also commonly employed in cross-linking strategies. These groups react with free cysteine residues on proteins.

Examples of thiol-reactive reagents:

- **Maleimides**
- Iodoacetamides
- Disulfides (e.g. dithiopyridyl)
- Alkyl halides

What factors affect amine reactivity?

- Redox environment and thiol/disulfide chemistry
- **Basicity (pK_a)**

Cys SH pK_a ≈ 8.2

Practical considerations for experiments:

There are many considerations to take into account when using a thiol-reactive reagent in cross-linking or other applications. These considerations include:

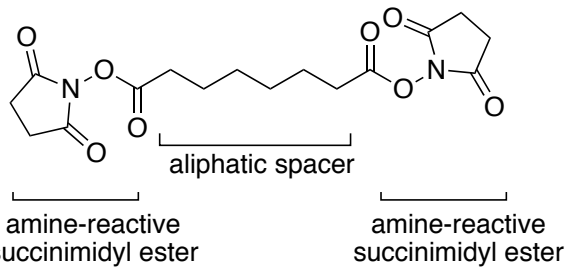
1. The redox environment. Is the cysteine of interest "free" or involved in a disulfide bond? Reducing agents (e.g. dithiothreitol, β-mercaptoethanol, tris(2-carboxyethyl)phosphine) can be added to samples to keep the thiols reduced. However, some of these reducing agents (DTT, BME) contain thiols and thus must be removed prior to introduction of the thiol-reactive reagent. If the reducing agent is removed, it is possible that the thiol will become oxidized (e.g. air oxidation). If TCEP is employed as the reducing agent, it is necessary to make sure the buffer has adequate buffering capacity or that the TCEP stock solution is pH adjusted. Solutions of TCEP in water are acidic.
2. Buffer pH – modification of the Cys will be pH dependent (thiolate needed)
3. Decomposition of the cross-linking reagents – it is important to know the properties of your reagent. Is it sensitive to pH? Light?
4. Purity of the cross-linking reagent – have healthy skepticism about reagents obtained from commercial suppliers, even if the reagents are expensive and claim high purity.
5. Cross-reactivity – do undesired reactions occur?

VI. Non-specific cross-linkers

These cross-linkers are either homobifunctional or heterobifunctional. They consist of three functional parts – the two reactive groups separated by a spacer.

Example of a non-specific, homobifunctional cross-linker:

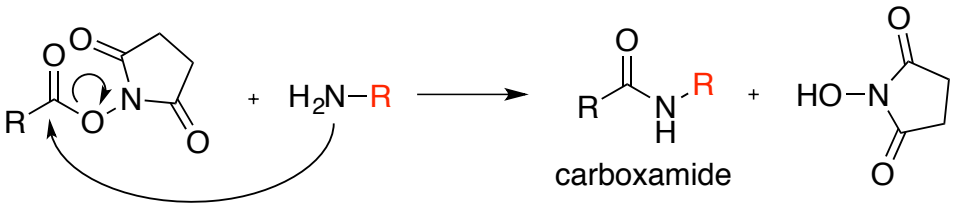
Disuccimidyl suberate (DSS)



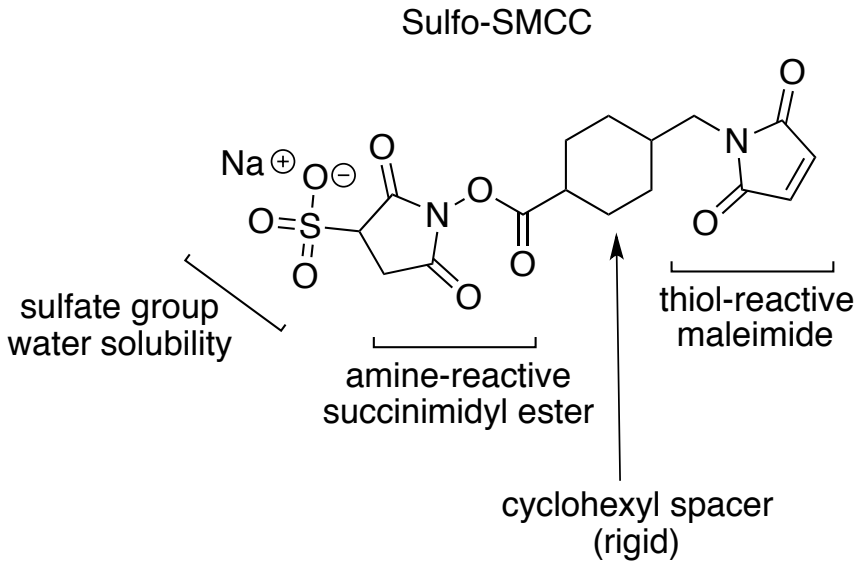
DSS exhibits:

Two amine-reactive groups, one at each terminus
An aliphatic spacer (or linker) that is stable (cannot be cleaved)
The linker provides a maximum distance between conjugates molecules of ≈11 Å

Chemistry (review NHS esters):



Example of a non-specific*, heterobifunctional cross-linker:

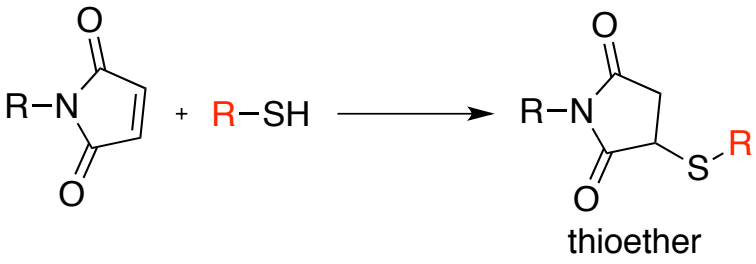


Sulfo-SMCC exhibits:

- One amine-reactive group (succinimidyl ester)
- One thiol-reactive group (maleimide)
- A cyclohexyl spacer (provides a maximum distance b/t conjugated molecules of ≈8.3 Å)
- A sulfate group to enhance water solubility

*Note that sulfo-SMCC could be used non-specifically or specifically. For instance, if the protein of interest has a single Cys residue, this residue can be modified with Sulfo-SMCC in one step and isolated. Then, cross-linking of this modified protein to another protein/biomolecule could occur. (→*What is the drawback of using Sulfo-SMCC in this type of approach?)*

Chemistry (review maleimide):



→ *Reaction mechanism?*

Consideration of the spacer/linker:

The composition of the spacer will affect the properties and behavior of the cross-linking reagents (these are general considerations for all types of reagents, including specific cross-linkers). Some considerations include:

1. Solubility – is the spacer hydrophobic (e.g. alkyl chain) or hydrophilic (e.g. PEG)?
2. For studies in live cells or tissues, will the linker facilitate or inhibit cellular uptake?
3. Is it necessary or helpful to be able to separate the two cross-linked species by using a cleavable linker? The examples above are stable, non-cleavable linkers. Introduction of a disulfide bond in the linker region provides means to cleavage the linker via addition of a reducing agent.
4. Length – how will the length of the linker influence your results? What length do you require?

Pros and cons of non-specific cross-linkers:

→ *What are the pros and cons of using non-specific cross-linkers?*

VII. Specific cross-linkers and photo-reactive cross-linking reagents

Strategies to obtain specific cross-linkers:

We define specific cross-linkers as cases where the cross-linking reagent is site-specifically positioned on a protein/biomolecule of interest. Methods to make a site-specific cross-linker include:

1. **Modify a single cysteine residue with a cross-linking reagent.** In this case, the protein should have only one cysteine. It may be a cysteine that is in the primary sequence or the cysteine may be introduced by site-directed mutagenesis. In cases where the protein has more than one cysteine, it will be necessary to mutate the "extra" cysteine residues to another amino acid like serine or alanine, and it is important to evaluate whether the mutations perturb function.
2. Use the **Schultz Method (or another method) for unnatural amino acid incorporation** by the ribosome to site-specifically install a cross-linking reagent.

→ *What are the pros and cons of each specific labeling approach?*

Photo-reactive reagents:

Proteins (and other biomolecules) can be site-specifically modified with photo-reactive cross-linking reagents. These reagents are chemically inert until irradiated with UV light, which activates the photo-reactive group and thus triggers cross-linking. Photo-reactive groups can be employed in the test tube and in live cells. They are often used to capture binding partners, and to determine the proximity between two sites.

There are several types of photo-reactive groups employed in cross-linking:

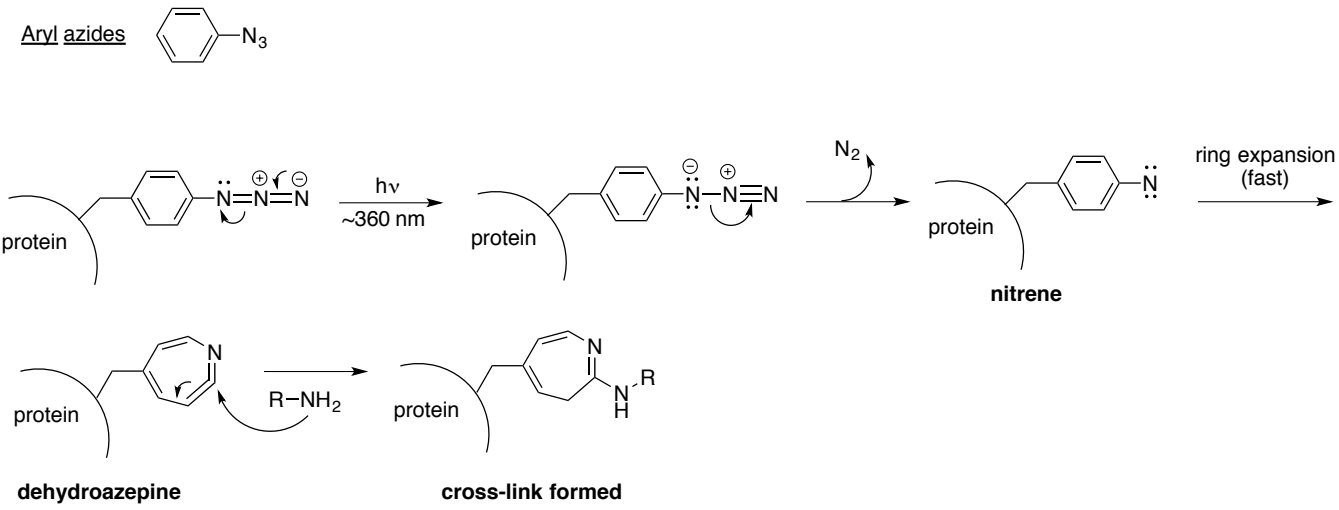
Aryl azides

Benzophenones

(recall we saw that a UAA with a benzophenone side-chain was used during studies of o-ribosome evolution to generate Ribo-X)

Diazirines

Aryl azide overview:



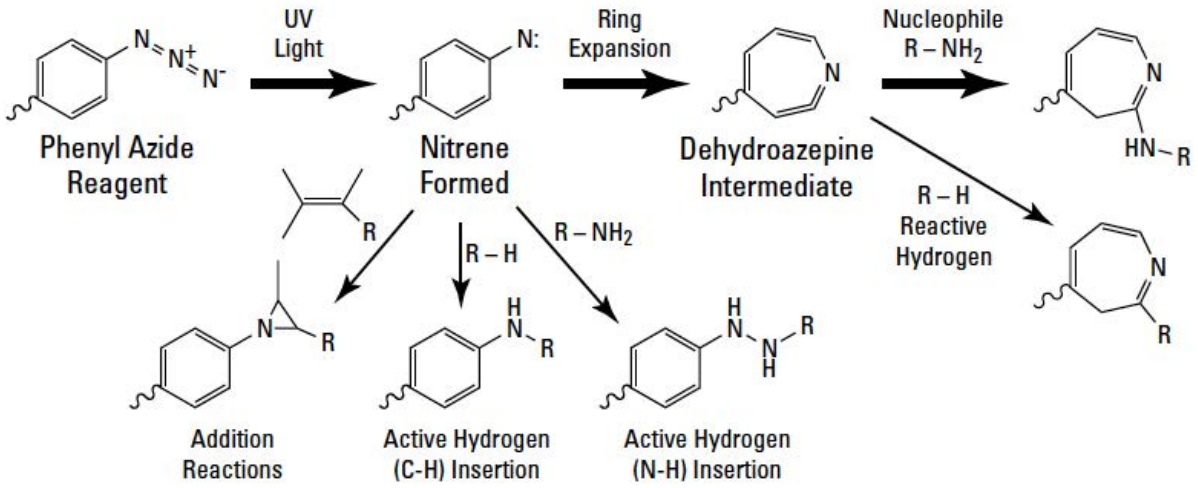
Nitrene group → can do many reactions

Addition into double bonds

Insertion into C–N, C–H bonds

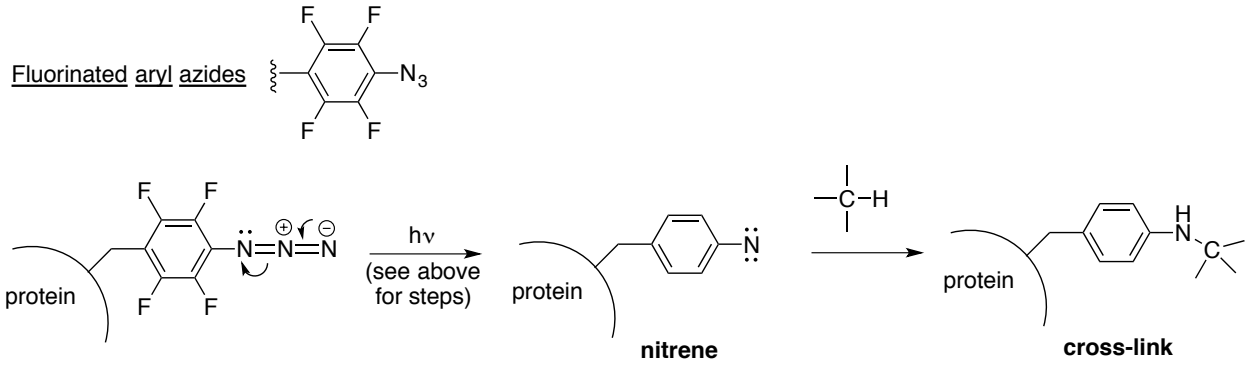
Ring expansion to form a dehydroazepine → react with a nucleophile (e.g. 1° amine)

Ring expansion is the dominant pathway. Other pathways are shown below (the bold arrows indicate the dominant pathway):



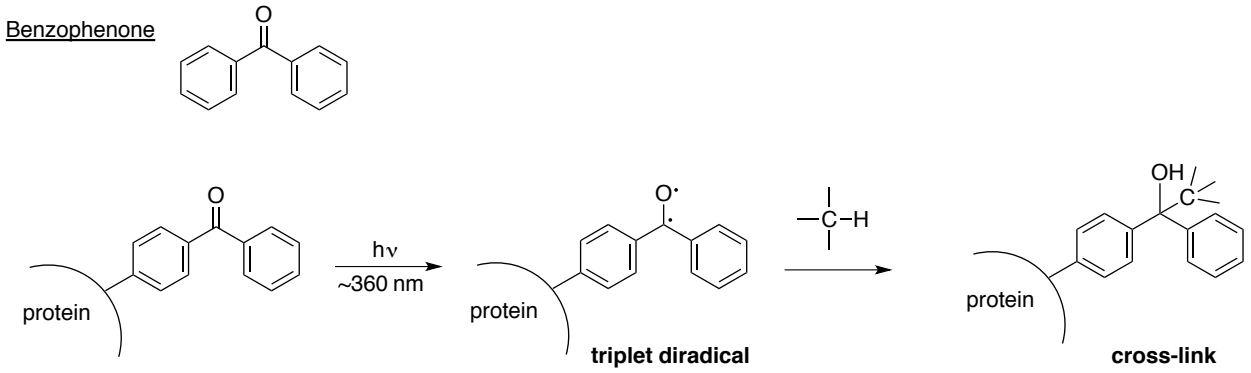
Fluorinated aryl azide overview:

Fluorinated aryl azides exhibit different chemistry than what is shown above for the simple phenyl azide. The fluorinated aryl azides ***do not*** undergo ring-expansion. Rather, cross-linking occurs from the nitrene via C–H insertion.



→ *What is a benefit of using the fluorinated aryl azide over the phenyl azide?*

Benzophenone overview:



→ *What is the relative cross-linking efficiency of these reagents?*

Benzophenone > fluorinated aryl azide > aryl azide

Note that the aryl azides prefer to react with nucleophiles (e.g. R-NH_2) via the dehydroazepine intermediate rather than with C–H bonds. Fluorinated aryl azides, in contrast, react with C-H bonds via the nitrene intermediate.

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