Protein Cross-Linking

Teacher's Guidebook

(Cat. # BE-605)

MATERIALS INCLUDED 3
SPECIAL HANDLING INSTRUCTIONS 3
ADDITIONAL EQUIPMENT REQUIRED 3
TIME REQUIRED 3
OBJECTIVES 3
BACKGROUND 4
SELECTION OF PROTEIN CROSS LINKERS 5
CROSS LINKING APPLICATION 5
PROTEIN STRUCTURAL & FUNCTIONAL STUDIES 5
PROTEIN INTERACTION AND RECEPTOR STUDIES 5
CONJUGATION FOR IMMUNOLOGICAL TOOLS 6
CELL MEMBRANE STRUCTURAL STUDIES 6
CELL SURFACE STUDIES 6
SOLID-PHASE IMMOBILIZATION 6
PREPARATION OF IMMUNOTOXINS 7
PROTEIN-PROTEIN CONJUGATION 7
PROTEIN TO DNA/RNA CROSS LINKING 7
REACTIVE GROUP TRANSFER 7
PRIMARY AMINE REACTIVE CROSS LINKERS 7
SULFHYDRYL REACTIVE CROSS LINKERS 8
CARBOHYDRATE REACTIVE CROSS LINKERS 8
CARBOXYL REACTIVE CROSS LINKERS 8
PHOTOREACTIVE CROSS LINKING REAGENTS 9
TEACHER'S PRE EXPERIMENT SET UP 10
MATERIALS FOR EACH GROUP 10
PROCEDURE 10
RESULTS, ANALYSIS & ASSESSMENT 11

MATERIALS INCLUDED

This kit has enough materials and reagents for 24 students (six groups of four students).

- 1 vial Protein Cross Linker
- 6 vials Protein: Lysozyme
- 1 bottle PBS
- 1 vial Cross Linker Solvent
- 1 bottle Non Reducing Sample Loading Buffer
- 1 vial Reducing Agent (TCEP)
- 1 vial Sterile Water
- 8 vials PAGEmark™ Blue PLUS Protein Marker
- 1 bottle LabSafe Gel Blue
- 110 Centrifuge Tubes (1.5ml)

SPECIAL HANDLING INSTRUCTIONS

- Store the kit at 4°C in a fridge upon arrival.

The majority of reagents and components supplied in the BioScience Excellence™ kits are non toxic and are safe to handle, however good laboratory procedures should be used at all times. This includes wearing lab coats, gloves and safety goggles.

For further details on reagents please review the Material Safety Data Sheets (MSDS).

ADDITIONAL EQUIPMENT REQUIRED

- Protein Electrophoresis Equipment
- Low speed centrifuge for 1.5-2ml tubes

TIME REQUIRED

- Day 1: 3-4 hours

OBJECTIVES

- Understand the principal of protein cross linkers.
- Couple a protein with a reversible protein cross linker.
- Reverse protein coupling using a reducing agent.

BACKGROUND

Cross linking agents contain at least two reactive groups that are reactive towards numerous groups, including sulfhydryls and amines, and create chemical covalent bonds between two or more molecules. Functional groups that can be targeted with cross linking agents are primary amines, carboxyls, sulfhydryls, carbohydrates and carboxylic acids. Protein molecules have many of these functional groups and therefore proteins and peptides can be readily conjugated using cross linking agents. Cross linking agents are used to study protein structure and function, to anchor proteins to solid supports, preparation of immunogens, immunotoxins, and other conjugated protein reagents.

Cross linking agents can be divided into groups dependent on the number and similarity of the reactive groups:

Homobifunctional have two reactive ends that are identical.

Heterobifunctional have two different reactive ends.

Homobifunctional cross linkers are used in one step reactions while the heterobifunctional cross linkers are used in two-step sequential reactions, where the least labile reactive end is reacted first. Homobifunctional cross linking agents have the tendency to result in self-conjugation, polymerization, and intracellular cross linking. On the other hand, heterobifunctional agents allow more controlled two step reactions, which minimizes undesirable intramolecular cross reaction and polymerization.

The most widely used heterobifunctional cross linking agents are used to couple proteins through amine and sulfhydryl groups. The least stable amine reactive NHS-esters couple first and, after removal of uncoupled reagent, the coupling to the sulfhydryl group proceeds. The sulfhydryl reactive groups are generally maleimides, pyridyl disulfides and α-haloacetyls. Other cross linkers include carbodiimides, which link between carboxyl groups (-COOH) and primary amines ($-NH_2$). There are heterobifunctional cross linkers with one photoreactive end. Photoreactive groups are used when no specific groups are available to react with as photoreactive groups react non-specifically upon exposure to UV light.

It is often desirable to minimize the degree of structural shift due to cross linking reactions, and more so if the protein molecule is biologically active. Therefore, cross linking is performed under mild buffer and pH conditions. Depending on the application, the degree of conjugation is also important and an optimal cross linker to protein ratio must be maintained. The number of target groups on the outer surface of a protein is also important. If the exposed target groups are readily available for conjugation; a lower cross linker to protein ratio can be used.

Cross linkers are available with different spacer arm lengths. A cross linker with a longer space arm may be used where two target groups are further apart. The availability of

cross linkers with different spacer arms allows optimization of cross reaction efficiency. Cross linkers with short space arms are suitable for intramolecular cross linking. Cleavable cross linkers are also available which extends the scope of protein analysis.

SELECTION OF PROTEIN CROSS LINKERS

The following features are taken into consideration when making selection of a cross linker:

1. Reagent solubility
2. The nature of reactive groups
3. Homobifunctional or heterobifunctional
4. Photoreactive or thermoreactive groups
5. The length of spacer arm
6. Conjugated product cleavable or not
7. Potential for further labeling
8. Reaction condition needed for conjugation

Cross Linking Application

Protein Structural & Functional Studies

Cross linking agents are used to study the structure and composition of protein molecules. Cross linking can answer questions about the subunit composition of a protein, protein conformations, various protein folding patterns, and so forth. Cross linkers can be used to stabilize protein conformational changes.

Use of heterobifunctional cross linkers may identify specific amino acids and their location within the molecules. Cleavable cross linkers may be used to identify subunit structures. After conjugation, the protein is subject to two-dimensional electrophoresis. When subunits are coupled with a cross linker, the protein molecules migrate as a single protein band, after cleaving the cross linked protein in second dimension, the single band will resolve into constituent subunits.

Cross linkers with short-to-medium spacer arms are suitable for intramolecular cross linking, while cross linkers with long spacer arms are suitable for intermolecular cross linking. Protein and reagent concentration may also effect intermolecular cross linking as high concentrations of homobifunctional cross linkers and dilute protein solution favors formation of intramolecular cross linking.

Protein Interaction and Receptor Studies

Protein cross linkers can be used to establish protein-to-protein association and ligand-receptor interactions. Since the distance between two potential molecules are known, it is often preferable to use a panel of similar cross linkers with different spacer arm lengths. Both cleavable and non-cleavable cross linkers can be used. Similarly, homo and heterobifunctional cross linkers can be used.

Conjugation for Immunological Tools

Antibody production routinely couples haptens, polypeptides and peptides to carrier proteins using a wide variety of cross linkers. The choice of cross linker is dictated by the functional groups present on the hapten and carrier proteins, with the amine groups the preferred group on carrier proteins. Peptides are often synthesized with terminal cysteines that are conjugated to carrier proteins using sulfhydryl-amine reactive heterobifunctional cross linkers. Carbodiimides are also a popular cross linker for producing protein-peptide conjugates, since both proteins and peptides usually contain several carboxyls and amines.

Cell Membrane Structural Studies

Cross linkers are useful for studying structure and function of membrane proteins. Cross linking will locate various proteins on both sides of a membrane. Suitable cross linkers for membrane study can penetrate the lipid bilayer environment. Imidoester cross linkers are water soluble but they are able to penetrate a membrane. Water soluble cross linkers are suitable for establishing the location of molecules on the outer layer of a membrane. Any combination of hydrophobic and hydrophilic cross linkers may be used for a complete picture. Sulfhydryl reactive cross linkers are useful for targeting the molecules with cysteine.

Cell Surface Studies

Cross linkers have been successfully used for identifying receptors on cell surfaces. Membrane impermeable cross linkers, when used carefully, only react with molecules on the cell surface. The sulfo-NHS-esters are membrane impermeable and are a good choice for cross linking proteins on cell surfaces. For determination of whether a protein is located on the cell surface, cell membrane preparation is conjugated with a known protein or a radioactive tag using a membrane impermeable cross linker. After conjugation, the cell membrane preparation is analyzed by SDS-polyacrylamide gel electrophoresis.

Solid-Phase Immobilization

A wide variety of affinity supports are prepared by cross linking proteins, peptides, and other molecules to a solid support. Nitrocellulose membrane, polystyrene, glass and agarose are amongst the most popular supports. Some of these supports can be activated for coupling and others are available with functional groups that can be coupled with proteins or other molecules.

Spacers can be attached to overcome steric hindrance. Useful spacer arms are diaminodipropylamine (DADPA), ethylenediamine, hexanediamine, and 6-amino-capronic. Amino acids and peptide can also be used as spacers.

Preparation of Immunotoxins

Toxic agents can be coupled to specific antibodies and used as a means to deliver toxins to a specific site within a cell. Immunotoxins are useful for killing specific cells such as tumor cells. These antibodies are often specific to tumor-associated antigens. For optimal immunotoxin effects, the immunotoxins often need to be released upon delivery. Cleavable disulfide-containing cross linkers have been found to be more useful than non-cleavable cross linkers. Cells are able to cleave the disulfide bond in the cross linker and release the toxin irreversibly.

Protein-Protein Conjugation

Protein-protein conjugation is one of the most common applications of a cross linker. Protein-protein cross linking is used for the preparation of enzyme coupled antibody probes; protein coupling to chromospheres, fluorophores, and other molecules. Enzymes such as alkaline phosphatase and peroxidase coupled to primary and secondary antibodies are among the most widely used protein-protein conjugation.

One of the widely used methods of protein-protein conjugation is through carbohydrate moieties, called reductive alkylation or amination. Carbohydrate moieties can be oxidized and then coupled with primary amines on enzymes. These conjugations are superior to glutaraldehyde conjugations, which tend to produce high background.

If two proteins contain sulfhydryls, homobifunctional sulfhydryl cross linkers may be used to couple them. Other homobifunctional cross linkers such as NHS-esters or imidoester may also be used. Homobifunctional cross linkers have the potential of producing self-conjugation or polymerization. Heterobifunctional cross linkers, on the other hand, do not pose the risk of self-conjugation and hence are the best choice for antibody-enzyme and other protein-protein conjugations. For example, cross linker SMCC or Sulfo-SMCC in a two-step reaction first conjugated with one protein. The second protein is thiolated with SATA and then conjugated with the first protein.

Protein to DNA/RNA Cross linking

DNA probes are synthesized with amine or thiol groups attached to specific bases, which act as target reactive sites for cross linking reactions.

Reactive Group Transfer

Cross linkers may be used to modify target groups and add spacer for subsequent coupling reactions. For example, amine activated support can be converted to sulfhydryl with NHS-ester maleimide.

PRIMARY AMINE REACTIVE CROSS LINKERS

Amines, lysine ε-amines and N-terminal α-amines, are the most abundant group in protein molecules and represent the most common target for cross linking. For example, BSA contains 59 primary amines, of which up to 35 are available on the surface of the molecules and can be reacted with amine reactive esters.

Amine reactive groups consist of imidoesters and N-hydroxysuccinimide (NHS) esters.

SULFHYDRYL REACTIVE CROSS LINKERS

Sulfhydryl reactive reagents are more specific and react only with free sulfhydryl residues (-SH or thiol groups). The side chain of the amino acid cysteine is the most common source of free sulfhydryl groups. If free sulfhydryl residues are not available, they can be generated by either the reduction of disulfides (-S-S-) with reducing agents such as mercaptoethylamine; or by modifying lysine ε-amines with Traut's reagent or SATA. If disulfide bond reduction is used then excess reducing agent must be removed before reaction with sulfhydryl reactive reagents and addition of a metal chelating agent (EDTA) as an anti-oxidant reduces the chances of reoxidation of sulfhydryls to disulfides.

There are three different reactions employed to cross link to sulfhydryl residues and involve either maleimides, haloacetyls or pyridylthiol groups.

CARBOHYDRATE REACTIVE CROSS LINKERS

Some cross linking reagents do not bind directly to the protein itself but conjugate to the carbohydrate residues of glycoproteins. Carbohydrate reactive cross linking reagents contain hydrazides (-NH-NH_2) as a reactive group. The hydrazide reactions require carbonyl groups, such as aldehydes and ketones, which are formed by oxidative treatment of the carbohydrates. Hydrazides react spontaneously with carbonyl groups, forming a stable hydrazone bond. These reagents are particularly suitable for labeling and studying glycosylated proteins, such as antibodies and receptors.

For reaction with glycoproteins, the first step is to generate carbonyl groups that react with hydrazide, under mild oxidizing conditions with sodium periodate ($NaIO_4$). At 1mM periodate and at 0°C, sialic acid residues on the glycoproteins can be specifically oxidized converting hydroxyls to aldehydes and ketones. At higher concentrations of 6-10mM periodate, other carbohydrates in protein molecules will be oxidized. Such oxidation reactions are performed in the dark to minimize unwanted side reactions.

Aldehydes can also be generated by enzymatic reactions. For example, neuraminidase treatment will generate galactose groups from sialic acid residues on glycoproteins and galactose oxidase converts primary hydroxyl groups on galactose and N-acetylgalactosamine to their corresponding aldehydes.

CARBOXYL REACTIVE CROSS LINKERS

Cross linking to carboxyl groups is mediated by a water-soluble carbodiimide. Carbodiimides effect conjugation of carboxyl to primary amines or hydrazides and result in formation of amide or hydrazone bonds. The conjugation is performed between pH4.5 to 7.5; however, reaction conditions of pH4.5-5.0 is generally recommended. The reaction takes only a few minutes to complete. The carboxyl termini of proteins, glutamic acid and aspartic acid side chain are targets. Since there is an abundance of both carboxyl and primary amine groups in protein, in the presence of excess of

carbodiimides, polymerization may occur. Since there is no spacer between the reacting groups, carbodiimides are called zero spacer arm cross linkers and the resulting bond is the same as a peptide bond.

PHOTOREACTIVE CROSS LINKING REAGENTS

On exposure to ultraviolet light photoreactive agents become active and bind non-specifically with neighboring molecules. Photoreactive reagents are suitable for labeling molecules that do not contain easily reactable functional groups. There are a variety of photoreactive cross linking reagents for the coupling of proteins, peptides, nucleic acids, and other molecules.

Photoreactive reagents contain any aryl azide group. Aryl azide groups are chemically inert until exposed to ultraviolet light. Highly reactive and short-lived aryl nitrenes are formed, which rapidly and non-specifically react with electron-rich sites by inserting into double bonds or active hydrogen bonds (insertion into C-H and N-H sites). Uncreated aryl nitrenes undergo ring expansion and become reactive toward primary amines and sulfhydryls.

This kit uses the homobifunctional N-hydroxysuccinimide ester (NHS-ester) DSP (Dithiobis[succinimidylpropionate]) to cross link lysozyme molecules through their amine groups. The DSP cross linker contains a disulfide bond and is therefore cleavable with a thiol reducing agent.

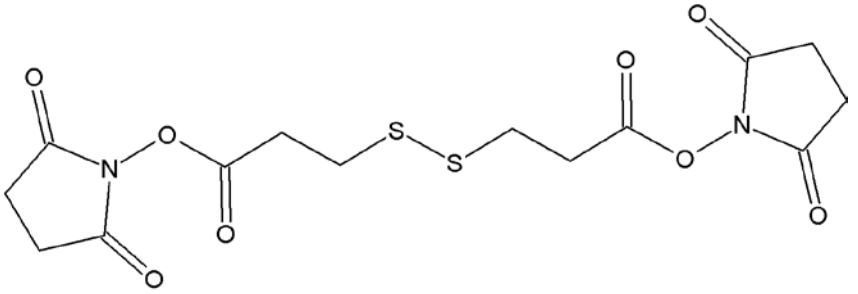


Figure 1: Structure of DSP.

TEACHER'S PRE EXPERIMENT SET UP

Acrylamide/Bis-acrylamide is toxic. Always wear gloves and protective clothing when handling the chemicals.

1. Prepare one 10-12% polyacrylamide gel containing 1% SDS (sodium dodecyl sulfate) or use premade electrophoresis gel with 2 sample lanes for each student and 2 reference wells for each group. G-Biosciences Protein Electrophoresis Kit is recommended for making your own gel.
2. Prepare the Protein Cross Linker the day of the experiment. Add 1ml Cross Linker Solvent to the Protein Cross Linker vial. Vortex or pipette up and down to completely dissolve. Aliquot 50 ☐l Protei
   "Cross Linker" and supply each group with one vial. Discard any remaining cross linker.
3. Transfer 50µl Reducing Agent (TCEP) to a clean Centrifuge Tube and add 450µl Sterile Water and thoroughly mix. Aliquot 50µl diluted Reducing Agent (TCEP) into six 1.5ml tubes and supply each group with one vial.
4. Aliquot 1ml PBS into six 1.5ml tubes and supply each group with one vial.
5. Aliquot 0.6ml Non Reducing Sample Loading Buffer (2X) into six 1.5ml tubes and supply each group with one vial.

MATERIALS FOR EACH GROUP

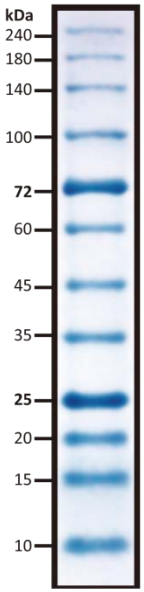
Supply each group with the following components. Several components are shared by the whole class and should be kept on a communal table.

- 1 vial Protein Cross Linker
- 1 vial Protein: Lysozyme
- 1 vial PBS
- 1 vial Non Reducing Sample Loading Buffer (2X)
- 1 vial Reducing Agent
- 1 vial PAGEmark™ Protein Marker (do not boil)
- 13 Centrifuge Tubes (1.5ml)

PROCEDURE

1. As a group, add 700µl PBS to the vial containing the Protein: Lysozyme. Incubate at room temperature for 5 minutes and then vortex or pipette up and down until all the protein has dissolved.
2. Label a 1.5ml Centrifuge Tube with your name and transfer 100µl Lysozyme solution to the tube.
3. Add 10µl Protein Cross Linker and quickly mix by pipetting or vortexing.
4. Incubate the vial at room temperature for 30 minutes.
5. In the meantime, as a group, label a tube "Control" and add 5µl Lysozyme solution from step 1 and add 5µl Non Reducing Sample Buffer.

6. After incubation has finished, add 110µl Non Reducing Sample Buffer to the vial of protein and cross linker.
7. Label two 1.5ml Centrifuge Tubes with your name and either “Non Reduced” and “Reduced”.
8. Transfer 10µl of your solution from step 6 to each tube.
9. Add 5µl Reducing Agent to the “Reduced” tube to reduce the cross linker’s disulfide bond.
10. Boil both samples and the control for 5 minutes. After boiling briefly centrifuge the tubes to bring down the condensation.
11. Follow your Teacher’s instructions for loading and running the protein gels. You will be required to load all of your samples. Load the group’s control and then unreduced and reduced samples. Remember to load 5µl Protein Marker. The PAGEmark™ Protein Marker consists of a mix of twelve Prestained proteins of molecular weight 240, 180, 140, 100, 72, 60, 45, 35, 25, 20, 15 and 10kDa.
12. Following gel electrophoresis, wash the gel twice in distilled water, five minutes each.
13. Remove all free water from the gel.
14. Add 50ml LabSafe GelBlue to cover the gel. Gently shake the gel for 60 minutes at room temperature.
15. Decant the LabSafe GelBlue and rinse the gel with distilled water. The gel can be stored in water. Longer destaining in water will give a clearer view of the protein bands.



4-20% Tris-Glycine

RESULTS, ANALYSIS & ASSESSMENT

Calculate the size of the bands seen in the:

- Control: ~14kDa
- Unreduced: Multiple bands of molecular weight ~14, 28, 42 and 56kDa
- Reduced: ~14kDa

Explain your results below:

The protein cross linker, DSP, couples amine groups. The lysozyme protein appears to have numerous amine groups available for coupling as the protein bands produce are indicative of a 14kDa monomer of lysozyme that is cross linked into a dimer, trimer and tetramer.

Upon reduction with a reducing agent the cross link is broken and the lysozyme oligomers resort back to the 14kDa monomer.

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OBJECTIVES 3
BACKGROUND 3
SELECTION OF PROTEIN CROSS LINKERS 4
CROSS LINKING APPLICATION 4
PROTEIN STRUCTURAL & FUNCTIONAL STUDIES 4
PROTEIN INTERACTION AND RECEPTOR STUDIES 5
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CELL MEMBRANE STRUCTURAL STUDIES 5
CELL SURFACE STUDIES 5
SOLID-PHASE IMMOBILIZATION 5
PREPARATION OF IMMUNOTOXINS 6
PROTEIN-PROTEIN CONJUGATION 6
PROTEIN TO DNA/RNA CROSS LINKING 6
REACTIVE GROUP TRANSFER 6
PRIMARY AMINE REACTIVE CROSS LINKERS 7
SULFHYDRYL REACTIVE CROSS LINKERS 7
CARBOHYDRATE REACTIVE CROSS LINKERS 7
CARBOXYL REACTIVE CROSS LINKERS 8
PHOTOREACTIVE CROSS LINKING REAGENTS 8
MATERIALS FOR EACH GROUP 9
PROCEDURE 9
RESULTS, ANALYSIS & ASSESSMENT 10

OBJECTIVES

- Understand the principal of protein cross linkers.
- Couple a protein with a reversible protein cross linker.
- Reverse protein coupling using a reducing agent.

BACKGROUND

Cross linking agents contain at least two reactive groups that are reactive towards numerous groups, including sulfhydryls and amines, and create chemical covalent bonds between two or more molecules. Functional groups that can be targeted with cross linking agents are primary amines, carboxyls, sulfhydryls, carbohydrates and carboxylic acids. Protein molecules have many of these functional groups and therefore proteins and peptides can be readily conjugated using cross linking agents. Cross linking agents are used to study protein structure and function, to anchor proteins to solid supports, preparation of immunogens, immunotoxins, and other conjugated protein reagents.

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Heterobifunctional have two different reactive ends.

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must be maintained. The number of target groups on the outer surface of a protein is also important. If the exposed target groups are readily available for conjugation; a lower cross linker to protein ratio can be used.

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SELECTION OF PROTEIN CROSS LINKERS

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2. The nature of reactive groups
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CROSS LINKING APPLICATION

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Solid-Phase Immobilization

A wide variety of affinity supports are prepared by cross linking proteins, peptides, and other molecules to a solid support. Nitrocellulose membrane, polystyrene, glass and agarose are amongst the most popular supports. Some of these supports can be activated for coupling and others are available with functional groups that can be coupled with proteins or other molecules.

Spacers can be attached to overcome steric hindrance. Useful spacer arms are diaminodipropylamine (DADPA), ethylenediamine, hexanediamine, and 6-amino-capronic. Amino acids and peptide can also be used as spacers.

Preparation of Immunotoxins

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Protein-Protein Conjugation

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If two proteins contain sulfhydryls, homobifunctional sulfhydryl cross linkers may be used to couple them. Other homobifunctional cross linkers such as NHS-esters or imidoester may also be used. Homobifunctional cross linkers have the potential of producing self-conjugation or polymerization. Heterobifunctional cross linkers, on the other hand, do not pose the risk of self-conjugation and hence are the best choice for antibody-enzyme and other protein-protein conjugations. For example, cross linker SMCC or Sulfo-SMCC in a two-step reaction first conjugated with one protein. The second protein is thiolated with SATA and then conjugated with the first protein.

Protein to DNA/RNA Cross linking

DNA probes are synthesized with amine or thiol groups attached to specific bases, which act as target reactive sites for cross linking reactions.

Reactive Group Transfer

Cross linkers may be used to modify target groups and add spacer for subsequent coupling reactions. For example, amine activated support can be converted to sulfhydryl with NHS-ester maleimide.

PRIMARY AMINE REACTIVE CROSS LINKERS

Amines, lysine ε-amines and N-terminal α-amines, are the most abundant group in protein molecules and represent the most common target for cross linking. For example, BSA contains 59 primary amines, of which up to 35 are available on the surface of the molecules and can be reacted with amine reactive esters.

Amine reactive groups consist of imidoesters and N-hydroxysuccinimide (NHS) esters.

SULFHYDRYL REACTIVE CROSS LINKERS

Sulfhydryl reactive reagents are more specific and react only with free sulfhydryl residues (-SH or thiol groups). The side chain of the amino acid cysteine is the most common source of free sulfhydryl groups. If free sulfhydryl residues are not available, they can be generated by either the reduction of disulfides (-S-S-) with reducing agents such as mercaptoethylamine; or by modifying lysine ε-amines with Traut's reagent or SATA. If disulfide bond reduction is used then excess reducing agent must be removed before reaction with sulfhydryl reactive reagents and addition of a metal chelating agent (EDTA) as an anti-oxidant reduces the chances of reoxidation of sulfhydryls to disulfides.

There are three different reactions employed to cross link to sulfhydryl residues and involve either maleimides, haloacetyls or pyridylthiol groups.

CARBOHYDRATE REACTIVE CROSS LINKERS

Some cross linking reagents do not bind directly to the protein itself but conjugate to the carbohydrate residues of glycoproteins. Carbohydrate reactive cross linking reagents contain hydrazides (-NH-NH_2) as a reactive group. The hydrazide reactions require carbonyl groups, such as aldehydes and ketones, which are formed by oxidative treatment of the carbohydrates. Hydrazides react spontaneously with carbonyl groups, forming a stable hydrazone bond. These reagents are particularly suitable for labeling and studying glycosylated proteins, such as antibodies and receptors.

For reaction with glycoproteins, the first step is to generate carbonyl groups that react with hydrazide, under mild oxidizing conditions with sodium periodate ($NaIO_4$). At 1mM periodate and at 0°C, sialic acid residues on the glycoproteins can be specifically oxidized converting hydroxyls to aldehydes and ketones. At higher concentrations of 6-10mM periodate, other carbohydrates in protein molecules will be oxidized. Such oxidation reactions are performed in the dark to minimize unwanted side reactions.

Aldehydes can also be generated by enzymatic reactions. For example, neuraminidase treatment will generate galactose groups from sialic acid residues on glycoproteins and galactose oxidase converts primary hydroxyl groups on galactose and N-acetylgalactosamine to their corresponding aldehydes.

CARBOXYL REACTIVE CROSS LINKERS

Cross linking to carboxyl groups is mediated by a water-soluble carbodiimide. Carbodiimides effect conjugation of carboxyl to primary amines or hydrazides and result in formation of amide or hydrazone bonds. The conjugation is performed between pH4.5 to 7.5; however, reaction conditions of pH4.5-5.0 is generally recommended. The reaction takes only a few minutes to complete. The carboxyl termini of proteins, glutamic acid and aspartic acid side chain are targets. Since there is an abundance of both carboxyl and primary amine groups in protein, in the presence of excess of carbodiimides, polymerization may occur. Since there is no spacer between the reacting groups, carbodiimides are called zero spacer arm cross linkers and the resulting bond is the same as a peptide bond.

PHOTOREACTIVE CROSS LINKING REAGENTS

On exposure to ultraviolet light photoreactive agents become active and bind non-specifically with neighboring molecules. Photoreactive reagents are suitable for labeling molecules that do not contain easily reactable functional groups. There are a variety of photoreactive cross linking reagents for the coupling of proteins, peptides, nucleic acids, and other molecules.

Photoreactive reagents contain any aryl azide group. Aryl azide groups are chemically inert until exposed to ultraviolet light. Highly reactive and short-lived aryl nitrenes are formed, which rapidly and non-specifically react with electron-rich sites by inserting into double bonds or active hydrogen bonds (insertion into C-H and N-H sites). Uncreated aryl nitrenes undergo ring expansion and become reactive toward primary amines and sulfhydryls.

This kit uses the homobifunctional N-hydroxysuccinimide ester (NHS-ester) DSP (Dithiobis[succinimidylpropionate]) to cross link lysozyme molecules through their amine groups. The DSP cross linker contains a disulfide bond and is therefore cleavable with a thiol reducing agent.

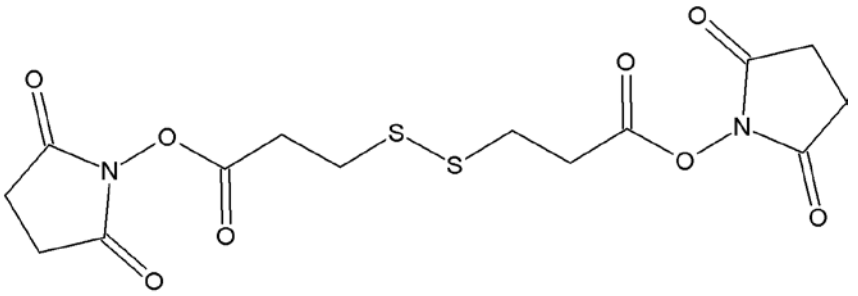


Figure 1: Structure of DSP.

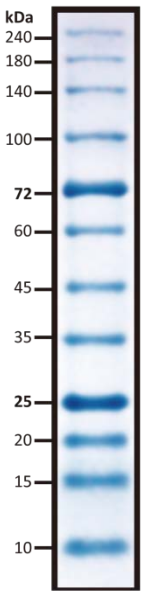
MATERIALS FOR EACH GROUP

Supply each group with the following components. Several components are shared by the whole class and should be kept on a communal table.

- 1 vial Protein Cross Linker
- 1 vial Protein: Lysozyme
- 1 vial PBS
- 1 vial Non Reducing Sample Loading Buffer (2X)
- 1 vial Reducing Agent
- 1 vial PAGEmark™ Protein Marker (do not boil)
- 13 Centrifuge Tubes (1.5ml)

PROCEDURE

1. As a group, add 700µl PBS to the vial containing the Protein: Lysozyme. Incubate at room temperature for 5 minutes and then vortex or pipette up and down until all the protein has dissolved.
2. Label a 1.5ml Centrifuge Tube with your name and transfer 100µl Lysozyme solution to the tube.
3. Add 10µl Protein Cross Linker and quickly mix by pipetting or vortexing.
4. Incubate the vial at room temperature for 30 minutes.
5. In the meantime, as a group, label a tube "Control" and add 5µl Lysozyme solution from step 1 and add 5µl Non Reducing Sample Buffer.
6. After incubation has finished, add 110µl Non Reducing Sample Buffer to the vial of protein and cross linker.
7. Label two 1.5ml Centrifuge Tubes with your name and either "Non Reduced" and "Reduced".
8. Transfer 10µl of your solution from step 6 to each tube.
9. Add 5µl Reducing Agent to the "Reduced" tube to reduce the cross linker's disulfide bond.
10. Boil both samples and the control for 5 minutes. After boiling briefly centrifuge the tubes to bring down the condensation.
11. Follow your Teacher's instructions for loading and running the protein gels. You will be required to load all of your samples. Load the group's control and then unreduced and reduced samples. Remember to load 5µl Protein Marker. The PAGEmark™ Protein Marker consists of a mix of twelve Prestained proteins of molecular weight 240, 180, 140, 100, 72, 60, 45, 35, 25, 20, 15 and 10kDa.
12. Following gel electrophoresis, wash the gel twice in distilled water, five minutes each.
13. Remove all free water from the gel.
14. Add 50ml LabSafe GelBlue to cover the gel. Gently shake the gel for 60 minutes at room temperature.
15. Decant the LabSafe GelBlue and rinse the gel with distilled water.



4-20% Tris-Glycine

The gel can be stored in water. Longer destaining in water will give a clearer view of the protein bands.

RESULTS, ANALYSIS & ASSESSMENT

Calculate the size of the bands seen in the

Control: _____

Unreduced: _____

Reduced:_____

Explain your results below:

_____

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