Bioanalytical chemistry

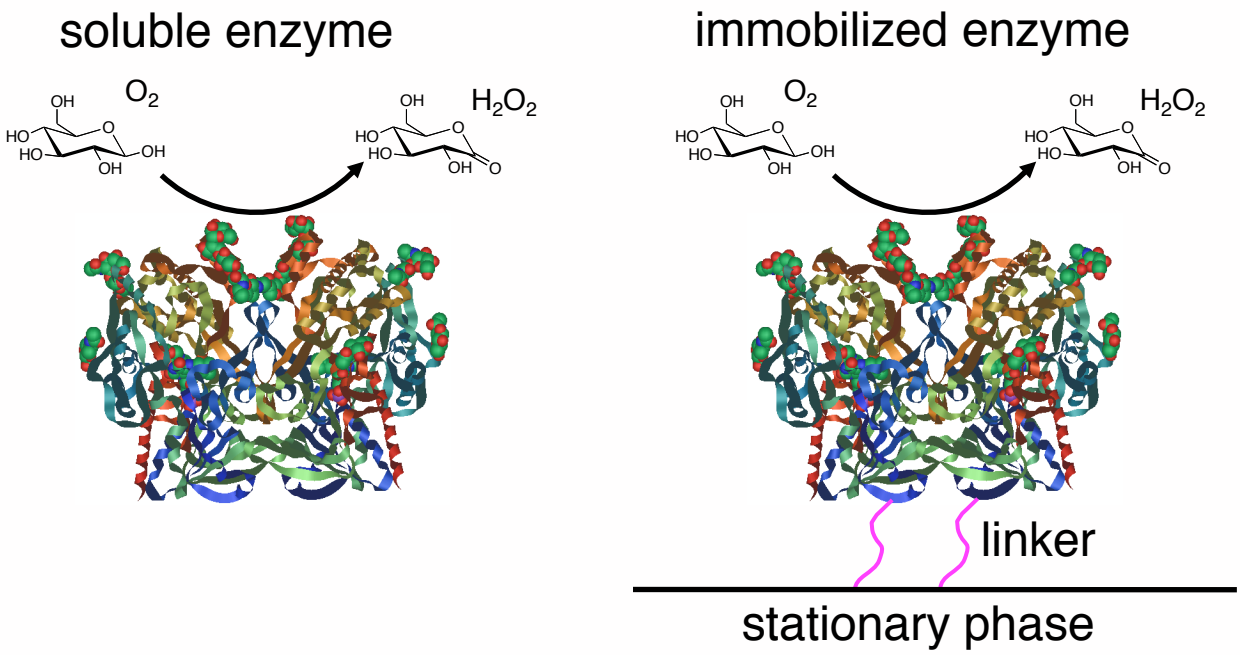
3. Protein conjugation and immobilization

Suggested reading: **Sections 4.1 to 4.3** of Mikkelsen and Cortón, *Bioanalytical Chemistry*

Primary Source Material

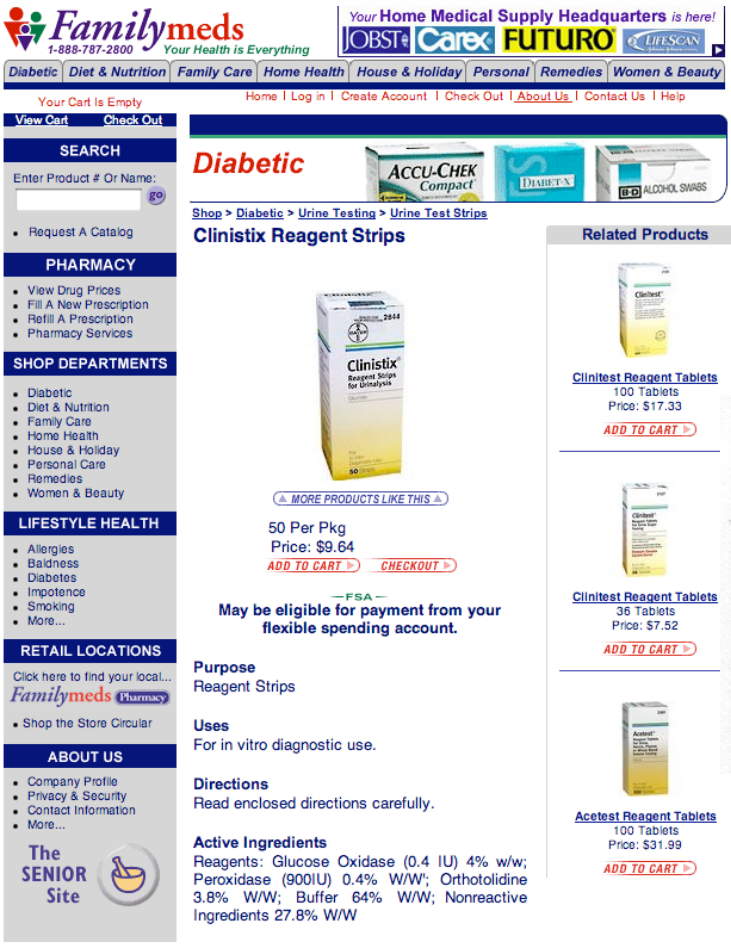
- Chapter 8 of Biochemistry: Berg, Jeremy M.; Tymoczko, John L.; and Stryer, Lubert (NCBI bookshelf).
- Chapter 4 of Mikkelsen, S.R. and Corton, E., Bioanalytical Chemistry (2004) John Wiley and Sons p. 61-71.
- Gary Walsh, Proteins: Biochemistry and Biotechnology, John Wiley & Sons; 2nd edition (2002)
- http://chem.ch.huji.ac.il/~eugeniik/index.htm
- http://chem.ch.huji.ac.il/~eugeniik/electron_mediators.htm

Soluble vs. immobilized enzymes



- Soluble enzymes are extremely useful analytical reagents but there are some disadvantages with their use:
  - Generally not reused or recycled
  - Activity decreases with time (due to oxidation, denaturing, sticking to glass, etc.)
- A solution is to use immobilized enzymes. The enzyme is somehow incorporated onto (or into) the stationary phase and then substrate is introduced in the mobile buffer phase which passes over the enzyme.
  - Can often be reused many times
  - More amenable to automation/robotic handling
  - Greater stability over time

Coupled assays with *immobilized enzymes* are used in glucose-sensing 'dipsticks'



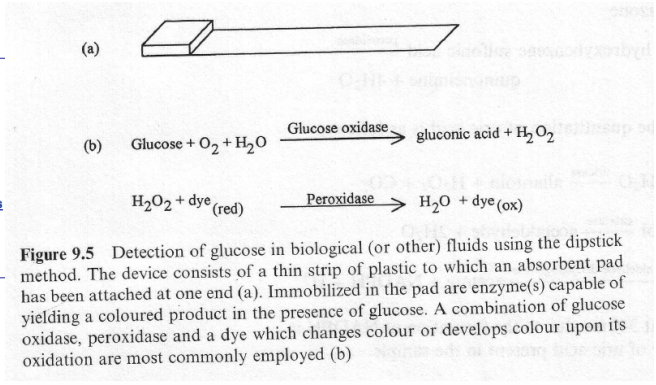


Figure 9.5 Detection of glucose in biological (or other) fluids using the dipstick method. The device consists of a thin strip of plastic to which an absorbent pad has been attached at one end (a). Immobilized in the pad are enzyme(s) capable of yielding a coloured product in the presence of glucose. A combination of glucose oxidase, peroxidase and a dye which changes colour or develops colour upon its oxidation are most commonly employed (b)

Gary Walsh, Proteins: Biochemistry and Biotechnology, John Wiley & Sons; 2nd edition (2002)

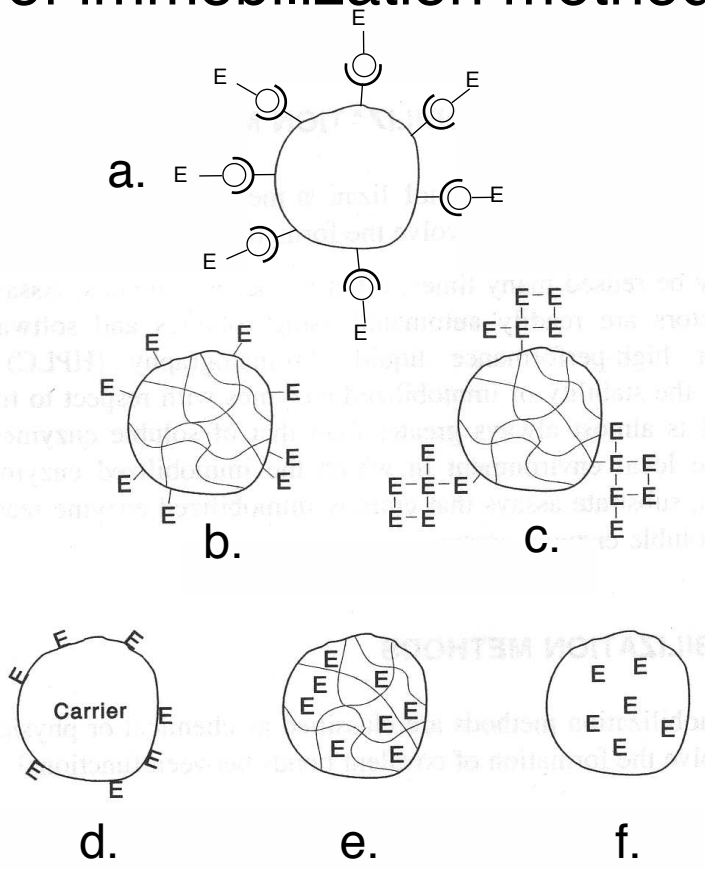
- CLINISTIX™ sticks contain the enzyme glucose oxidase immobilized onto the paper pad at the end of the stick. This enzyme oxidizes glucose to yield gluconic acid and hydrogen peroxide. Peroxidase is also immobilized on the paper pad and it uses the hydrogen peroxide to oxidize a colored dye to a form with a different color (or no color).
- The degree of color change is proportional to the amount of glucose. The color can either be estimated by eye or, more quantitatively, using a simple colorimeter. Why not use fluorescence in this case
- *Q: On most of the slides, you mentioned glucose oxidase converts the glucose to gluconolactone. But here you said the product of this reaction is gluconic acid. Although lactone can be opened to form a gluconic acid, but it requires acid or base as catalyst. I'm quite confused about this.*
- *A: To the best of my knowledge, the product of the enzymatic reaction is gluconolactone. Water will add to the gluconolactone to give gluconic acid. If you were asked what the product of the enzyme reaction is, the correct answer is gluconolactone.*

Overview of immobilization methods

Non-covalent receptor-mediated methods

Chemical (covalent) methods

Physical methods



Mikkelsen, S.R. and Corton, E., Bioanalytical Chemistry (2004)

- a. Receptor-mediated. Non-covalent immobilization based on specific protein-ligand interactions (I.e. avidin-biotin) with an immobilized small molecule or protein binding partner.

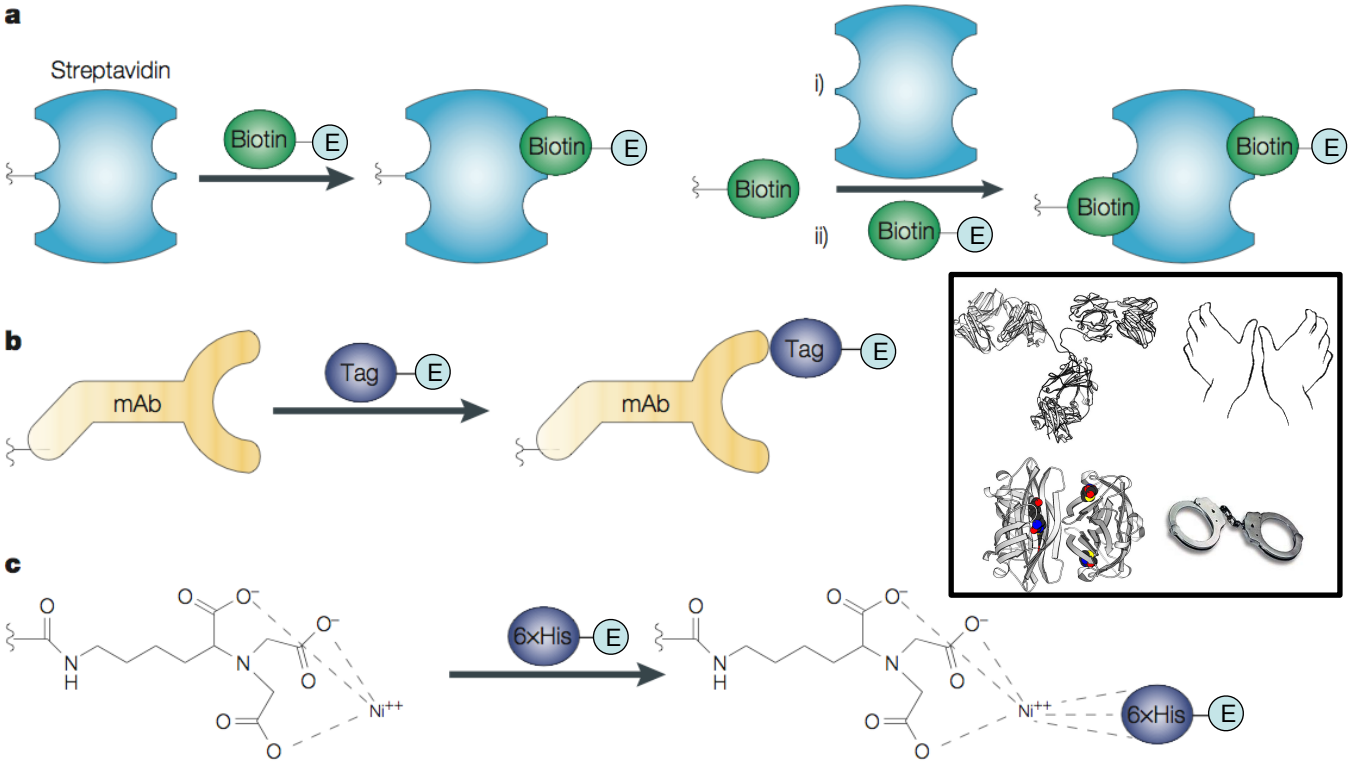
Chemical. Chemical methods involve the formation of a covalent bond between the protein and the stationary phase. Chemical methods can be further classified as either non-polymerizing or cross-linking.

- b. 'Non-polymerizing' means that covalent bonds are only formed between the enzyme and the stationary phase.
- c. Crosslinking means that covalent bonds are formed enzyme-stationary phase and also enzyme-enzyme.

Physical. Physical methods do not change the chemical structure of the enzyme because no new covalent bonds are formed. These methods can be sub-classified as adsorption, entrapment, and encapsulation.

- d. Adsorption can be thought of as relatively non-specific 'sticking' to the stationary phase.
- e. Entrapment means that the enzyme is trapped in a cross-linked polymer but there are no covalent bonds to the enzyme itself. Entrapment is accomplished by performing the polymerization reaction in the presence of the enzyme.
- f. Encapsulation means that the enzyme is separated from the bulk solution by a semipermeable membrane. Because the enzyme is big and the substrate is small, the substrate (and product) can diffuse across the membrane but the enzyme can not.

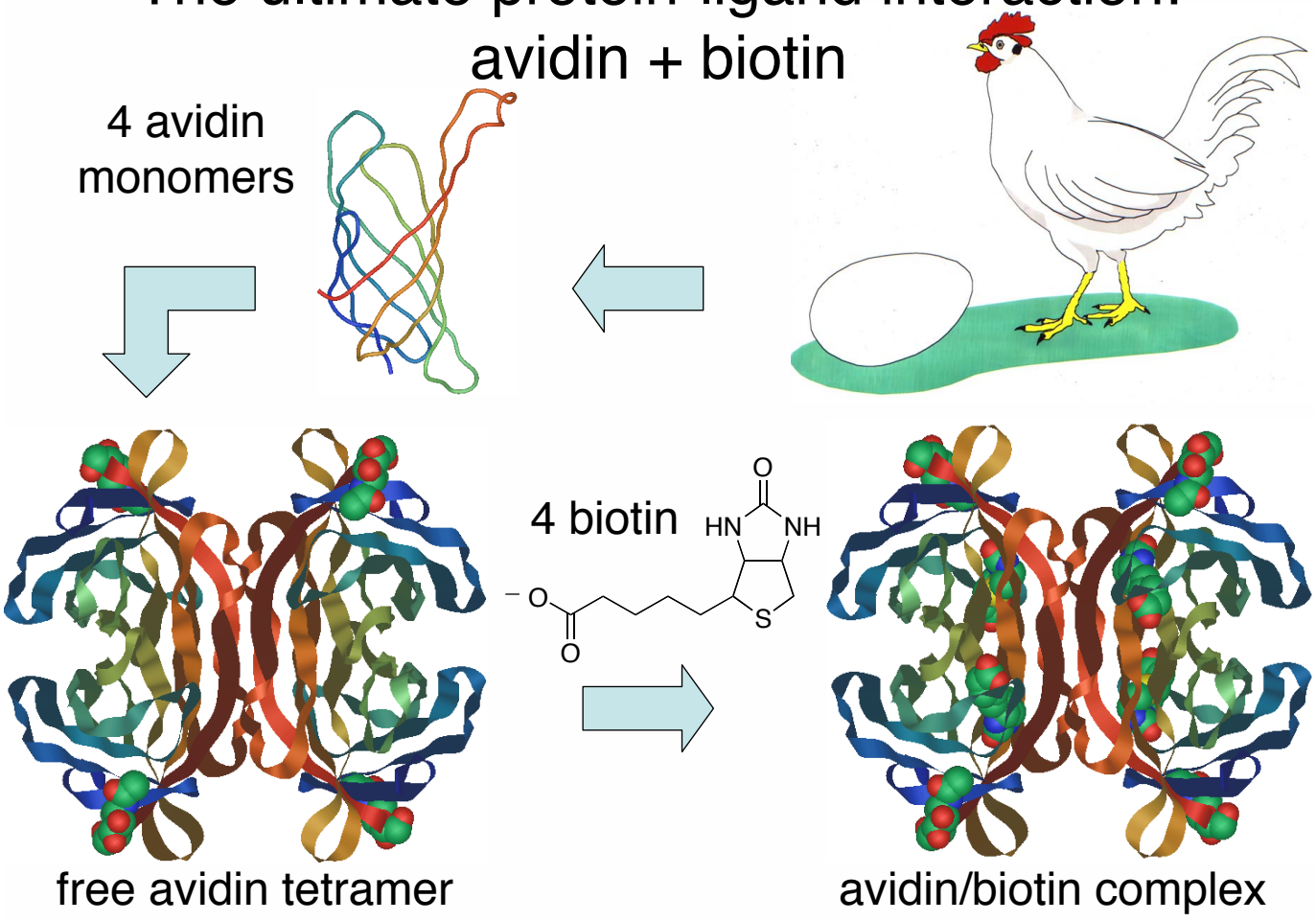
Methods for protein immobilization: non-covalent receptor mediated



Matthew A. Cooper, *Nature Reviews Drug Discovery* **1**, 515 - 528.

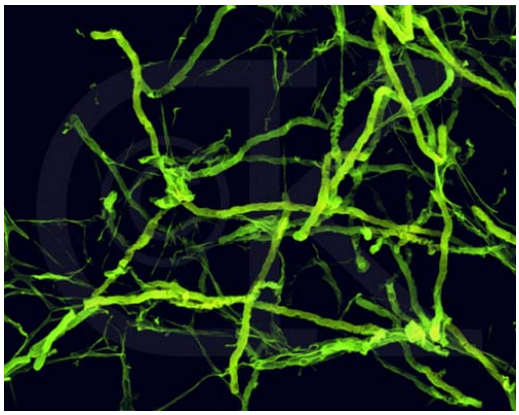
- **Biotin- or streptavidin-presenting surfaces:** These can be used to capture biotinylated-receptors . The multiple biotin-binding sites of streptavidin on each face of the molecule allow biotinylated ligands to be crosslinked by the streptavidin 'double adaptor'. This method is highly efficient and leads to stable complexes, but is effectively irreversible. It is commonly used to immobilize 5'-biotinylated oligonucleotides.
- **Monoclonal antibodies.** These can be covalently attached to a solid support by means of amine coupling. Epitope-tagged or fusion proteins can then be directly and reversibly coupled to the surface through the antibody–antigen interaction. Commonly used tags include, for example, glutathione *S*-transferase, herpes simplex virus glycoprotein D epiptope, FLAG epitope and 6-His.
- **Metal-coordinating groups.** Groups such as iminodiacetic acid (IDA) and nitrilotriacetic acid (NTA) have been widely used for direct immobilization of 6-His- and 10-His-tagged receptors. The moderate affinity of the chelate–Ni^{2+}–histidine ternary interaction means that there is sometimes considerable decay in the level of immobilized receptor. For this reason, anti-6-His monoclonal antibodies are often used to enable stable, oriented immobilization of His-tagged receptors.

The ultimate protein-ligand interaction: avidin + biotin

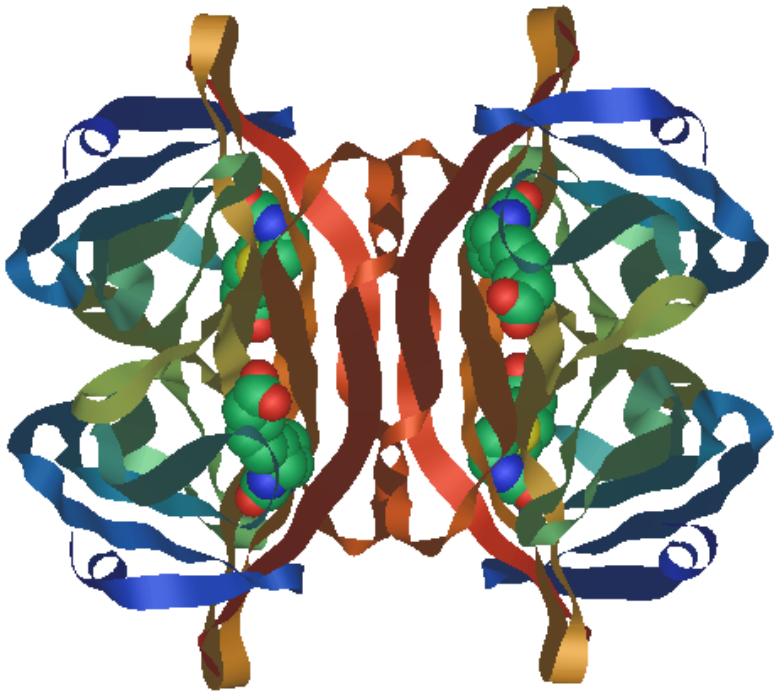


- Avidin is a tetrameric glycoprotein isolated from chicken egg white. The ability of avidin to bind biotin with exceptionally high affinity (K_d ~ 10^{-15} M) has been the basis for its exploitation as a molecular tool in biotechnological, diagnostic and therapeutic applications, collectively known as avidin-biotin technology. This interaction is one of the tightest non-covalent interactions known between proteins and their ligands and the overall stability approaches that *of a single covalent bond*. Biotin is a small water-soluble vitamin (otherwise known as vitamin H).
- The avidin tetramer consists of four identical subunits, all bearing 128 amino acids and possessing one biotin-binding site. The protein is basically charged (*pI* ~ 10.5), and each of its monomers possesses eight arginine and nine lysine residues.
- The polypeptide chain of avidin also contains a glycosylation site at residue Asn-17. The carbohydrate moiety accounts for about 10% of the molecular mass of avidin and exhibits extensive glycan microheterogeneity.
- Despite the utility of chicken avidin in the many applications of avidin-biotin technology, there are some drawbacks associated with its use. Its high p*I* and the presence of carbohydrate can cause non-specific binding to extraneous material in certain applications, and these properties, therefore, hinder its use. Due to these difficulties, streptavidin, a non-glycosylated and neutrally charged bacterial counterpart of avidin, has virtually replaced avidin in these applications, even though avidin contains more lysine residues for potential attachment of probes, is more hydrophilic, and is considerably more abundant and cheaper than streptavidin. (from FEBS Letters Volume 467, Issue 1 , 4 February 2000, Pages 31-36)
- Commercially available 'Neutravidin' (Pierce) is avidin which has had the carbohydrate portion removed enzymatically (glycosidase) and has been chemically treated to lower the isoelectric point. I have not been able to figure out what the chemical treatment is - it may be proprietary information. However, we can guess that the chemical treatment is either the blocking of arginines or the addition of more negative charges.

Streptavidin has largely replaced avidin in biotechnological applications

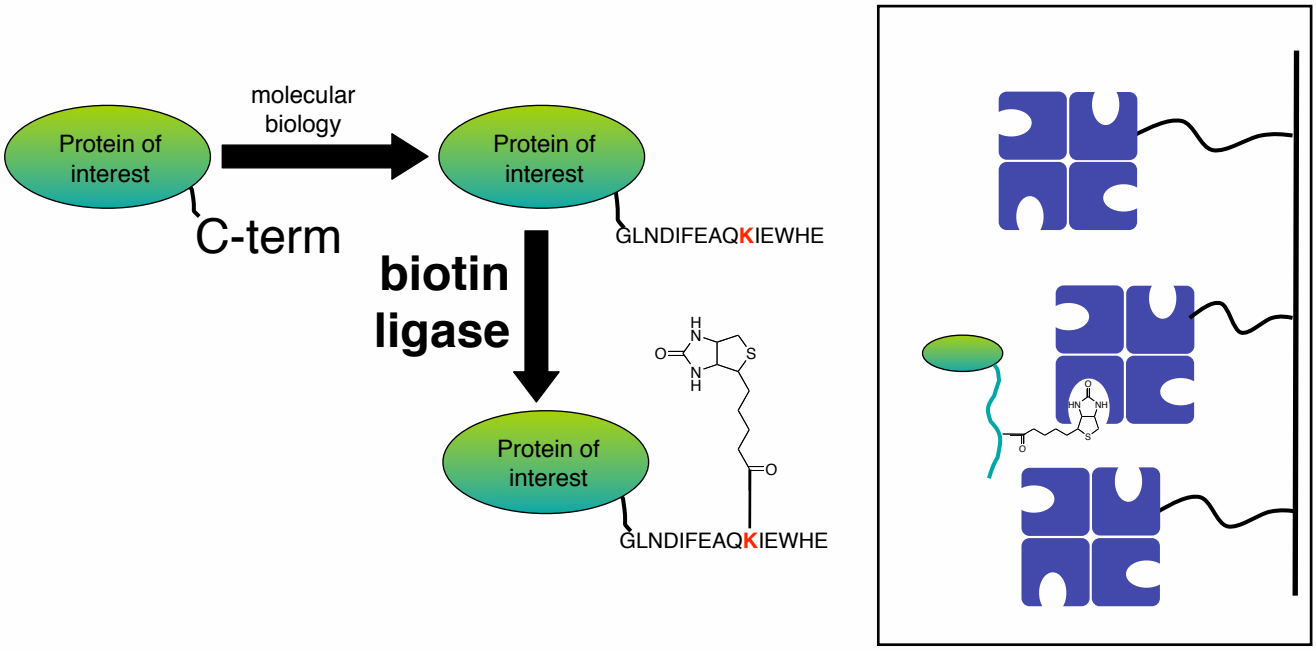


Streptomyces:
a bacteria that forms filamentous structures similar to those formed by fungus



- Streptavidin is a close homolog of avidin produced by the bacteria *Streptomyces avidinii*. The dissociation constant (*K*d) of the complex between streptavidin and biotin is similar to that of avidin and biotin,
- Streptavidin is also one of the most stable proteins known. For example, it can maintain its functional structure at high temperatures, extremes of pH, and in the presence of high concentrations of denaturants and organic solvents. This protein also has exceptional stability against proteolysis. These unique properties of streptavidin, along with the ability of biotin to be incorporated easily into various biological materials, allow streptavidin to serve as a versatile, powerful affinity tag in a variety of biological applications. In particular, streptavidin is one of the most frequently used proteins in clinical diagnostics.
- It is interesting to note that streptavidin is not Streptomycetes only claim to fame. These bacteria are masterful chemists and produce the majority of antibiotics applied in human and veterinary medicine and agriculture, as well as anti-parasitic agents, herbicides, pharmacologically active metabolites (e.g. immuno-suppressants) and several enzymes important in the food and other industries. [From Sano et al. Journal of Chromatography B, 715 (1998) 85-91.]

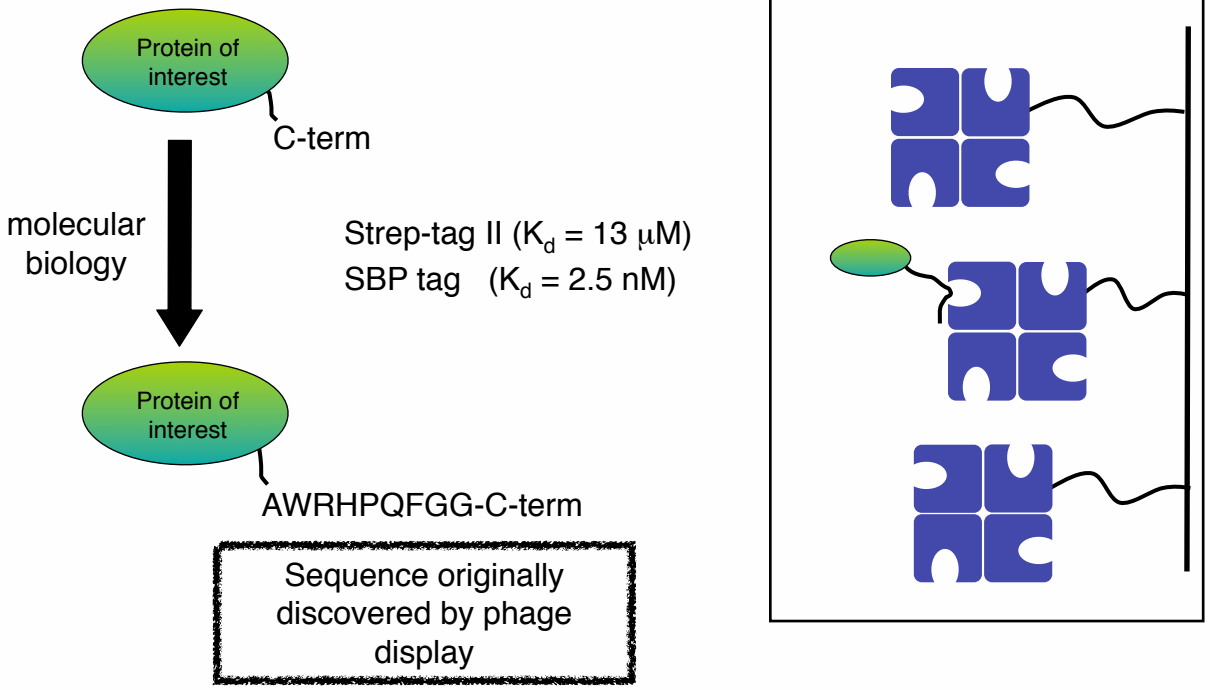
Adding a biotin tag to a macromolecule: The Avitag



Home page for the Avitag: http://www.avidity.com/

- The Biotin AviTag sequence is a peptide, just 15 residues long, that is recognized as a substrate by biotin ligase. In the presence of ATP, the ligase specifically attaches biotin to a specific lysine residue in this sequence. Can you propose a mechanism? Sounds like it would be similar to the same basic mechanism we have seen several times now: biotin-ligase activates biotin to form a biotinyl 5' adenylate and then transfers the biotin to biotin-accepting proteins.
- Using vectors developed by Avidity, the Biotin AviTag can be genetically fused to a much bigger protein. This feature effectively allows any protein that has been cloned to be tagged with a biotin molecule.
- The Biotin AviTag system affords several major advantages over the chemical labelling of proteins with biotin: Because the biotinylation is performed enzymatically, the reaction conditions are very gentle and the labelling is highly specific. Either *in vivo* or *in vitro* biotinylation of proteins is possible.
- *Q: Do we have to know the entire sequence for the Avitin tag and Streptag or can we just know the important pieces of both of them?*
- *A: You don't need to know the entire sequence, but you should understand how they work and how the binding to biotin or streptavidin (as the case may be) occurs.*

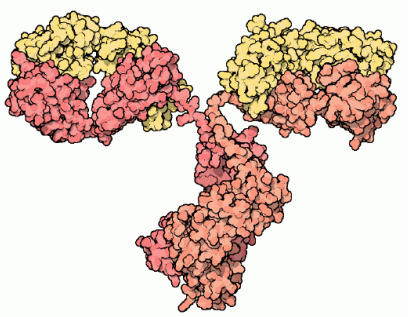
Adding a biotin tag to a macromolecule: The Streptag: low affinity interaction with streptavidin



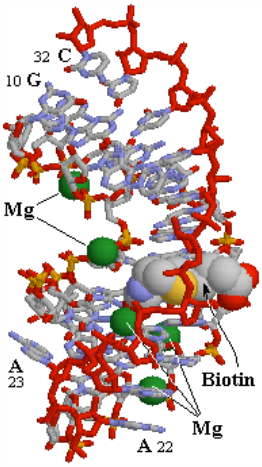
Original publications
http://www.lrz-muenchen.de/%7EBiologische-Chemie/Publikationen/Publikationen.html
http://genetics.mgh.harvard.edu/szostakweb/publications/framecontent/pubcontent.html

- The Strep-tag is a "tailor-made", 8 amino acid strepavidin binding sequence.
- This sequence was found through the systematic screening of random peptide libraries in order to identify a peptide binding sequence with optimal affinity tag properties. It turns out that phage display was not used for the original discovery of this peptide, but it is a suitable and practical method that could have been used. Phage display of random peptides with selection for binding to streptavidin reliably results in the enrichment for peptide sequences that contain the HPQ sequence. This tripeptide binds in the biotin binding site.
- The Strep-tag is not a replacement for biotin since it is much lower affinity. However, it does allow one to reversibly bind proteins to the many different resins and surfaces that are available with streptavidin coatings. For example, it can be used as an affinity tag for protein purification using columns with immobilized streptavidin. Highly specific elution of the bound protein from the column can be achieved through the addition of biotin or related compounds using a physiological buffer, ie. no high salt or extreme pH are required for chromatography.
- *Q. You said high specific elution can be achieved through the addition of biotin, my q is, does it (biotin) interacts with the protein and strips the protein from the strep tag (there is no indication of biotin ligase) ? Do u get biotin-E complexed with streptavidin? on the page 23, it seems that Strepavadin does makes a S-B-E complex. Also are their any major difference between Stretavadin binding protein tag (SBP) and Strep-tag besides sequence diff? I am kinda confused in this manner.*
- *A. The Streptag is just a peptide sequence that interacts with streptavidin. There is no biotin involved in this interaction. However, the Streptag can be displaced from streptavidin by adding biotin, since biotin and the streptag compete for the same binding site on the streptavidin. Streptag and SBP are just two different versions of the peptide that have different Kds due to differences in their sequences. Neither interaction involves biotin.*

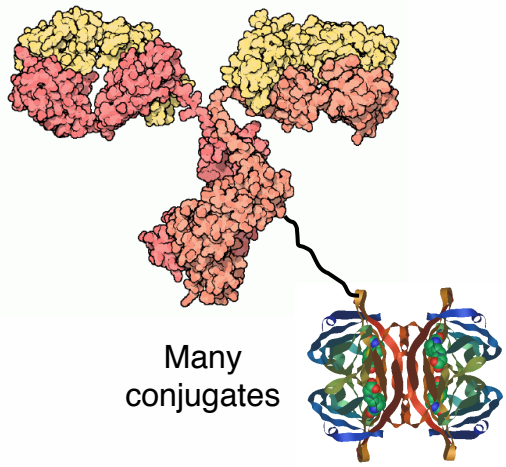
Many possibilities!!



Anti-biotin
Anti-(strept)avidin
Anti-strep-tag
Anti-FLAG



Biotin aptamer
Streptavidin aptamer



Many conjugates

many beads

- biotin beads
- (Strept)avidin beads

+ magnetic
+ fluorescence
+ other properties

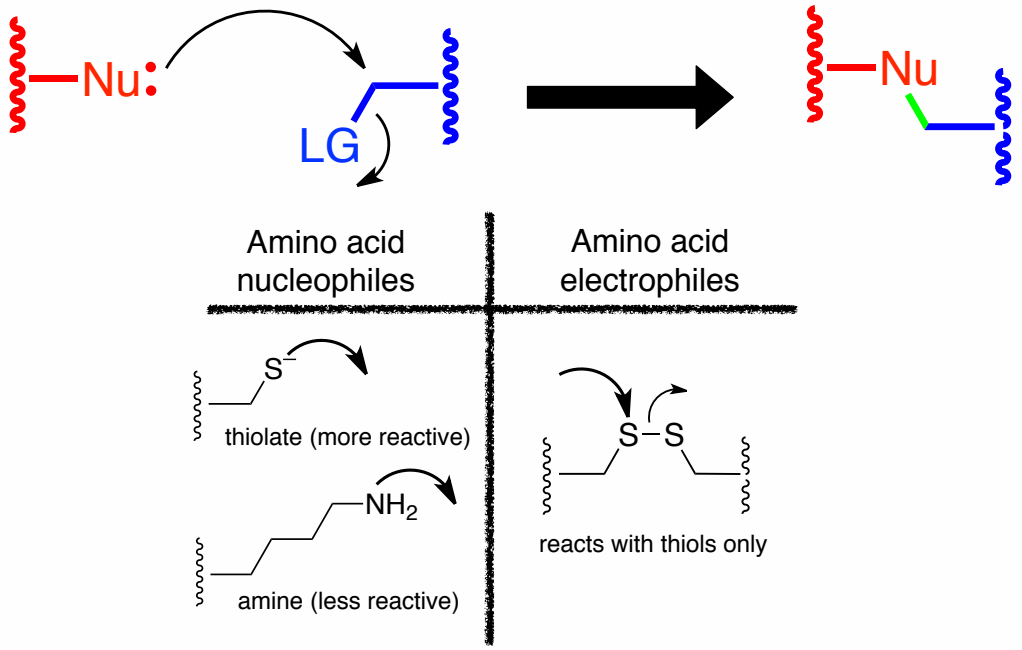
- Reactive labels
- Coated surfaces:
  - chips, slides, microplates
- The list goes on….

http://www.chembio.uoguelph.ca/educmat/chm730/k730.htm

- The biotin/(strept)avidin system has been employed in so many different types of applications that you can use it to link together basically any two molecules of interest.
- This slide is supposed to just hit some highlights but there many, many possibilities.
- *Q: what is the characteristic of magnetic beads?*
- *A: These are just beads that are strongly attached to a magnet and also have some other protein or functional group attached to them (depending on the application). By holding a magnet against the side of a plastic tube the beads can be held and washed to remove non-specific binders while retaining the specific binders.*

Covalent modification of proteins

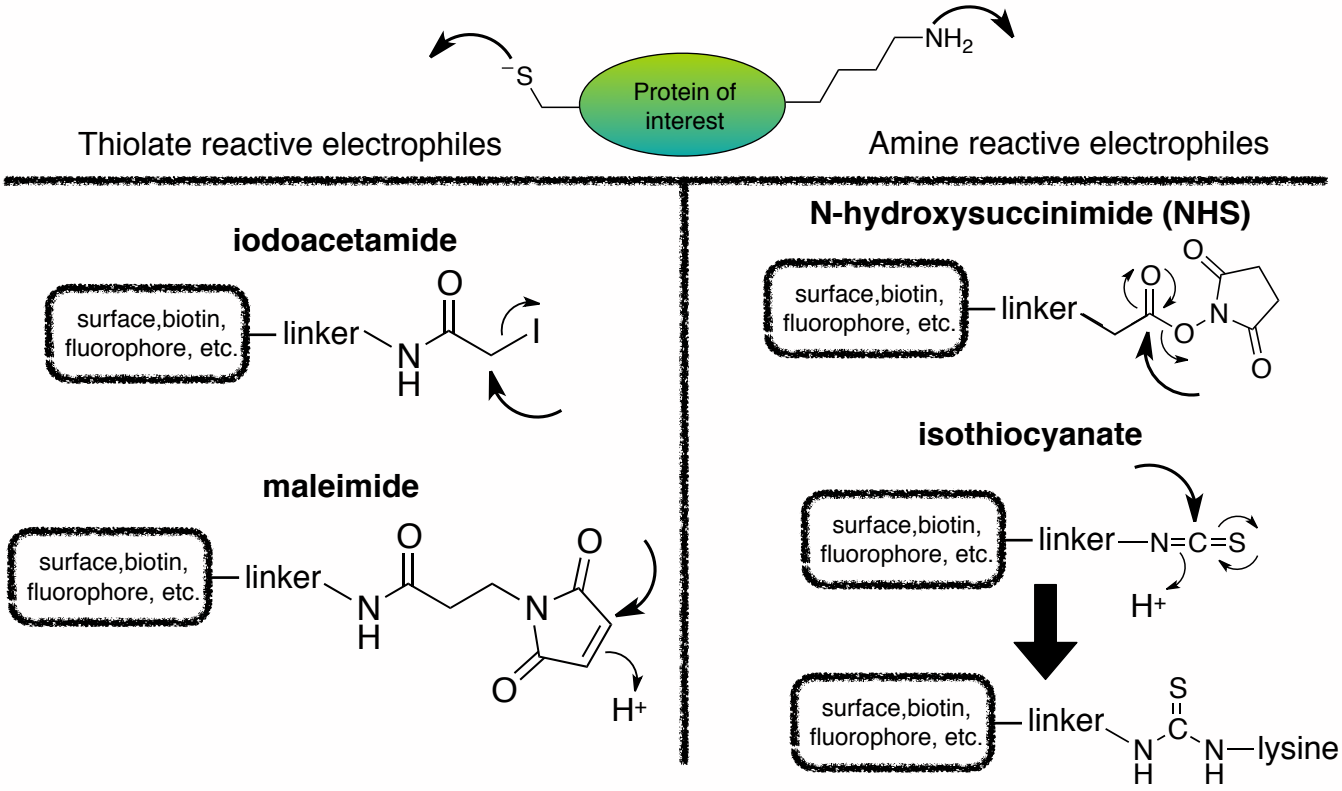
Tends to rely on very simple S_N2 chemistry, so requires an electrophile and a nucleophile.



Of the naturally occuring amino acids, only cysteine (as the $-S^-$) and lysine (as $-NH_2$) are good nucleophiles. The only good electrophile is a disulphide bond.

Covalent modification of proteins

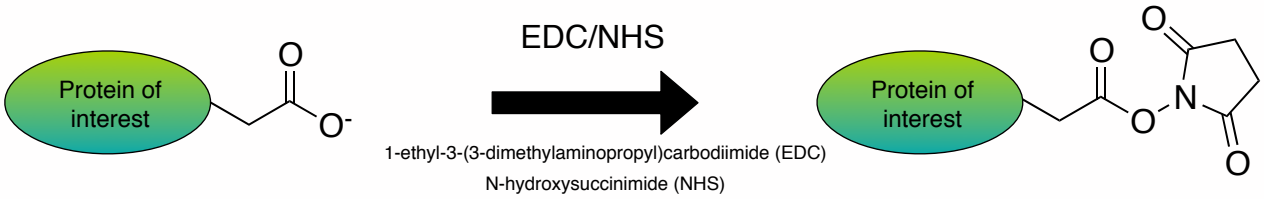
When we want to covalently attach something to a protein, we most commonly react with the cysteine or lysine nucleophiles on the surface of the protein



- The goal of the covalent non-polymerizing immobilization strategy is often to link the protein directly to the surface using chemistry. Typically this is done through the formation of a amide bonds between amines and carboxylic acids or thioether bonds with good electrophiles and free thiols (the only good nucleophile in proteins)
- *Q. What do the "linker" on page 31 and the "S-linker" on page 26 refers to? are they a covalent bond, such as a disulfide bond? how did those linkers attached to the surface, biotin or fluorophores?*
- *A. In these cases linker refers to a linear covalent chain of carbon, oxygen and nitrogen atoms. Slides 32 and 33 contain several examples of linkers. For example, BM(PEG)2 and Maleimide-PEG2-Biotin both have polyethylene glycol (PEG) linkers. PEG (of various lengths) is generally the preferred linker.*

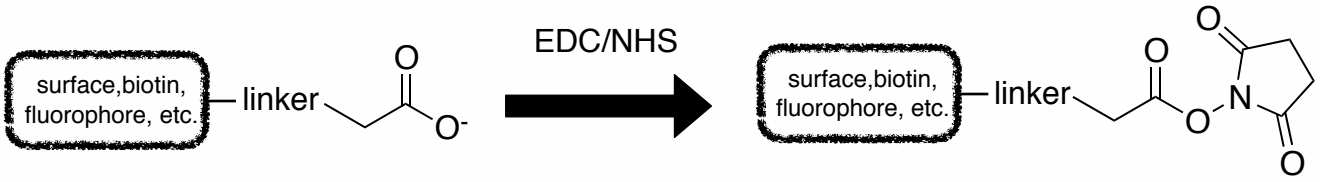
Covalent modification of proteins

The carboxylic acid groups on proteins (Asp, Glu, C-term) can be converted into a good electrophile by **activating** them as an NHS ester



The activated carboxylic acid could then be reacted with surfaces or probes that have a free amine nucleophile.

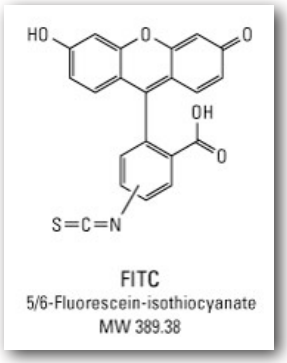
Of course, it could also be done the other way around, with the surface or probe being activated such that it is reactive towards a lysine on a protein

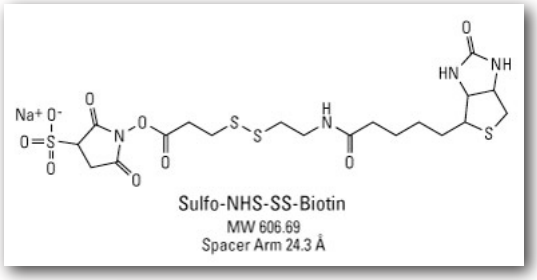


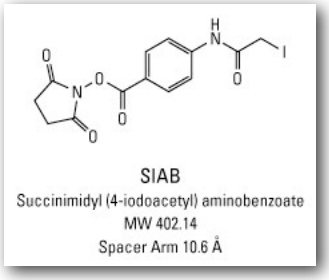
- It is quite common to start with a surface that presents either free amines or free carboxylic acids for derivatization.
- To link proteins to a surface that presents carboxylic acid groups, the acid moieties can be made reactive towards amines using water-soluble EDC/NHS mediated activation. The resultant reactive NHS ester can then be coupled directly with available amino moieties of a protein (lysine or N-terminus) to form a stable amide linkage.
- Amino-presenting surfaces can be treated with commercially available bifunctional linking reagents to effect coupling with free amino or sulphydryl groups on the receptor

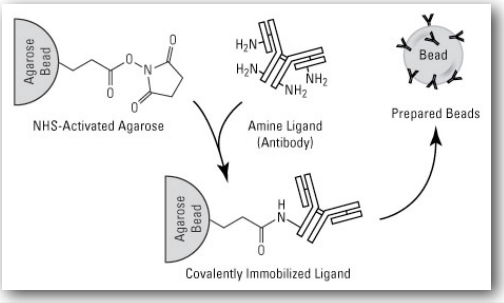
Examples of reagents used for covalent modification of proteins

What would each of these be useful for?



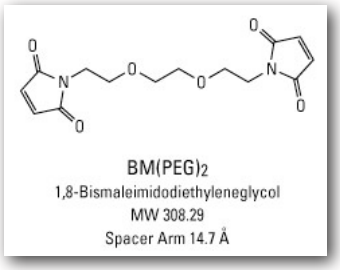


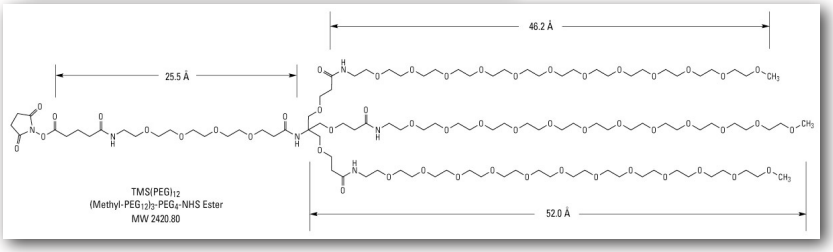




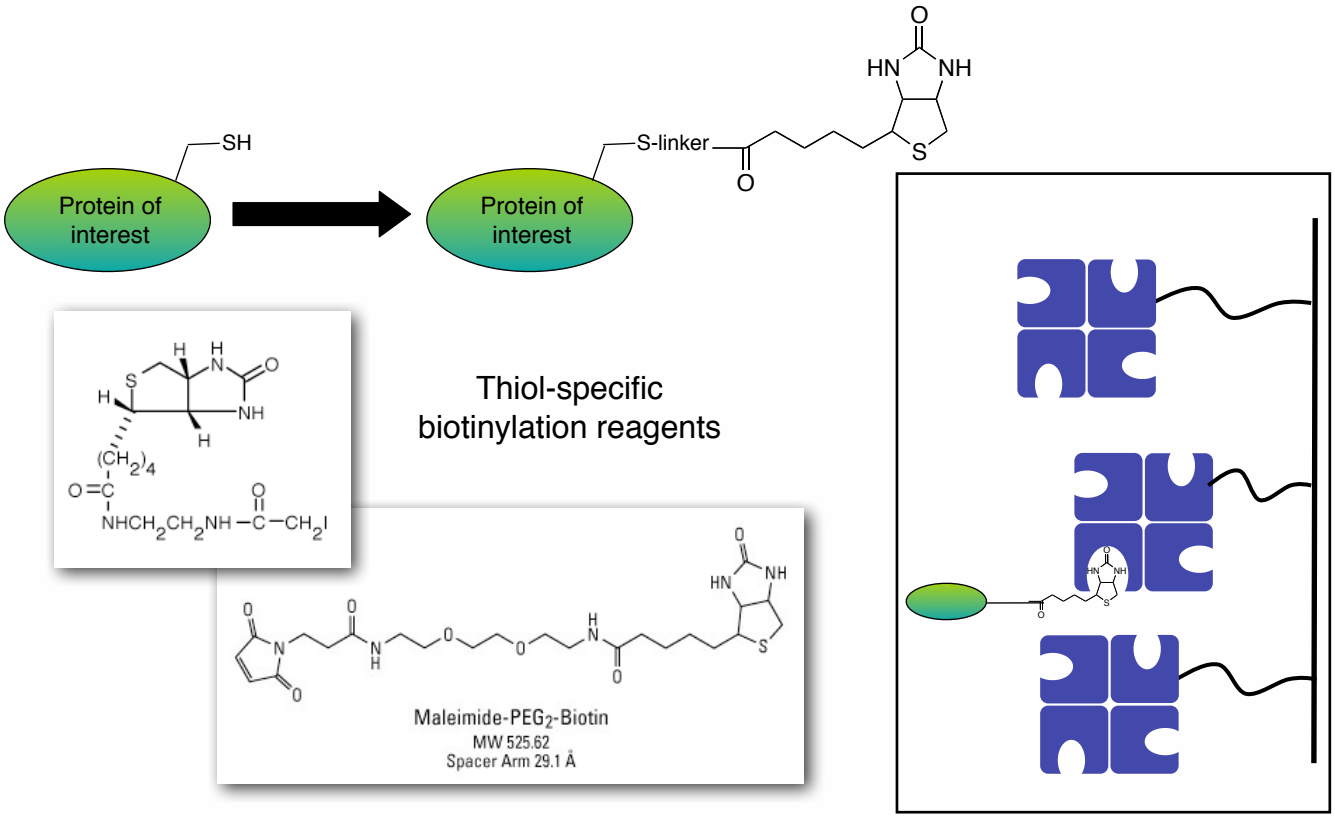
http://www.piercenet.com

(just a few of hundreds of examples)





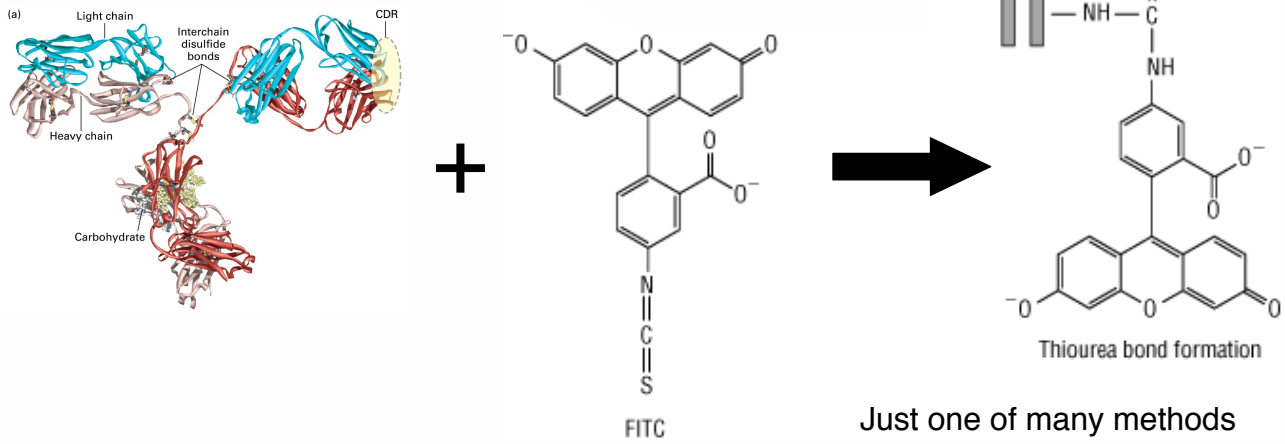
Adding a biotin tag to a macromolecule with thiol reactive biotin labels



Biotinylation and Haptenylation Reagents http://www.probes.com/handbook/sections/0402.html

Q. What does "S-linker" refer to?
A. The S is just for the sulphur atom from cysteine.

Preparing a fluorescently labeled antibody



Just one of many methods

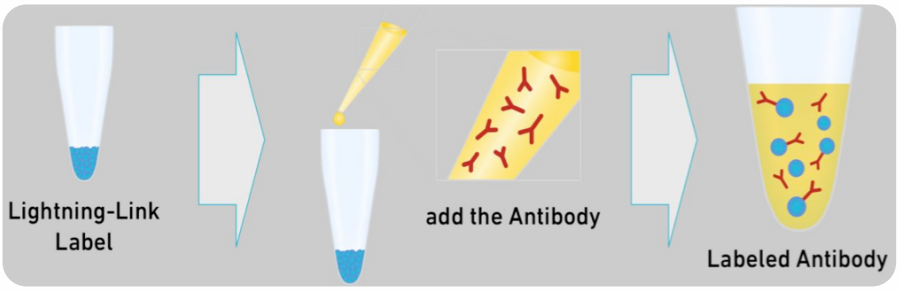
- as with enzyme labelling, there are many commercially available kits that make this process very simple to carry out

http://www.piercenet.com/

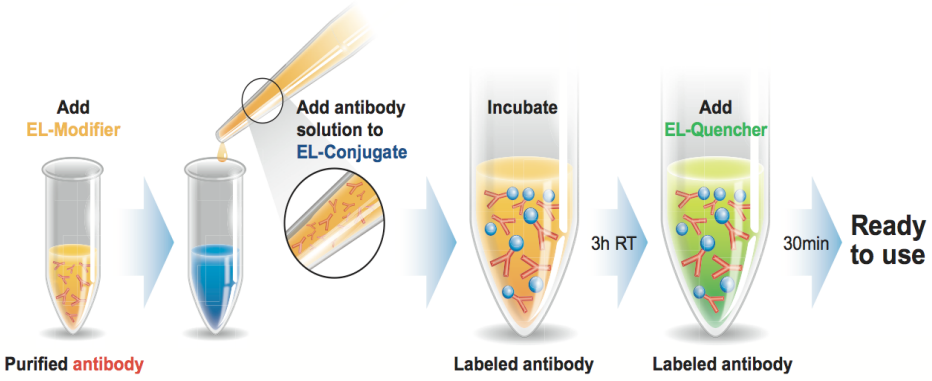
- Antibody molecules can be labeled with any of a number of different fluorescent probes currently available from commercial sources. Each probe option has its own characteristic spectral signals of excitation (or absorption) and emission (or fluorescence). Many derivatives of these fluorescent probes possess reactive functional groups convenient for covalently linking to antibodies and other molecules.
- Each of the main fluorophore families contains at least a few different choices in coupling chemistry to direct the modification reaction to selected functional groups on the molecule to be labeled. These choices include amine-reactive, sulfhydryl-reactive and aldehyde-reactive.
- Perhaps the most common fluorescent tags with application to immunoassays include derivatives of fluorescein, rhodamine, aminomethylcoumarin (AMCA) and phycoerythrin. Instrumentation is widely available for measuring the fluorescent response of any of these probes, including standard filter selections that match their excitation and emission patterns. Such fluorescently labeled antibodies can be used in immunohistochemical staining, in flow cytometry or cell sorting techniques, for tracking and localization of antigens and in various double-staining methods.
- In choosing a fluorescent tag, the most important factors to consider are good absorption, stable excitation, and efficient, high-quantum yield of fluorescence. All fluorophores, to various extents, exhibit photobleaching which lowers the quantum yield over time. Up to 50% of the fluorescent intensity observed on a fluorescein-stained slide can be lost within 1 month of storage.

Preparing an enzyme labeled antibody

Example: Lightning-link kit from Innova Biosciences



Example: Easylink kit from Abcam

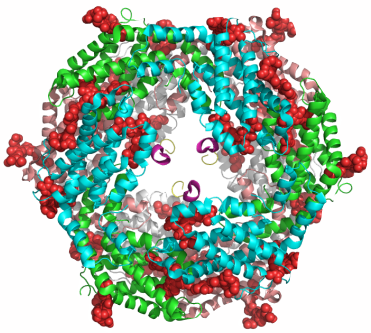


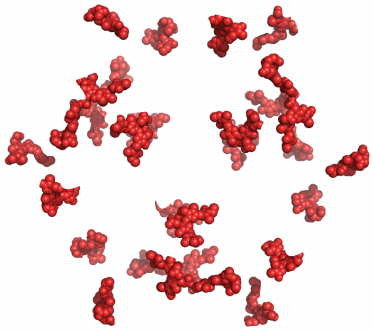
http://www.biomol.de/details/IN/Innova_Guide_Antibody_Labeling_biomol.pdf

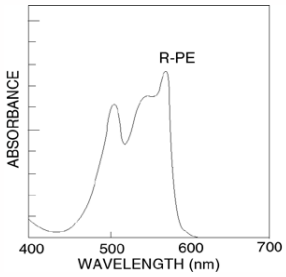
- preparing an enzyme labeled antibody involves using the chemical conjugation methods discussed in the previous section of the notes.
- as with most things in biology, kits are available that make this labelling process quite easy.
- a typical kit would contain the enzyme (e.g. HRP or alkaline phosphatase) that has already been ‘activated’ for reaction with lysines on the antibody. That is the enzyme in the kit has been modified to have NHS esters attached to its own lysines (or possibly cysteines)

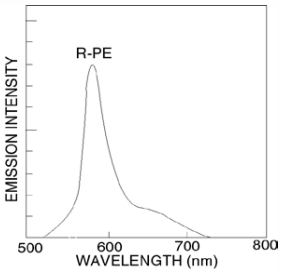
R-Phycoerythrin - one of the most important fluorophores for protein labelling

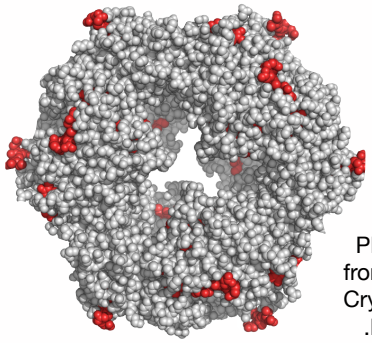
Red marine algae



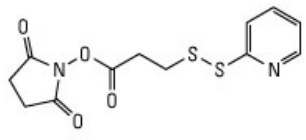








PDB ID 1EYX, from (2001) Acta Crystallogr.,Sect .D **57:** 52-60



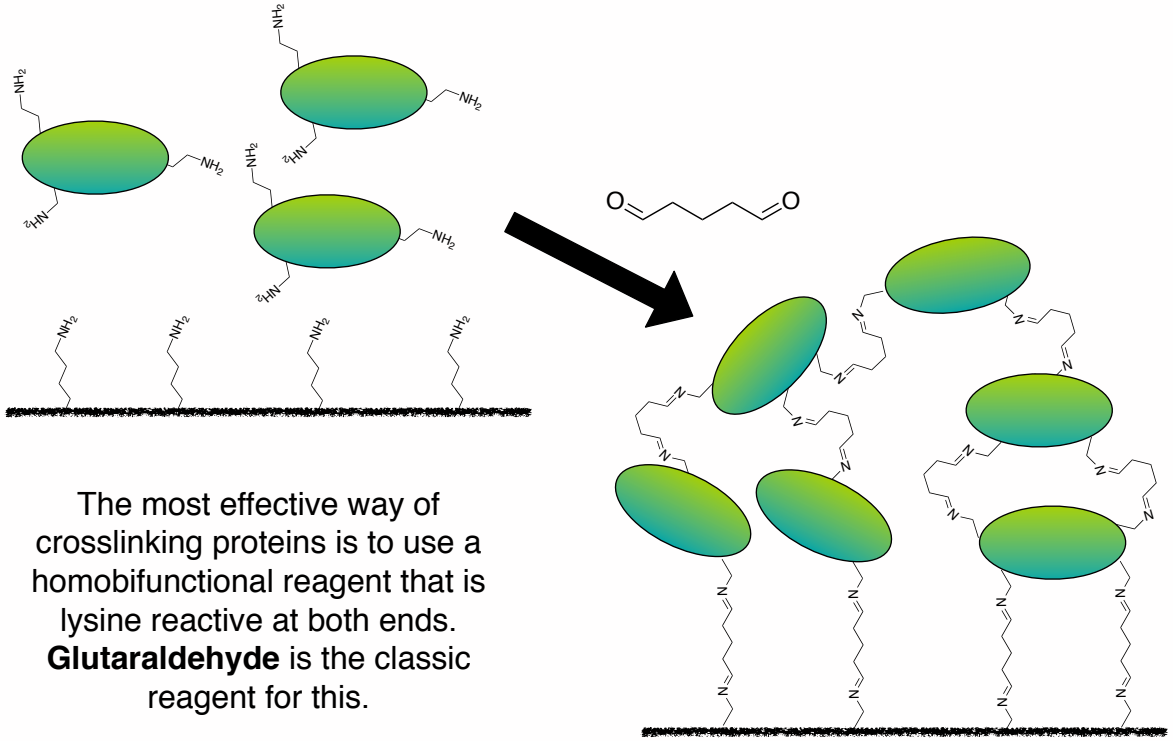
SPDP
Succinimidyl 3-(2-pyridyldithio)propionate

Pierce recommends SPDP for linking R-PE to other proteins. R-PE is reacted first with SPDP. The protein of interest, with a free sulfhydryl is then added to the R-PE-SPDP conjugate.

- R-phycoerythrin is a a red fluorescent protein (MW 240k) from a red marine algae. The protein contains 30 copies of a bilin chromophore, giving it a very high extinction coefficient of 2 x 10^6 M-1cm-1.
- The protein is a hexamer of ab heterodimers (ab)6
- Due to its high extinction coefficient and high quantum yield, it is one of the brightest fluorophores known. It's brightness is comparable to that of a quantum dot.
- It is widely used as a fluorescent label for proteins and antibodies.
- http://www.piercenet.com/instructions/2160350.pdf

Crosslinking of proteins

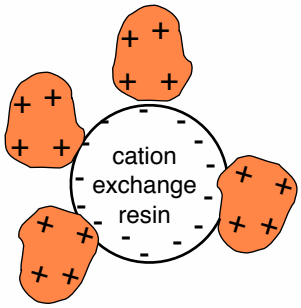
Covalent “crosslinking” or proteins typically refers to the relatively non-specific formation of covalent bonds between protein molecules and also between protein molecules and surfaces. It is the “quick and dirty” way to immobilize proteins.



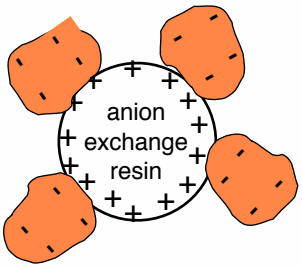
The most effective way of crosslinking proteins is to use a homobifunctional reagent that is lysine reactive at both ends. **Glutaraldehyde** is the classic reagent for this.

- Covalent cross-linking is a poorly controlled method so would only be used in cases where it is not important to retain a high percentage of enzyme activity and where the enzyme is abundant.
- During the cross-linking reaction bonds will form between the enzyme and the stationary phase but also between multiple copies of the enzyme.
- The enzyme polymerization will generally result in lower enzymatic activity than the more controlled methods of receptor-mediated immobilization and nonpolymerizing covalent attachment. There are several reasons for this:
  - The polymeric network of enzyme molecules will obscure or covalently modify active sites of the enzyme.
  - The rate of substrate and product diffusion will be decreased.
  - The tertiary structure of the enzyme may be disrupted
- *Q: Could you use a homobifunctional cross-linking reagent to chemically react biotin to the protein of interest? One end for the protein of interest and other end to biotin?*
- A: *Homobifunctional cross-linking reagents are practically always molecules that contain two electrophilic centres. Accordingly, they are used to react with nucleophiles on the two molecules to be cross linked. The carboxylic acid group of biotin is neither nucleophilic nor electrophilic. In can be most easily made into an electrophile by forming the NHS ester. In principle, it could then be made into a nucleophile by reacting with a diamine.*
- *Q: to what extent is it necessary that we be able to draw chemical structures and reactions from all of the sections of this course?*
- *A: The exact structures of molecules talked about in class are not that important, though the methods of linking things together is quite important.*

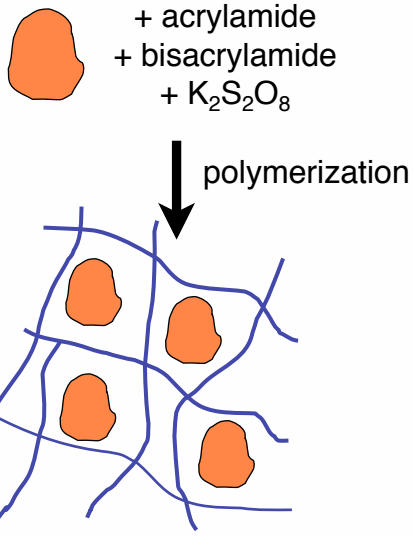
Methods for protein immobilization: adsorption, entrapment, and encapsulation



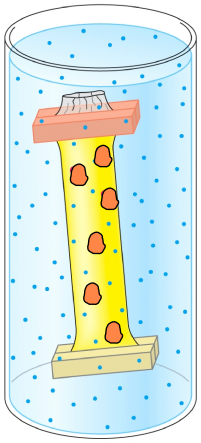
pH < pI of protein



pH > pI of protein



cross-linked polyacrylamide gel



Perhaps the simplest form of encapsulation is separate the enzyme from the bulk solution with a semipermeable membrane (just like in dialysis)

http://www.lsbu.ac.uk/biology/enztech/immethod.html

- Adsorption of enzymes onto insoluble supports is a very simple method of wide applicability and capable of high enzyme loading (about one gram per gram of matrix).
- Simply mixing the enzyme with a suitable adsorbent, under appropriate conditions of pH and ionic strength, followed, after a sufficient incubation period, by washing off loosely bound and unbound enzyme will produce the immobilized enzyme in a directly usable form. The driving force causing this binding is usually due to a combination of hydrophobic effects and the formation of several salt bridges per enzyme molecule.
- Examples of suitable adsorbents are ion-exchange matrices, porous carbon, clays, hydrous metal oxides, glasses and polymeric aromatic resins.
- Ion-exchange matrices, although more expensive than these other supports, may be used economically due to the ease with which they may be regenerated when their bound enzyme has come to the end of its active life; a process which may simply involve washing off the used enzyme with concentrated salt solutions and re-suspending the ion exchanger in a solution of active enzyme.
- Entrapment of enzymes within gels or fibres is a convenient method for use in processes involving low molecular weight substrates and products. Amounts in excess of 1 g of enzyme per gram of gel or fibre may be entrapped.
- Because large molecules have trouble approaching the catalytic sites of entrapped enzymes it is not practical to entrap enzymes with high molecular weight substrates.
- The enzyme is trapped by performing a polymerization reaction with acrylamide (CH_2=CH-CO-NH_2) and bisacrylamide (H_2N-CO-CH=CH-CH=CH-CO-NH_2) to form a gel. The polymer forms around the enzyme and the gel pores are too small for the enzyme to diffuse out. This approach does not covalently modify the enzyme. We will learn more about the formation of polyacrylamide gels in the section on electrophoresis.
- Encapsulation of enzymes may be achieved by a number of quite different methods, all of which depend on the semipermeable nature of the membrane. This must confine the enzyme whilst allowing free passage for the reaction products and, in most configurations, the substrates.
- The simplest of these methods is achieved by placing the enzyme on one side of the semipermeable membrane whilst the reactant and product stream is present on the other side.This is basically the same as dialysis, though done on an analytical scale.