BIOSENOSRS

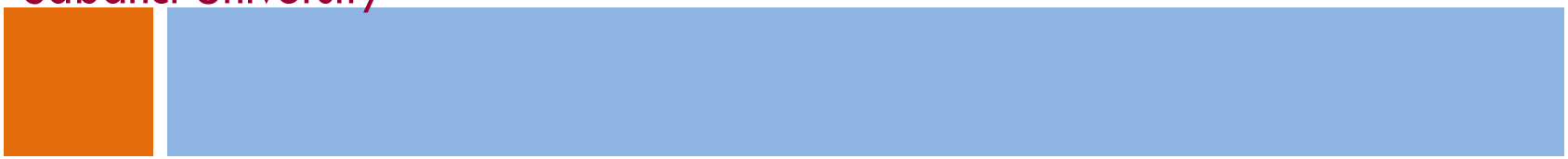
BIO 580

Modification of sensor surfaces and immobilization techniques - theory part 1

WEEK-11

Fall Semester

Faculty: Dr. Javed H. Niazi KM
Faculty of Engineering & Natural Sciences
Sabanci University



Topics that will be covered in the course

- ❑ History of biosensor development, applications and requirements of biosensors and classification
- ❑ Principles of molecular recognition and transduction signal acquisition
  - ✓Sources of Biological Recognition elements – enzymes/proteins, ssDNAs, antibody and Others
  - ✓Design considerations for use of recognition elements in biosensors
  - ✓Modeling of reactions for various biosensor applications- electrochemical, optical, piezoelectric, colorimetric, fluorometric and others.
- ❑ Modification of sensor surfaces and immobilization techniques
  - ✓Covalent modification of surfaces using surface chemistry
  - ✓Self Assembled Monolayers (SAM) and adsorptions
  - ✓Other ways to immobilize biological macromolecules on various solid surfaces
- ❑ Detection methods and Physical Sensors
  - ✓Electrodes/transducers – electrochemical (amperometric, potentiometric, and conductimetric transductions)
  - ✓Other sensors - for e.g., optical sensors (colorimetric/fluorimetric/luminometric sensors), Surface Plasmon Resonance (SPR) sensors, and piezoelectric resonators.
- ❑ Fabrication of biosensors
  - ✓Miniaturization-application of nano-materials, nanoparticles, carbon nanotubes (CNTs) and others
  - ✓Biocompatibility – stability, reproducibility and repeatability of biomolecules on transducer surfaces
- ❑ Data acquisition, statistical and error analysis
  - ✓Inter and Intra-assays and Coefficient of variation (CV)
  - ✓Signal to noise ratio
  - ✓Normalization/optimization and signal retrieval
- ❑ Examples of commercial biosensors

Chemical modifications of surfaces and their applications

Chemical modification - two major reasons

1. to attach selective groups (binding sites or catalysts) to the sensor surface in order to recognize target species in the sample.

2. to increase the selectivity of the sensor by reduction of interfacerences arising from non-specific interactions.

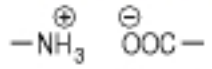
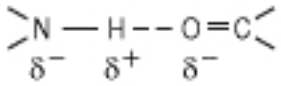
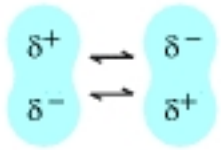
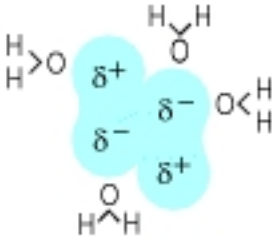
Surface modification - Covalent and non-covalent strategies

1. Covalent approaches- a chemical bond is made between the surface and the attached species - irreversible process.

2. Non-covalent approaches- weaker, non-bonding, interactions b/w the surface and the adsorbed species are utilized - reversible
Eg., charge-charge, charge-dipole, dipole-dipole, dipole-induced dipole, and induced dipole-induced dipole interactions (van der Waals interactions)

-these are less surface specfic than the covalent approaches - can be more readily achieved

Non covalent forces

Noncovalent forces	Origin	
Electrostatic forces	Attraction between opposite charges	
Hydrogen bonds	Hydrogen shared between electronegative atoms (N,O)	
Van der Waals forces	Fluctuations in electron clouds around molecules oppositely polarize neighboring atoms	
Hydrophobic forces	Hydrophobic groups interact unfavorably with water and tend to pack together to exclude water molecules. The attraction also involves van der Waals forces	

Covalent modification of sensor surface

The formation of a bond to some functional group on the surface.

The different surface modifications are as follows:

1. Reactive organo-silane surfaces are widely used sensor surfaces for modifcation

Metal oxide surfaces were silanized followed by linking chemical groups

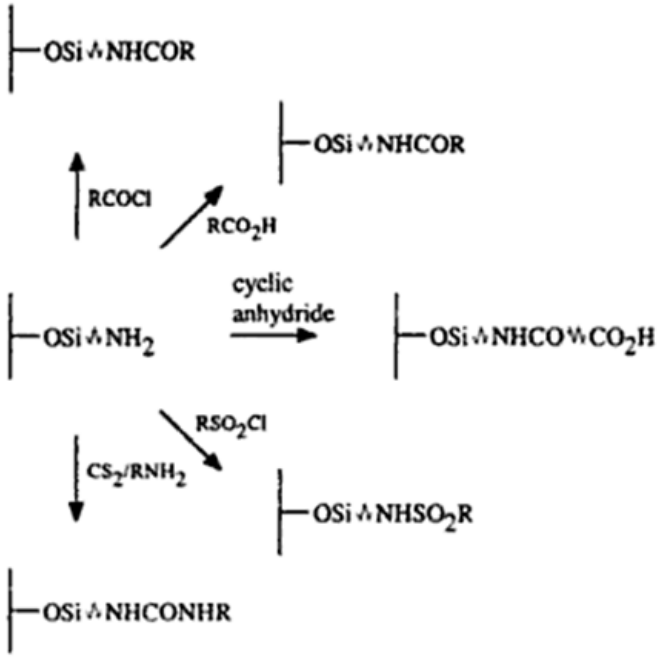


Figure 6.2 Development of surface chemistry starting from an alkylamine silane-modified surface. ∿ represents a generalized linking chemical group such as an alkyl chain.

Eg., Organo-silanization

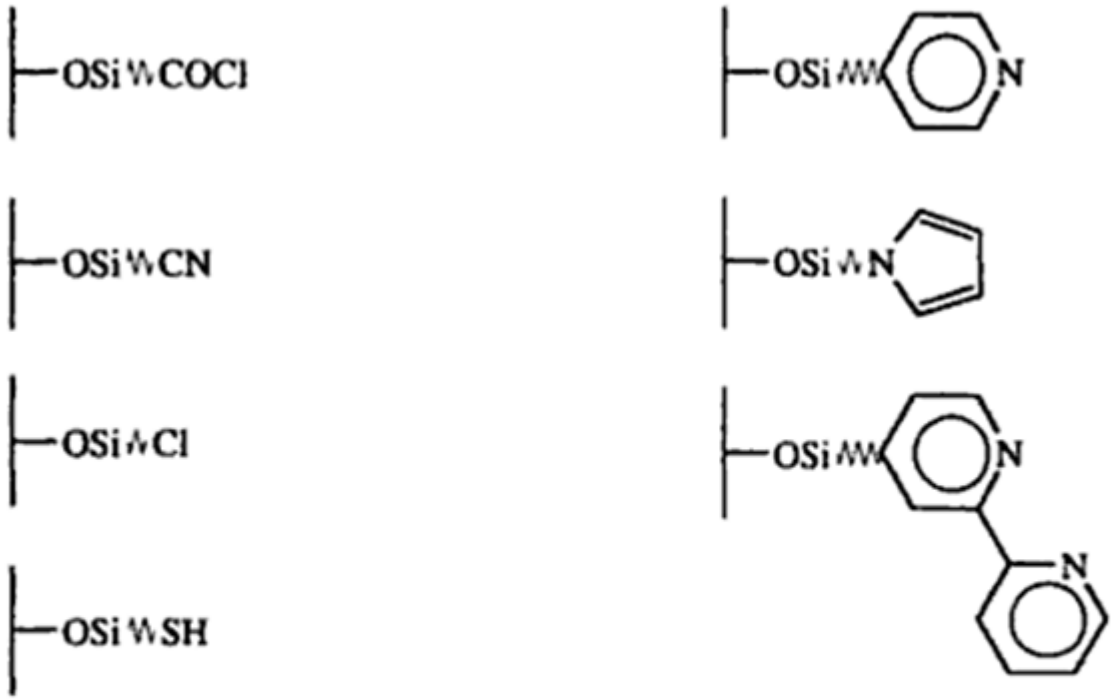


Figure 6.3 Some examples of functional groups which have been attached to surfaces by use of the corresponding reactive organosilanes. $\sim\!\!\wedge\!\!\vee\!\!\sim$ represents a generalized linking chemical group such as an alkyl chain.

Cyanuric chloride activation on hydroxyl surfaces

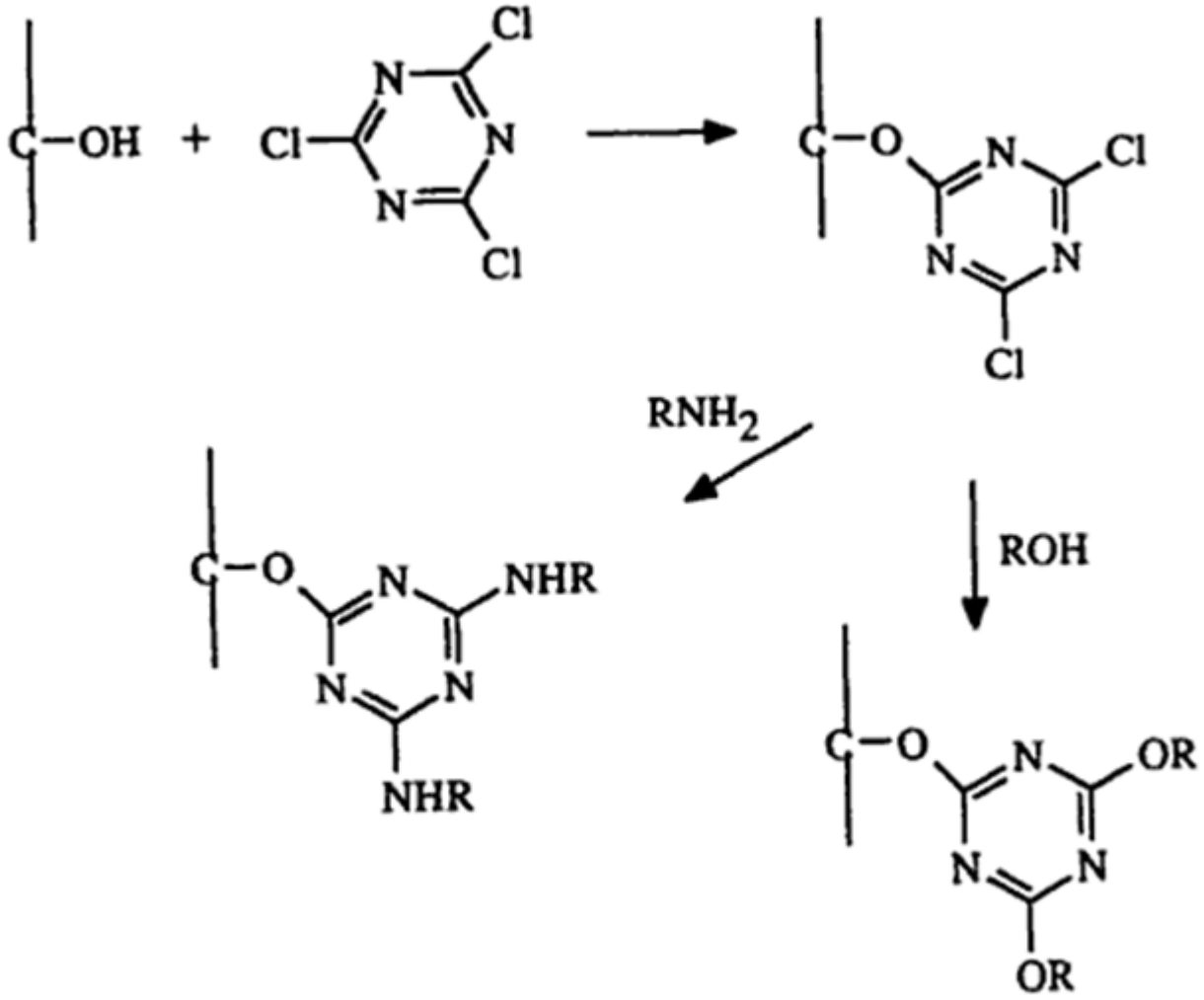


Figure 6.5 The use of cyanuric chloride to couple to hydroxyl groups on surfaces.

Covalent attachment on acrylic surfaces (plastic)

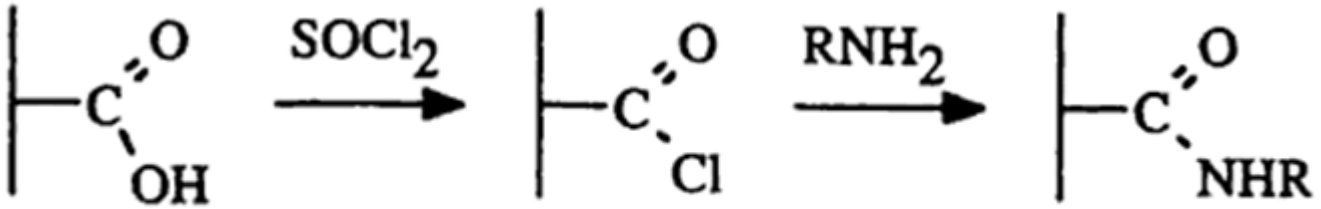


Figure 6.6 The use of thionyl chloride ($SOCl_2$) to activate polyacrylic acid surfaces.

Activation of carboxy surfaces by DCC

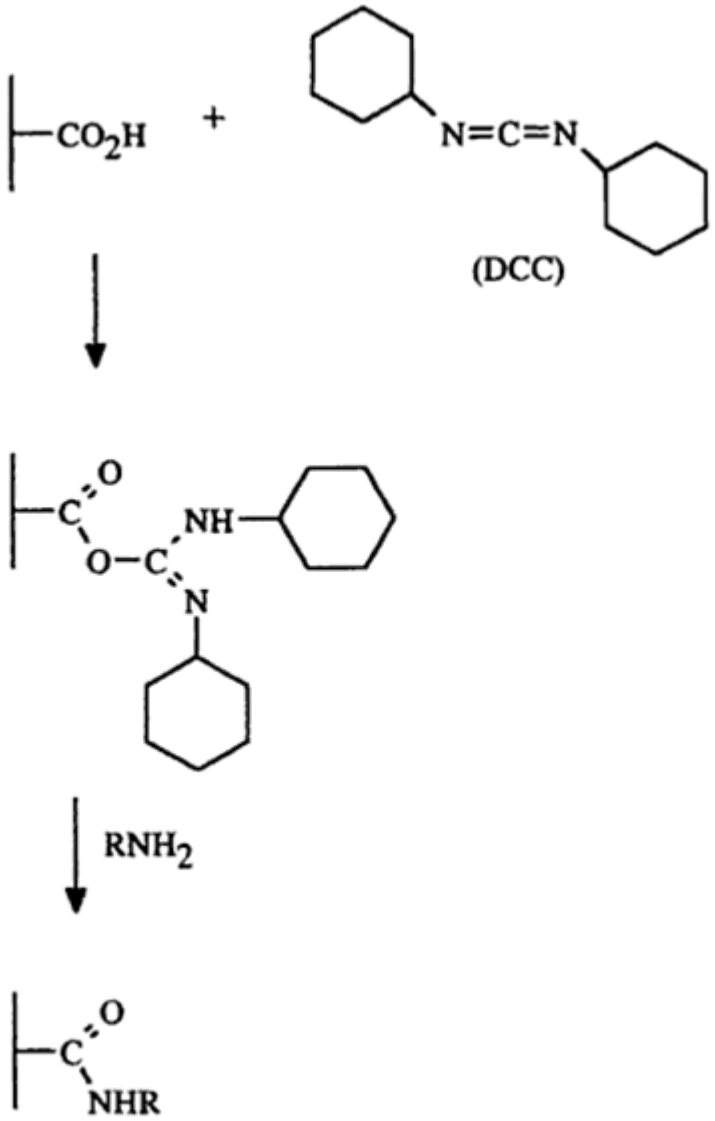
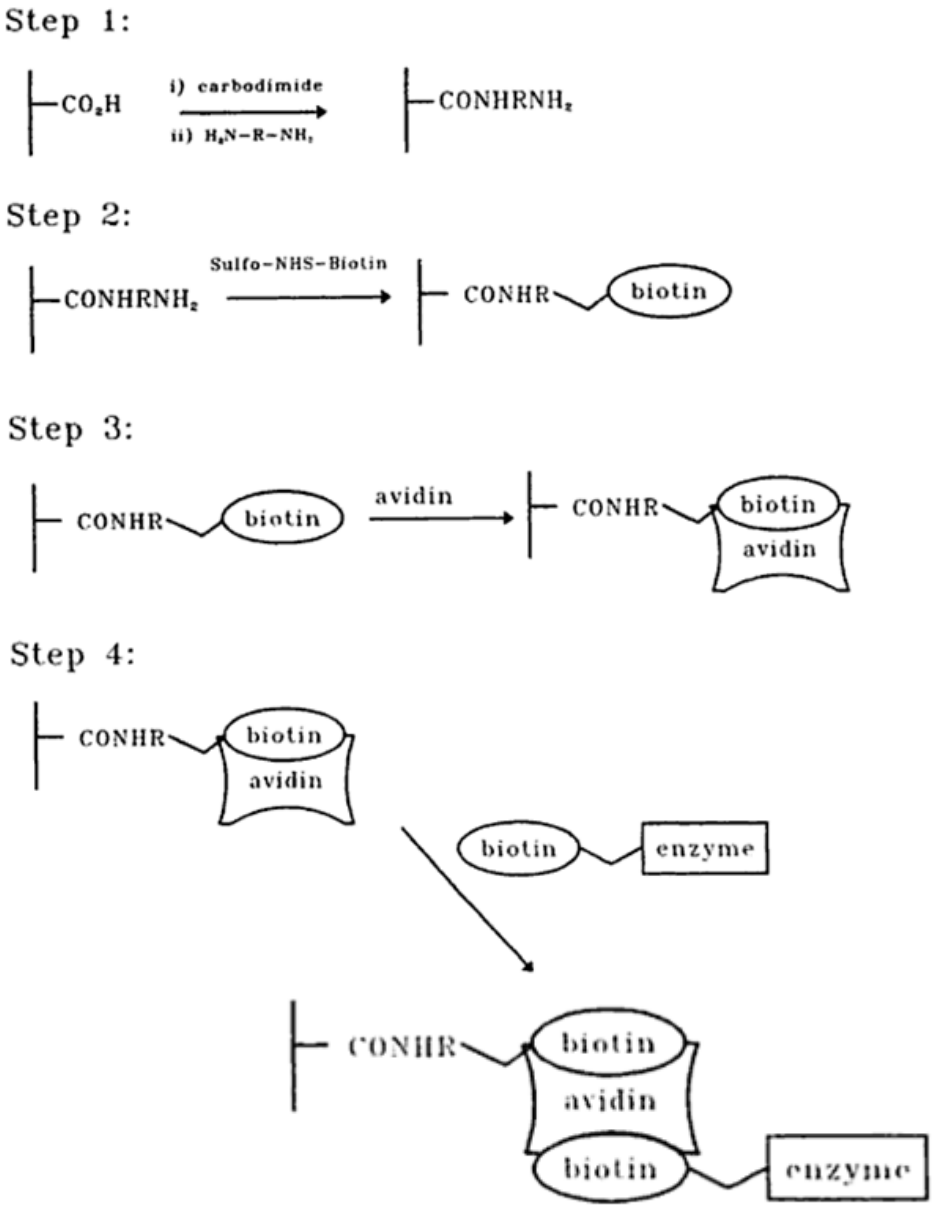


Figure 6.7 Activation of an oxidized carbon surface using a carbodiimide (in this case DCC).

Eg., Modification of surfaces (sensor/biomolecule) through carbodiimide, avidin-biotin interactions



EDC-NHS coupling (covalent)

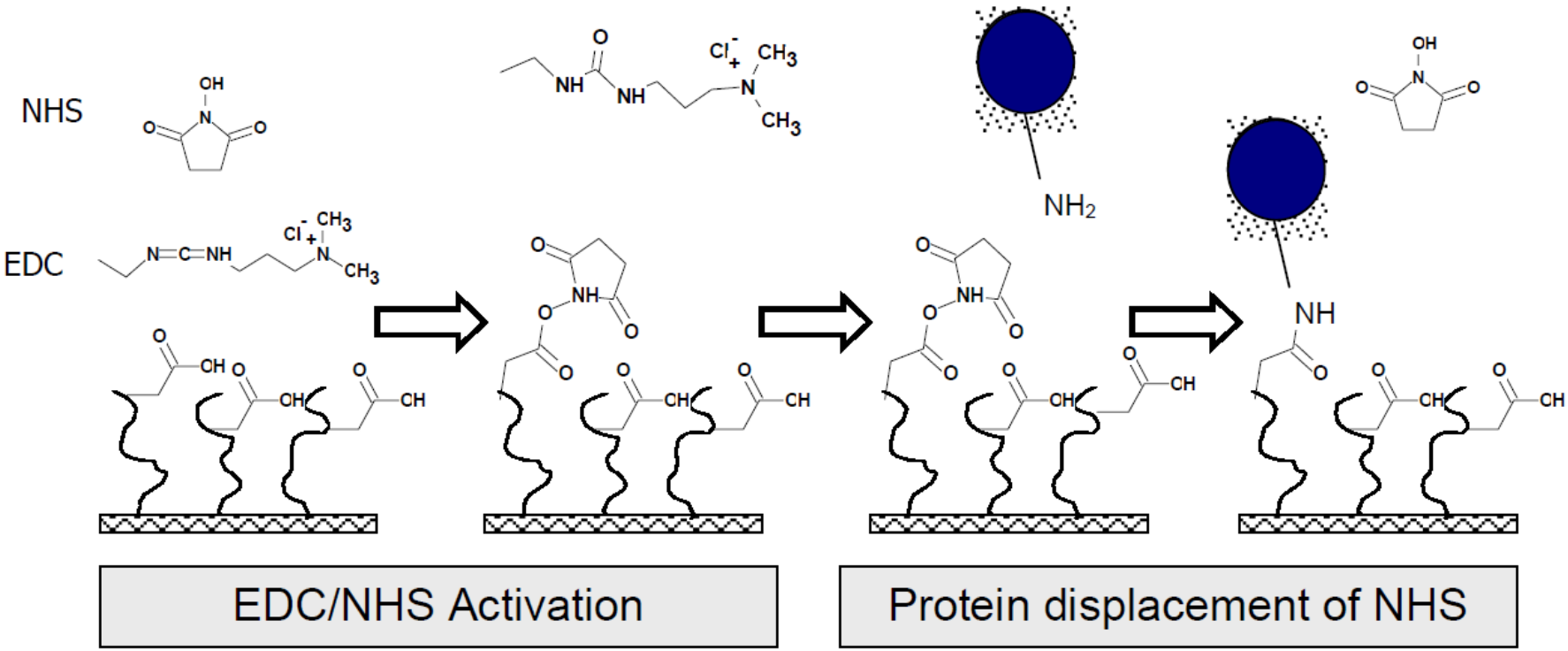
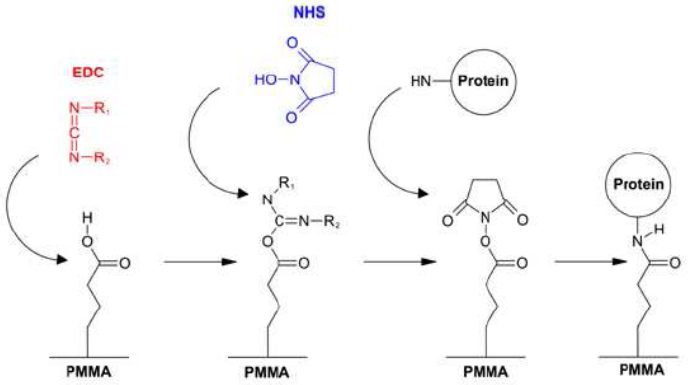


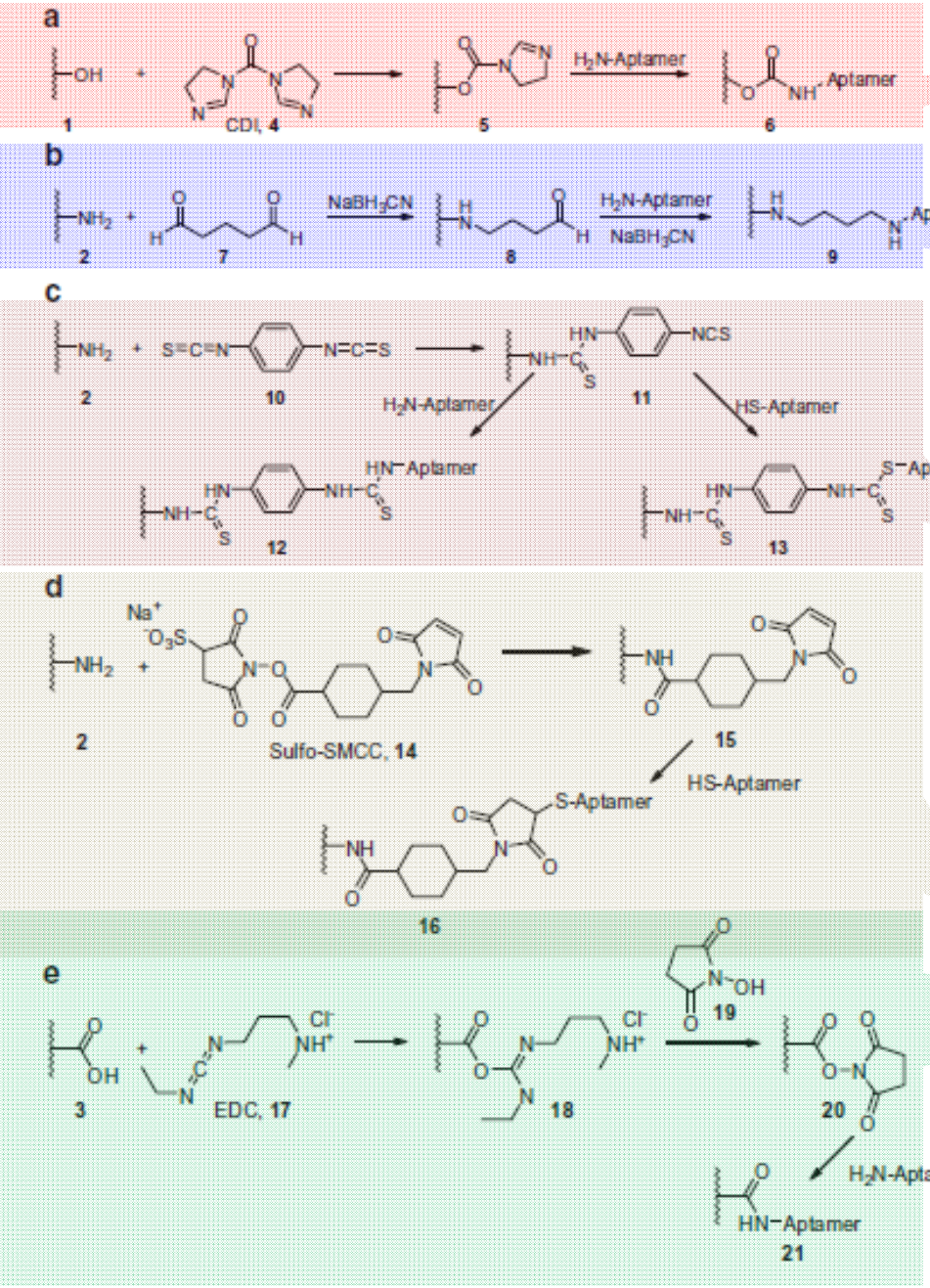
Figure 1 Reaction scheme for EDC/NHS mediated amine coupling to a CM Dextran cuvette.

covalent coupling of proteins and nucleic acids

- EDC/NHS affinity ligand coupling chemistry
  - EDC couples COC/PMMA with primary aminogroups of proteins
  - NHS enhances coupling, stabilizes intermediate stage



EDC/NHS protein coupling



a. -OH surfaces are first modified with carbonyldiimidazole (CDI) to form reactive intermediate- forms a stable carbamate bond to an amino terminated biomolecule

b. -NH2 gp can be modified with glutaraldehyde which forms imine bond (Schiff's base) with an aldehyde, leaving the other aldehyde free for repeating this chemistry with an amino-terminated molecule

c. Symmetric diisothiocyanates have also been used as bifunctional linkers for attachment of amine-functionalized surfaces to either thiol-terminated or amine-terminated molecules

d. Heterobifunctional linker sulfosuccinimidyl-4-(N-maleimidomethyl)-cyclohexane-1-carboxylate, which first reacts with surface-bound -NH2 through displacement of the NHS, subsequently, a thol-tethered molecule can be reacted with the pendant maleimide gp. To form surface-conjugated molecules

e. EDC/NHS reaction - the most common ones

Scheme 3 General modification of silanoxide surfaces for subsequent aptamer conjugation

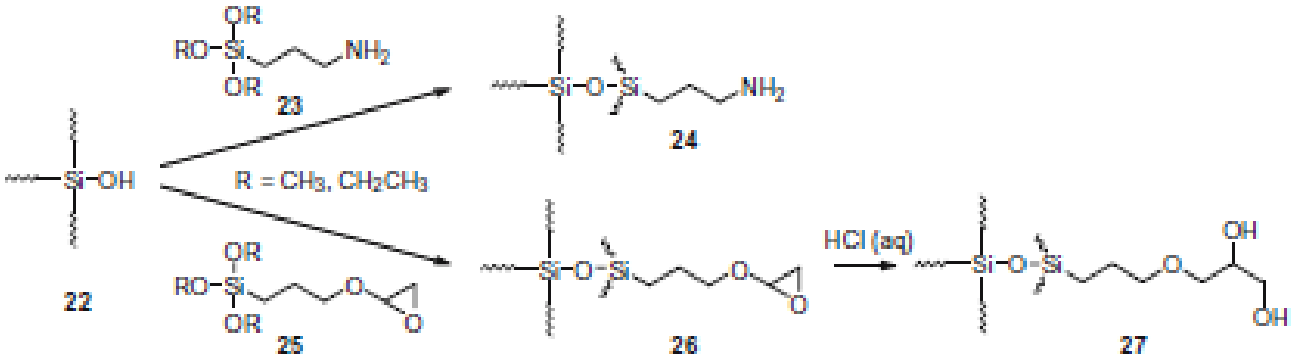


Table 1. Reactive Crosslinker Groups and Their Functional Group Targets

Reactive Group	Functional Group	Reactive Group	Functional Group
Aryl Azide	Non-selective (or primary amine)	Maleimide	Sulfhydryl
Carbodiimide	Amine/Carboxyl	NHS-ester	Amine
Hydrazide	Carbohydrate (oxidized)	PFP-ester	Amine
Hydroxymethyl Phosphine	Amine	Psoralen	Thymine (photoreactive intercalator)
Imidoester	Amine	Pyridyl Disulfide	Sulfhydryl
Isocyanate	Hydroxyl (non-aqueous)	Vinyl Sulfone	Sulfhydryl, amine, hydroxyl

How to Choose a Crosslinker

Crosslinkers are selected on the basis of their chemical reactivities (i.e., specificity for particular functional groups) and compatibility of the reaction with the application. The best crosslinker to use for a specific application must be determined empirically. Crosslinkers are chosen based on the following characteristics:

- Chemical specificity
- Spacer arm length
- Reagent water-solubility and cell membrane permeability
- Same (homobifunctional) or different (heterobifunctional) reactive groups
- Thermoreactive or photoreactive groups
- Reagent crosslinks cleavable or not
- Reagent contains moieties that can be radiolabeled or tagged with another label

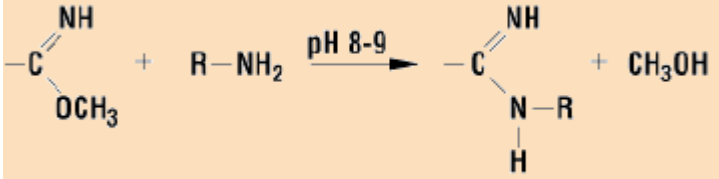


Figure 2. Imidoester reaction scheme.

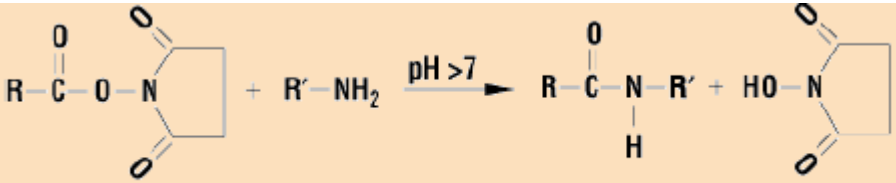


Figure 3. NHS-ester reaction scheme.

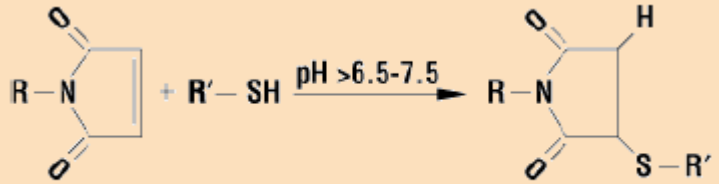


Figure 4. Maleimide reaction scheme.

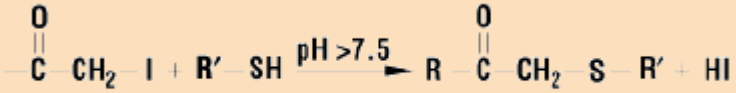


Figure 5. Active halogen reaction scheme.

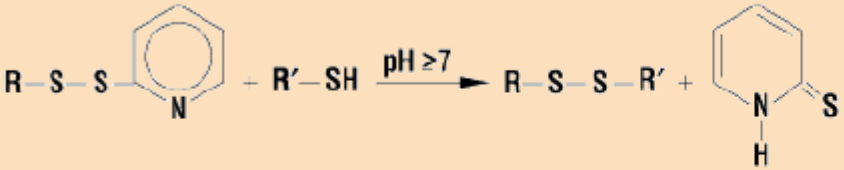
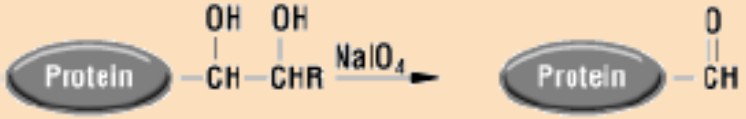


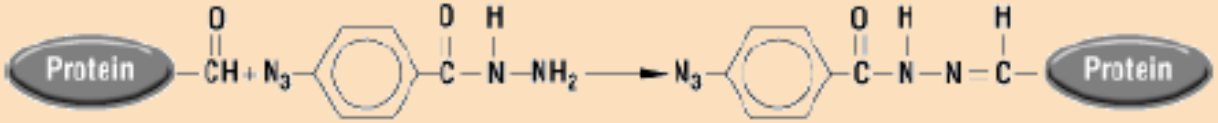
Figure 6. Pyridyl disulfide reaction scheme. Reaction efficiency can be monitored by determining the concentration of the released pyridine-2-thione by measuring the absorbance at 343 nm (molar extinction coefficient at 343 nm = 8.08 x 10^3 M^{-1} cm^{-1}).

Hydrazides

Carbonyls (aldehydes and ketones) react with hydrazides and amines at pH 5 - 7. The reaction with hydrazides is faster than with amines, making them useful for site-specific crosslinking. Carbonyls do not readily exist in proteins; however, mild oxidation of sugar glycols using sodium *meta*-periodate will convert vicinal hydroxyls to aldehydes or ketones (Figure 7). The oxidation is performed in the dark at 0-4°C to prevent side reactions. Subsequent reaction with hydrazides results in formation of a hydrazone bond. Carbohydrate modification is particularly useful for antibodies in which the carbohydrate is located in the Fc region away from binding sites. At 1 mM $NaIO_4$ and a temperature of 0°C, the oxidation is restricted to sialic acid residues. At concentrations of 6-10 mM periodate, other carbohydrates in proteins (including antibodies) will be targeted.



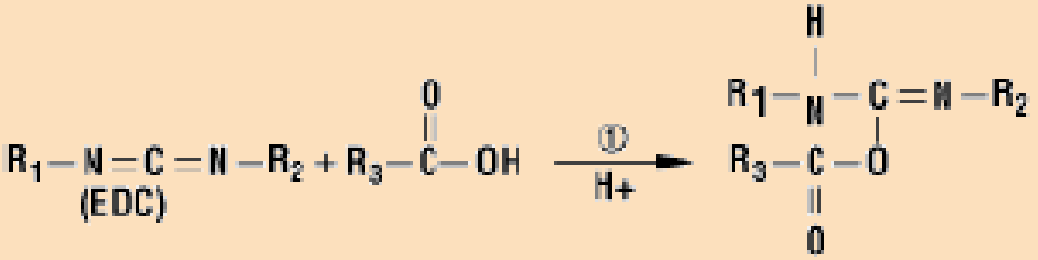
The oxidation of a Protein Carbohydrate (*cis*-diol) to an aldehyde.



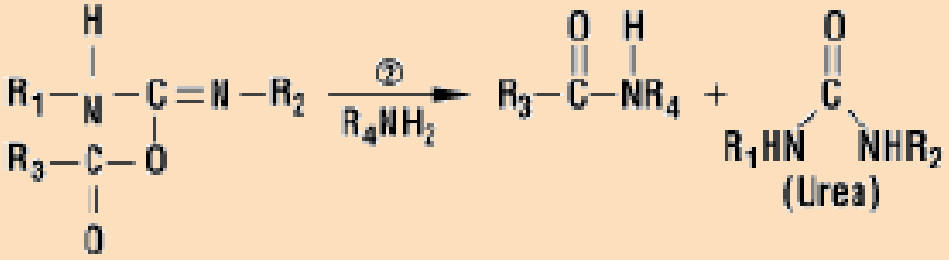
ABH, or Azidobenzoyl Hydrazide, reacts with the aldehyde on the protein to form an arylazide activated protein.

Figure 7. Hydrazide reaction scheme.

Carbodiimides



EDC reacts with carboxylic acid group and activates the carboxyl group, allowing it to be coupled to the amino group (R_4NH_2) in the reaction mixture.



EDC is released as a soluble urea derivative after displacement by the nucleophile, R_4NH_2.

Figure 8. EDC coupling reaction scheme.

Aryl azide cross-linkers

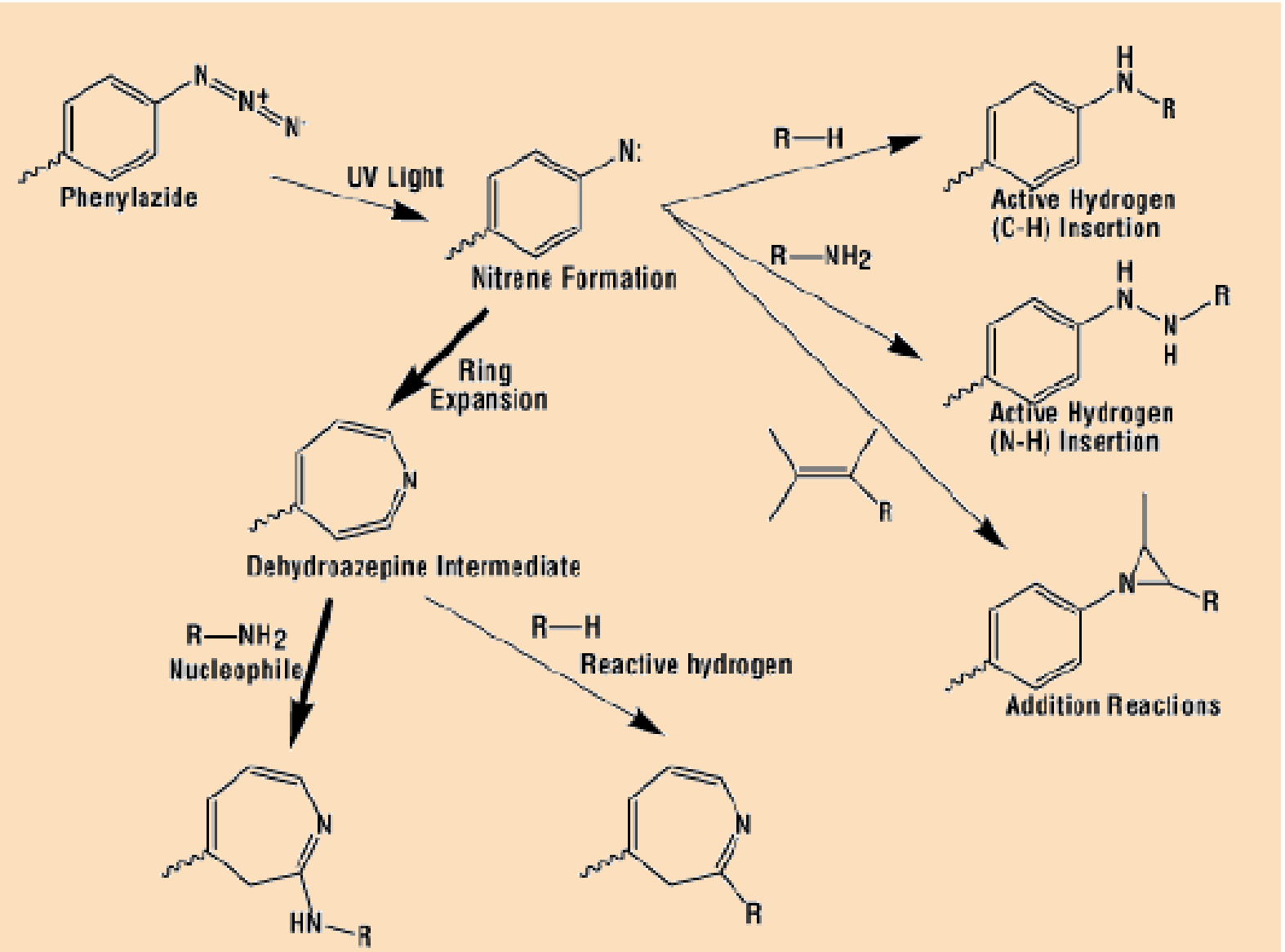


Figure 10. Possible reaction pathways of aryl azide crosslinkers.

SELF ASSEMBLED monolayer (SAM)

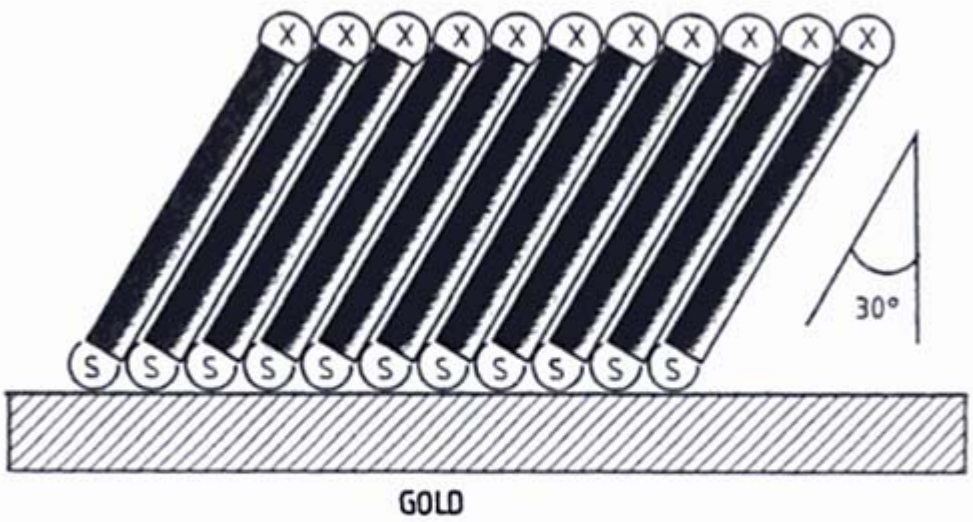
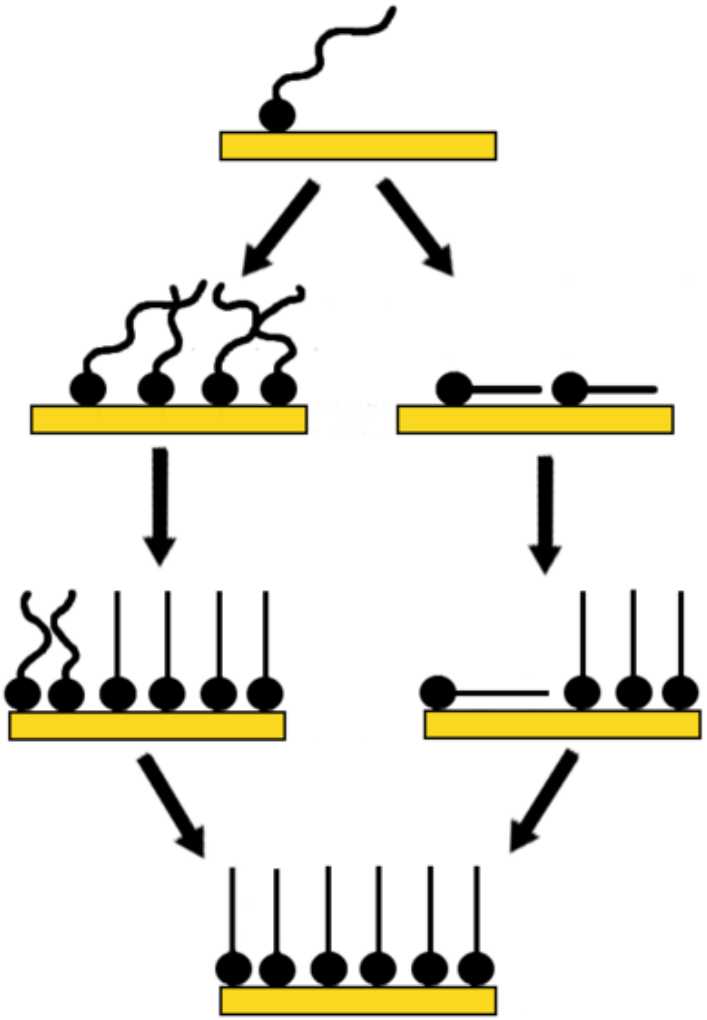
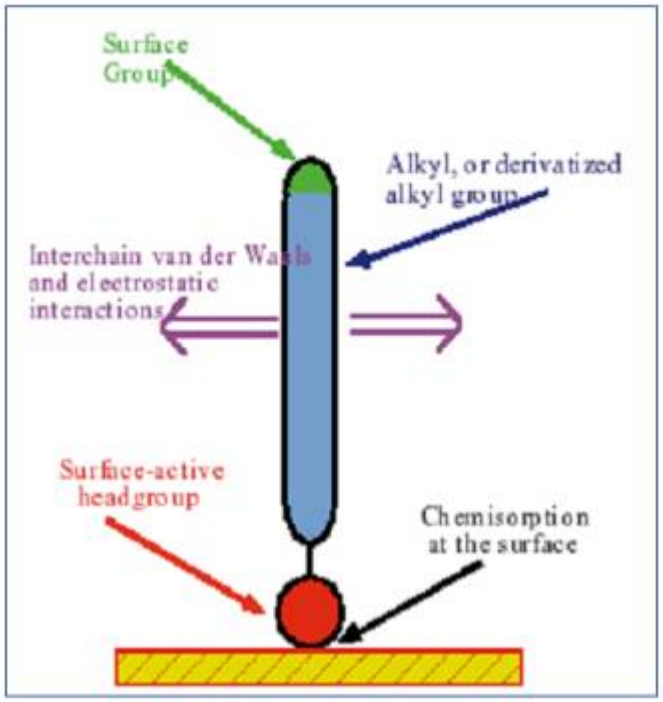
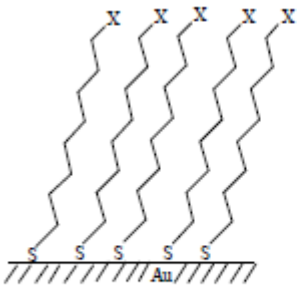


Figure 6.12 The structure of self-assembled alkylthiol films at a gold surface. In general the molecules form close-packed layers with the polymethylene chains tilted at about 30° from the vertical. The interfacial properties of the films are determined by the end groups, X, of the chains. (Adapted from [50].)

SAM







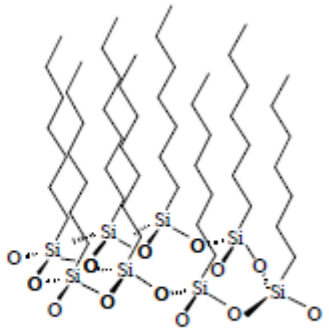
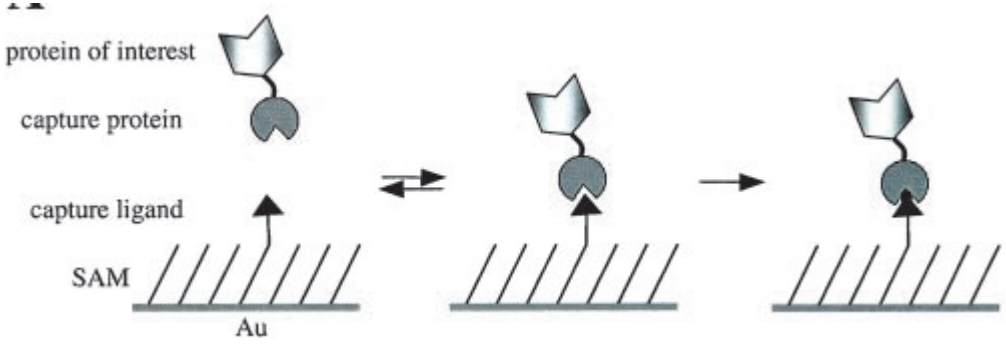


Figure 1. Gold thiolate monolayer and alkylsilane monolayer


protein of interest
capture protein
capture ligand
SAM
Au

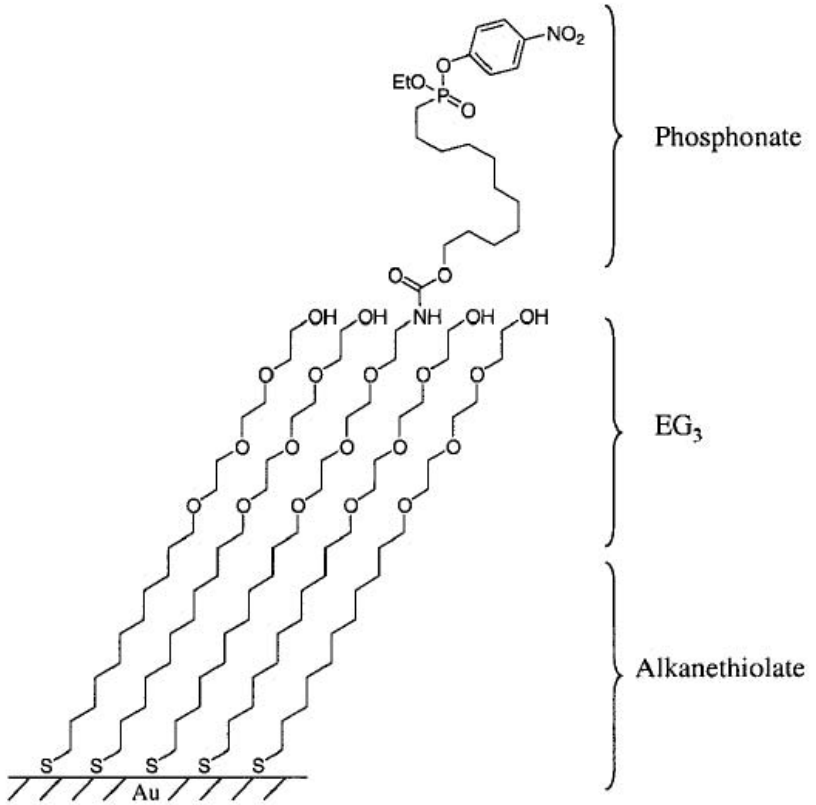


Fig. 3. Structure of a monolayer designed to covalently immobilize cutinase. The phosphonate capture ligand is present at a density of ≈1% of total alkanethiolate. After cutinase binds to the phosphonate capture ligand, an active-site serine residue displaces *p*-nitrophenol, resulting in irreversible covalent immobilization of the protein. The tri(ethylene glycol) groups prevent nonspecific protein adsorption to the monolayer.

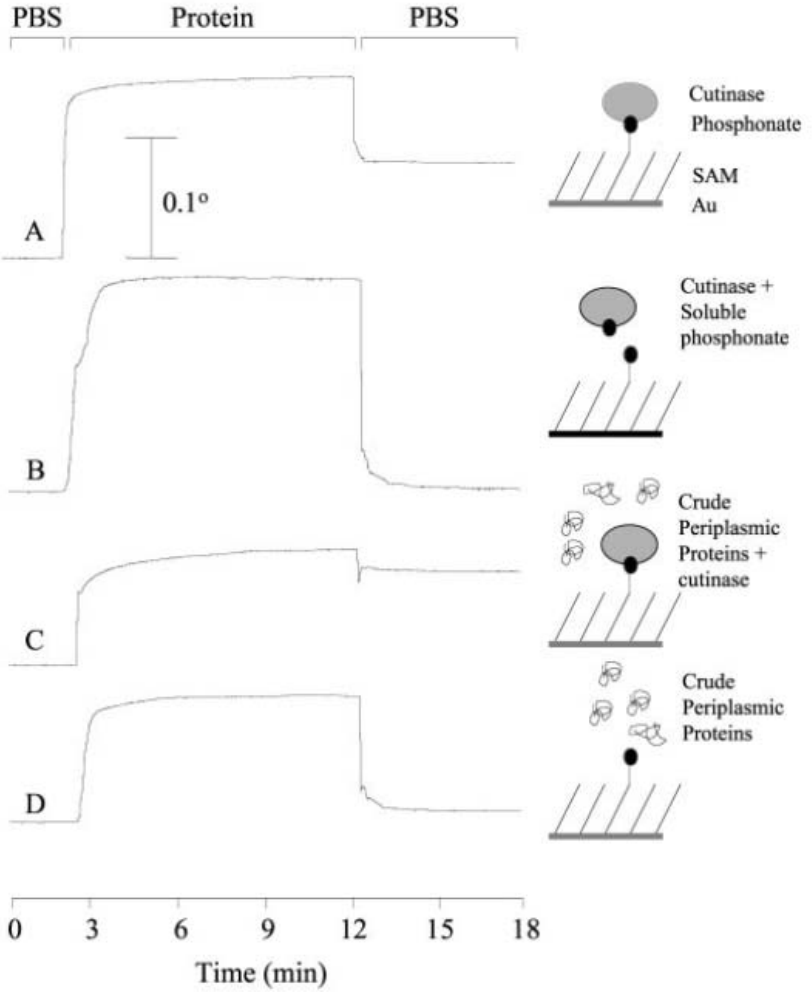
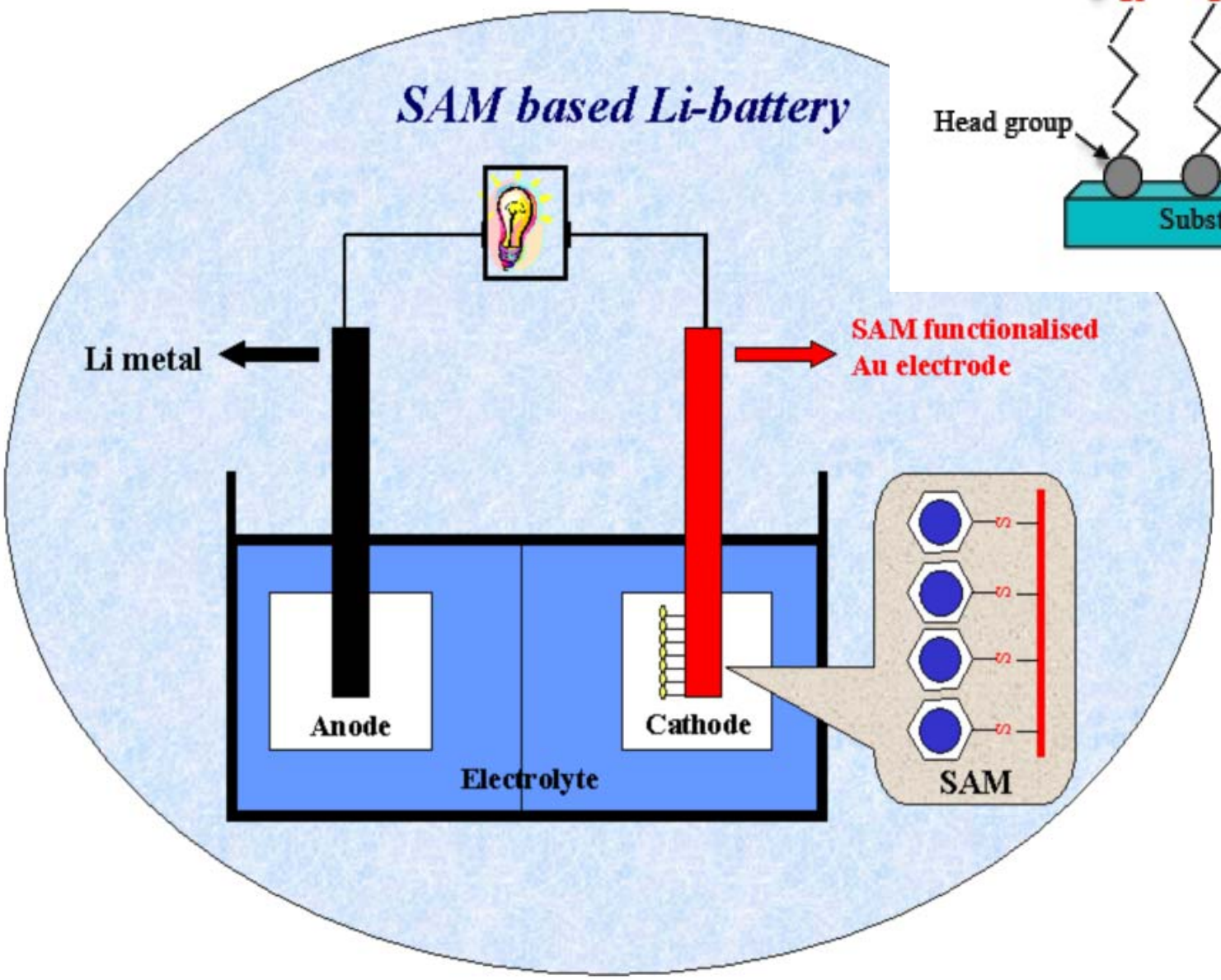
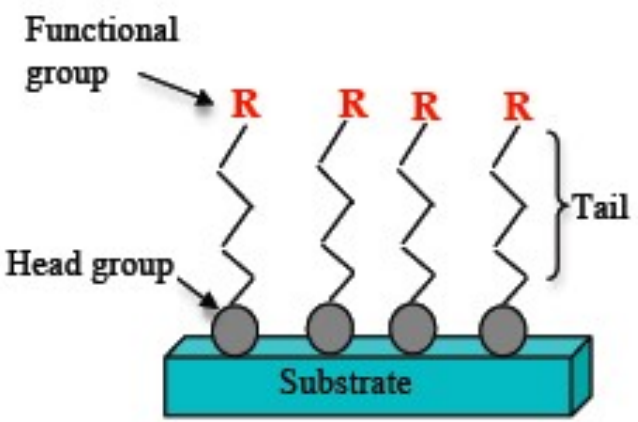
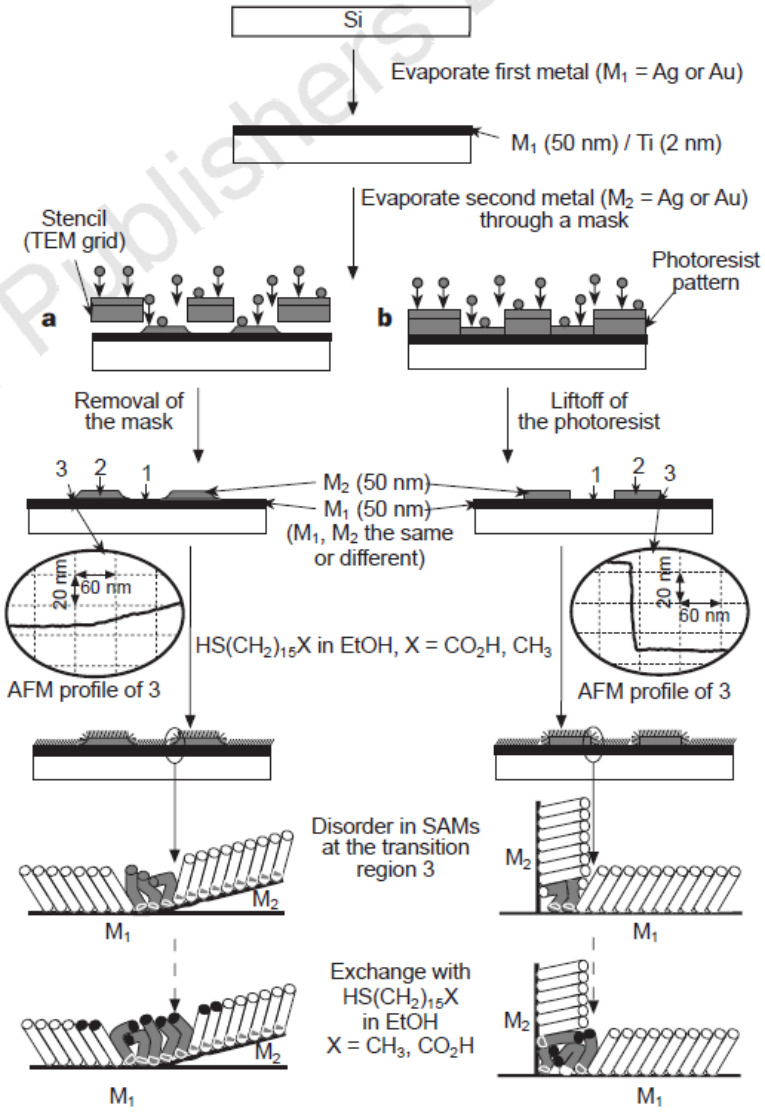


Fig. 4. SPR spectroscopy data showing the biospecific and irreversible immobilization of cutinase to the phosphonate-tethered SAM shown in Fig. 3. Conditions are described in *Materials and Methods*. The change in resonance angle ($\Delta\theta$) is plotted on the vertical axis. The scale bar applies to all data, which are offset for clarity. (*A*) Cutinase (25 μM) was irreversibly immobilized to the monolayer. (*B*) Incubation of cutinase with four equivalents of soluble inhibitor **1** before immobilization completely inhibited immobilization of the protein. (*C*) Cutinase within crude *E. coli* periplasmic lysate also underwent efficient immobilization, demonstrating that the protein does not need to be purified before immobilization. (*D*) An analogous experiment using periplasnstrates

www.pnas.org/cgi/doi/10.1073/pnas.072685299


SAM based Li-battery
Li metal
SAM functionalised
Au electrode
Anode
Cathode
Electrolyte
SAM


Functional
group
R
R
R
R
Tail
Head group
Substrate



Controlling local disorder in self-assembled monolayers by patterning the topography of their metallic supports

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Micropatterning is a powerful method for controlling surface properties, with applications from cell biology to electronics[1–8]. Self-assembled monolayers (SAMs) of alkanethiolates on gold and

Soft-Lithography-Mediated Submicrometer Patterning of Self-Assembled Monolayer of Hemoglobin on ITO Surfaces

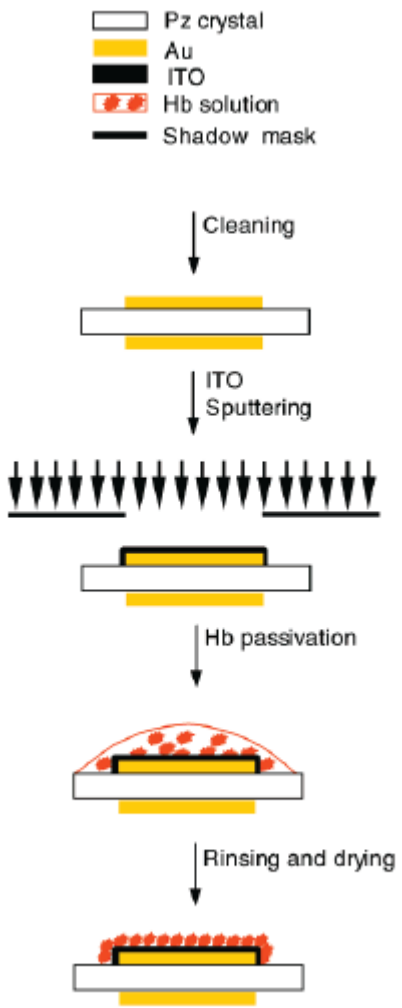


Figure 1. Process flow for preparation of an ultrathin layer of ITO on Au electrode of a Pz crystal for Hb passivation.

ITO-Indium tin oxide

Langmuir **2000**, *16*, 5221−5226

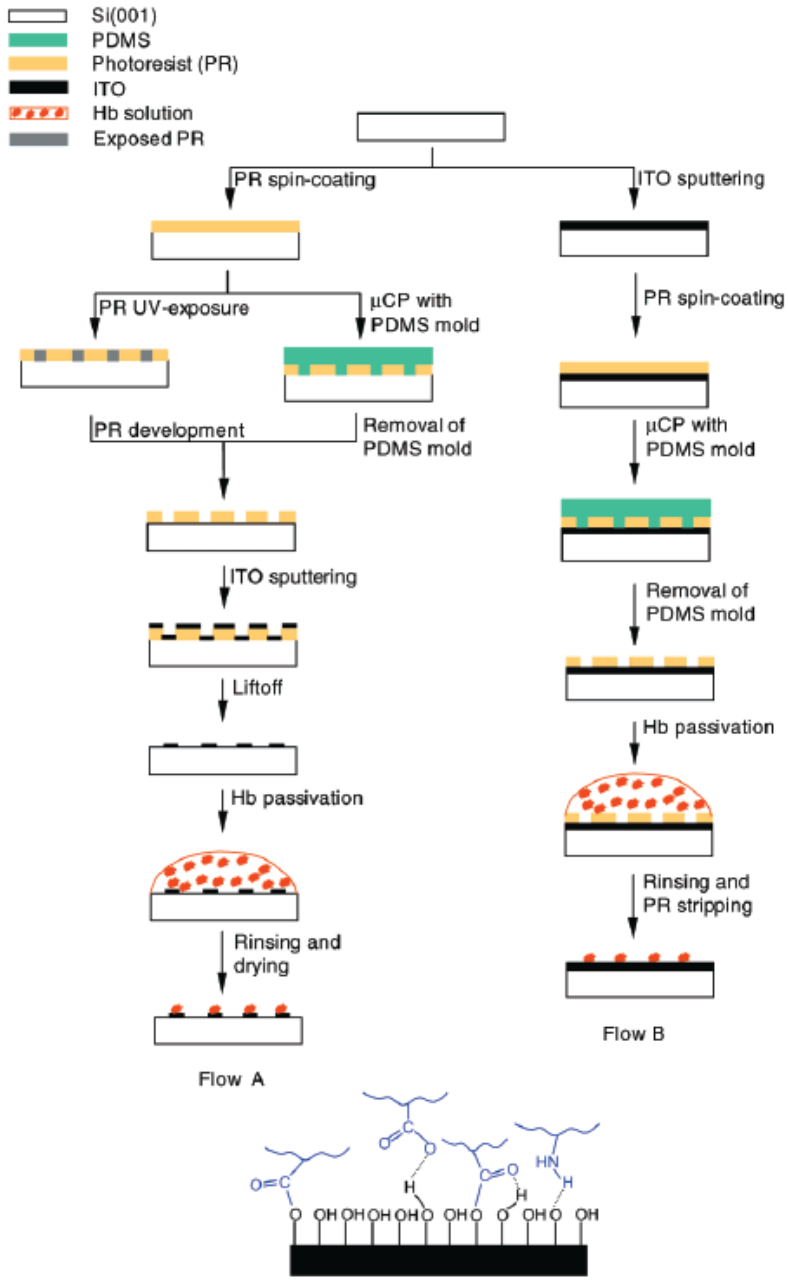


Figure 2. Two process flows for submicrometer patterning of Hb SAM on ITO/Si(001) surfaces. Schematic representations of proposed ester bonds and hydrogen bonding are also shown.

Immobilization Technology

Must be oriented in a specific direction - immobilization technology

Five major immobilization methods used in the preparation of Chemical and bio-sensors.

1. Covalent binding - attachment of the active component to the transducer surface using a chemical reaction such as peptide bond formation or linkage to activated surface groups (thiol, epoxy, amino, carboxylic, etc)

2. Entrapment - physical trapping of the active component into a film or coating.

3. Cross-linking - similar to entrapment, only a polymerization agent (such as gluteraldehyde) is used to provide additional chemical linkages between the active, entrapped component and the film or coating.

4. Adsorption - association of the active component with a film or coating through hydrophobic, hydrophilic, and/or ionic interactions.

5. Biological binding - association of an active biomolecule to a film or coating through specific, biochemical binding

The **covalent binding** method is based on the binding of enzymes and water-insoluble carriers by covalent bonds. The functional groups that may take part in this binding are listed below:

Amino group	Carboxyl group	Sulfhydryl group,
Hydroxyl group	Imidazole group	Phenolic group
Thiol group	Threonine group	Indole group

Covalent attachment to a support matrix must involve only functional groups of the enzyme that are not essential for catalytic action. Higher activities result from prevention of inactivation reactions with amino acid residues of the active sites. A number of protective methods have been devised:

Covalent attachment of the enzyme in the presence of a competitive inhibitor or substrate.

1. A reversible, covalently linked enzyme-inhibitor complex.
2. A chemically modified soluble enzyme whose covalent linkage to the matrix is achieved by newly incorporated residues.
3. A zymogen precursor.

Zymogen: Any of a group of compounds that are inactive precursors of enzymes and require some change (such as the hydrolysis of a fragment that masks an active enzyme) to become active

Hence, covalent binding can be brought about by the following:

Diazotization : SUPPORT--N=N--ENZYME.

Amide bond formation : SUPPORT--CO-NH--ENZYME

Alkylation and Arylation: SUPPORT--CH_2-NH-ENZYME

SUPPORT--CH_2-S--ENZYME

Schiff's base formation : SUPPORT--CH=N--ENZYME

Amidation reaction : SUPPORT--CNH-NH--ENZYME

Thiol-Disulfide interchange : SUPPORT--S-S--ENZYME

UGI reaction

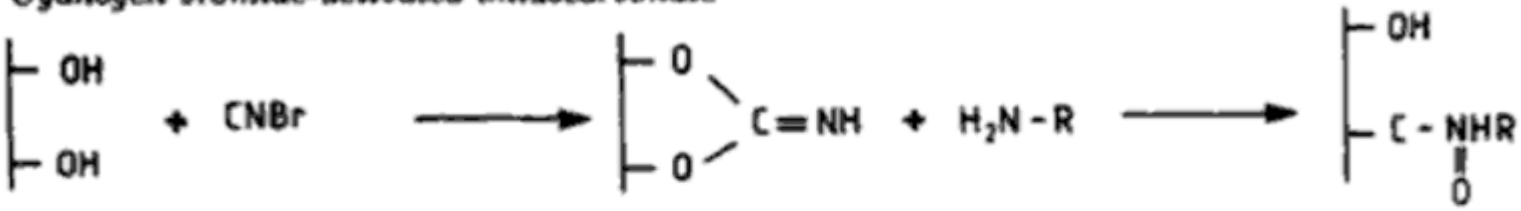
Gamma-Irradiation induced coupling

Carrier binding with bifunctional reagents :

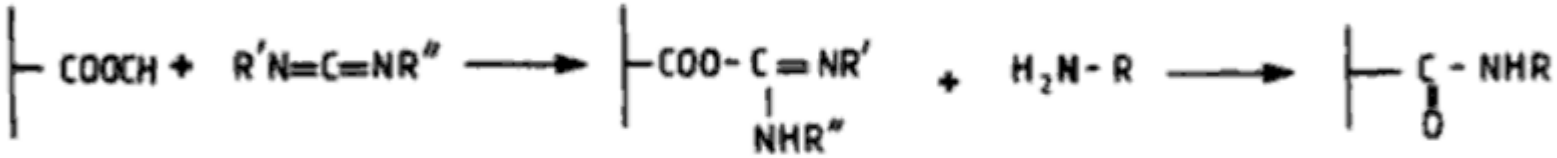
SUPPORT-O$(CH_2)_2$ N=CH$(CH_2)_3$ CH=N-ENZYME

Table 8.1 Commonly used methods for immobilization of active sensor components. (i) Peptide bond formation. (ii) Other immobilization methods.

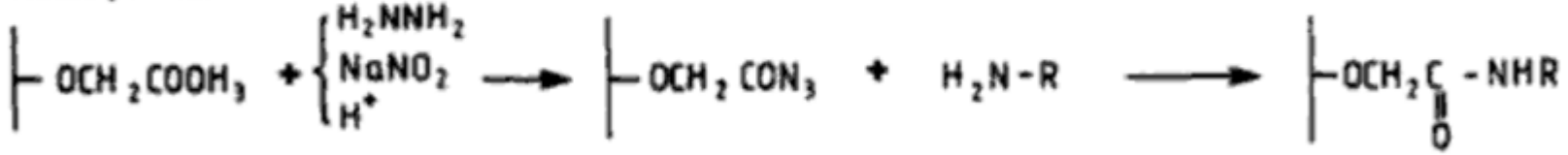
Cyanogen bromide-activated imidocarbonate



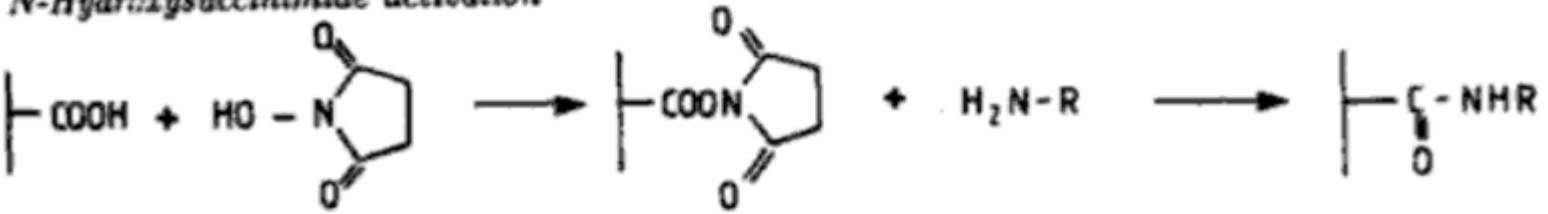
Carbodiimide condenstation



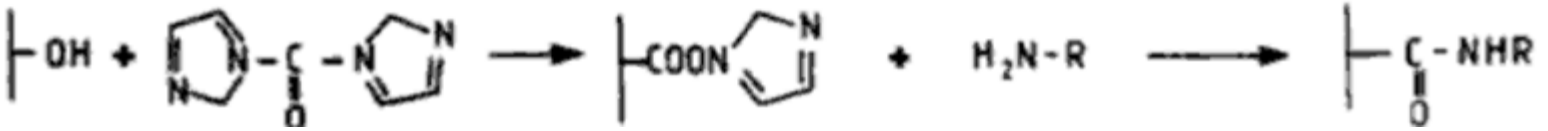
Azide formation



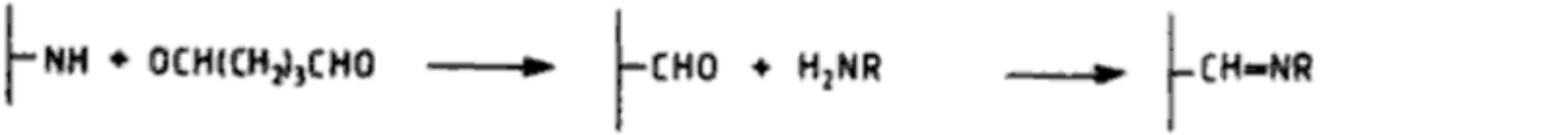
N-Hydroxysuccinimide activation



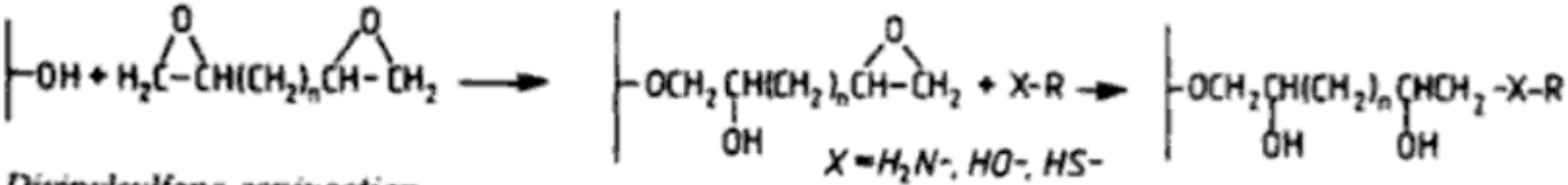
Carbonyldiimidazole activation



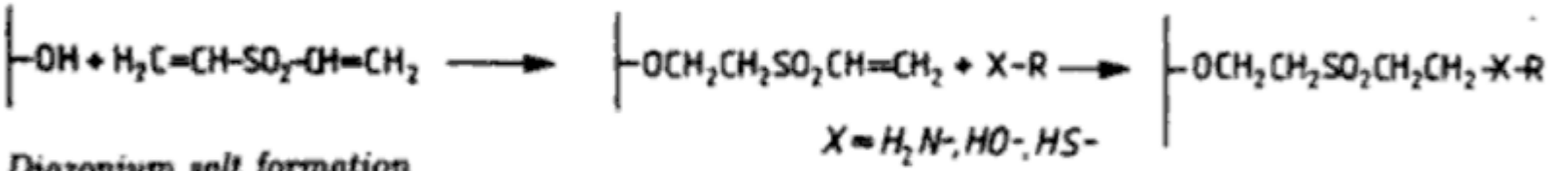
Glutaraldehyde coupling/cross-linking



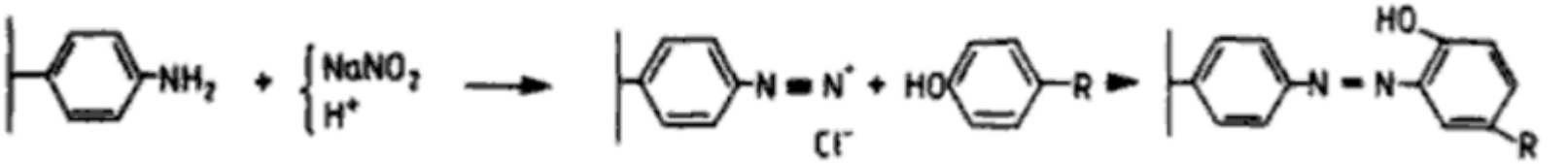
Epoxy (bisoxirane) activation



Divinylsulfone conjugation



Diazonium salt formation



Cyanuric chloride arylation

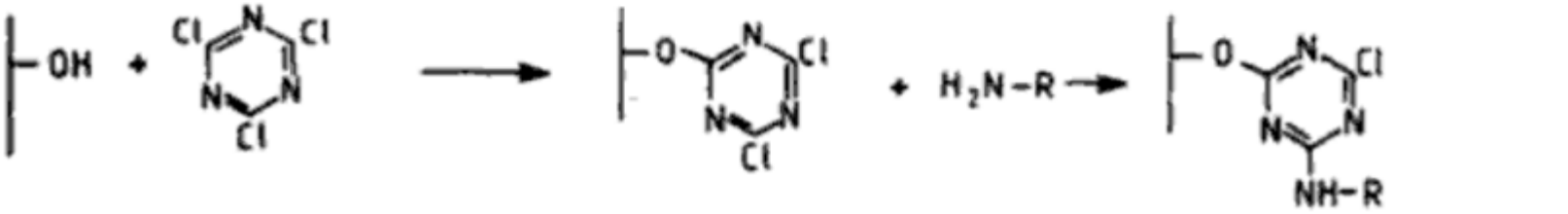


Table 1: Methods of Immobilization

Attachment	
Without support	Aggregation of floc for formation of cross-linking
With support	Co-valent binding
	Adsorption to ion-exchangers or inorganic
	Biofilm formation
Entrapment	Organic polymer
	Inorganic polymer
	Semi-permeable membrane

Glutaraldehyde- The surfaces (proteins) of microorganisms are linked with the surfaces of other microorganisms by aldehyde groups of glutaraldehyde

Immobilization of Microorganisms by Covalent Coupling

By these methods microorganisms are crossliked by chemical substances, e.g., by glutardialde aldehyde groups of glutardialdehyde. Yeast cells, for instant, react with free ε-amino group or N-term groups to double linkages of ε-,β-unsaturated oligomers, which are present in commercial aqueous solu and toxic influences on the microorganisms are very intensive. These reactions are only partly understoo

Table 2: Microbial cells covalently linked to various supports

Species	Support	Product
Actobacter	Metal hydroxides	Acetic acid
Aspergillus niger	Glycidyl methacrylate	Gluconic acid
Micrococcus luteus	CM-cellulose	Urocanic acid
Saccharomyces cerevisiae	Aminopropyl silica	Ethanol
Saccharomyces cerevisiae	Hydrocyalkyl methacrylate	Killer toxin
Saccharomyces cerevisiae	Cellulose	Ethanol
Zygosaccharomyces lactis	Hydroxyalkyl methacrylate	β-galactosidase

Table 3: Experimental studies of diffusion in immobilized cell systems and their associat

Cell Type	Immobilization	Solute
Saccharomycess cerevisiae	Ca-alginate	glucose
Baker's yeast	Ca-alginate	glucose
Ehrlich ascites tumor	agar, collagen	glucose
Zymomonas mobilis	k-carrageenan, Ca-alginate	glucose
Pseudomonas aeruginosa	Ca-alginate	glucose
Saccharomycess cerevisiae	Ca-alginate	glucose
Plant	Ca-alginate	sucrose
Baker's yeast	Ca-alginate	galactose
Zymomonas mobilis	Ca-alginate	galactose
Baker's yeast	Ca-alginate	lactose
Ehrlich ascites tumor	agar, collagen	lactic acid
Clostridium butyricum	polyarylamide, agar collagen	hydrogen
Escherichia coli	natural aggregates	nitrous oxide
Saccharomycess cerevisiae	fermentation media	oxygen
Saccharomycess cerevisiae	Ca-alginate, Ba-alginate	oxygen
Escherichia coli	fermentation media	oxygen
Penicillium chrysogenum	fermentation media	oxygen
Bacillus amilaliquefaciens	Ca-alginate, PVA-SbQ gel	oxygen
Saccharomycess cerevisiae	Ca-alginate	ethanol
Baker's yeast	Ca-alginate	ethanol

Table 1. Immobilization procedures for enzymes

Method	Advantages	Disadvantages
Adsorption on insoluble matrices (e.g. by van der Waals forces, ionic binding or hydrophobic forces)	Simple, mild conditions, less disruptive to enzyme protein	Enzyme linkages are highly dependent on pH, solvent and temperature; insensitive
Entrapment in a gel (eventually behind a semipermeable membrane)	Universal for any enzyme, mild procedure	Large diffusional barriers, loss of enzyme activity by leakage, possible denaturation of the enzyme molecules as a result of free radicals
Crosslinking by a multifunctional reagent (such as glutaraldehyde *bis*-isocyanate derivatives or *bis*-diazobenzidine)	Simple procedure, strong chemical binding of the biomolecules; widely used in stabilizing physically adsorbed enzymes or proteins that are covalently bound onto a support	Difficult to control the reaction, requires a large amount of enzyme, the protein layer has a gelatinous nature (lack of rigidity), relatively low enzyme activity
Covalent bonding onto a membrane, insoluble supports	Stable enzyme-support complex, leakage of the biomolecule is very unlikely, ideal for mass production and commercialization	Complicated and time-consuming; possibility of activity losses due to the reaction involving groups essential for the biological activity (can be minimized by immobilization in the presence of the substrate or inhibitor of the enzyme)

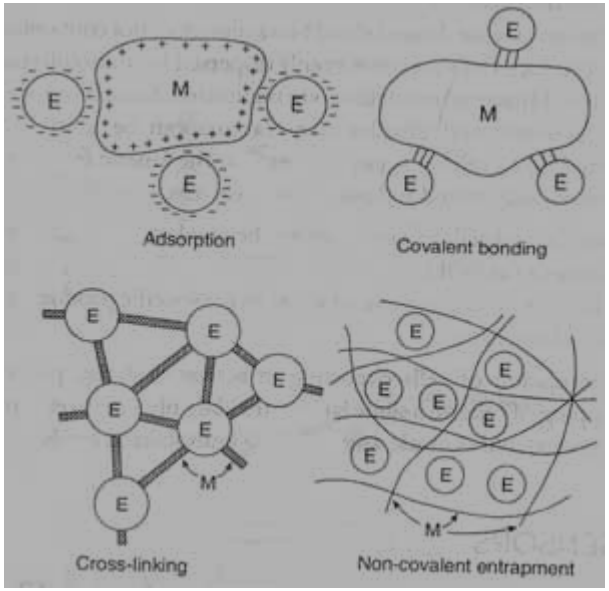
Entrapment and encapsulation

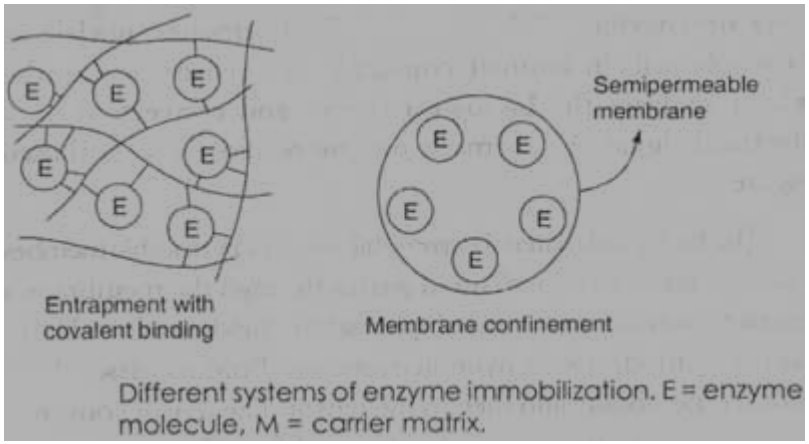
The immobilized enzyme can be classified into four types:
particles, membranes, tubes, and filters. Most immobilized enzymes are in particle form for ease of handling and ease of application.

Particles - The particle form of immobilized enzyme on solid particles.
Membranes - Enzyme membranes can be prepared by attaching enzymes to membrane-type carriers, or by molding into membrane form. The molding is done after the enzymes have been enclosed within semi-permeate membranes of polymer by entrapment.
Tubes - Enzyme tubes are produced using Nylon and polyacrylamide tubes as carriers. The polymer tube is first treated in a series of chemical reactions and the enzyme is bound by diazo coupling to give a tube in a final step.
Fibers - Enzymes that have been immobilized by entrapment in fibers to form enzyme fibers.

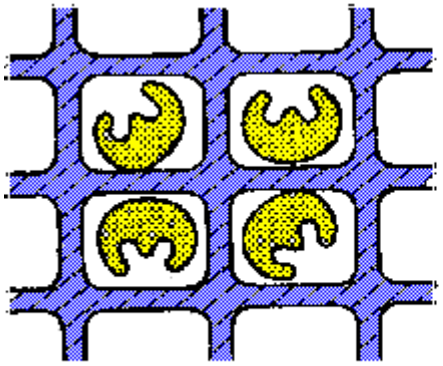
The solid supports used for enzyme immobilization can be *inorganic* or *organic* . Some organic supports include: **Polysaccharides, Proteins, Carbon, Polystyrenes, Polyacrylates, Maleic Anhydride based Copolymers, Polypeptides, Vinyl and Allyl Polymers, and Polyamides.**

Immobilization through Entrapment

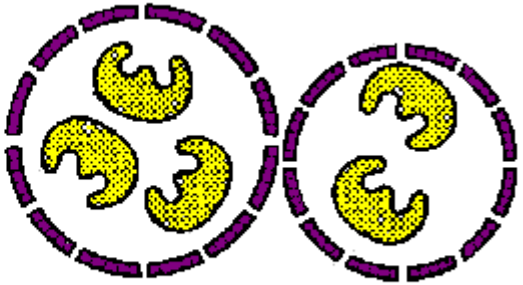




Different systems of enzyme immobilization. E = enzyme molecule, M = carrier matrix.



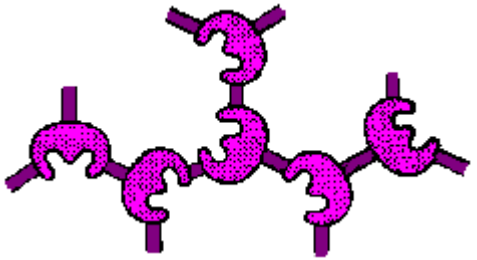
entrapped in a matrix



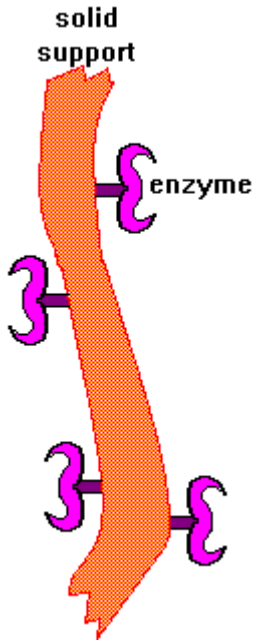
entrapped in droplets

The entrapment method of immobilization is based on the localization of an enzyme within the lattice of a polymer matrix or membrane. It is done in such a way as to retain protein while allowing penetration of substrate. It can be classified into **lattice** and **micro capsule** types

Cross-linking is based on the formation of chemical bonds, as in the covalent binding method. The immobilization is performed by the formation of intermolecular cross-linkages between the enzyme molecules by means of bi or multifunctional reagents.
The most common reagent used for cross-linking is glutaraldehyde. Cross-linking reactions are carried out under relatively severe conditions. These harsh conditions can change the conformation of active center of the enzyme; and so may lead to significant loss of activity.



The **carrier-binding** method is the oldest immobilization technique for enzymes. In this method, the amount of enzyme bound to the carrier and the activity after immobilization depend on the nature of the carrier. The following picture shows how the enzyme is bound to the carrier:
The selection of the carrier depends on the nature of the enzyme itself, as well as the:
Particle size
Surface area
Molar ratio of hydrophilic to hydrophobic groups
Chemical composition -polysaccharide derivatives such as cellulose, dextran, agarose, and polyacrylamide gel.



Physical Adsorption Mode

Based on the physical adsorption of enzyme protein on the surface of water-insoluble carriers.

The processes available for physical adsorption of enzymes are: **Static Procedure**
Electro-deposition
Reactor Loading Process
Mixing or Shaking Bath Loading

Adsorption tends to be less disruptive to the enzymatic protein than chemical means of attachment because the binding is mainly by hydrogen bonds, multiple salt linkages, and Van der Waal's forces.

Because of the weak bonds involved, desorption of the protein resulting from changes in temperature, pH, ionic strength or even the mere presence of substrate, is often observed. Another disadvantage is non-specific, further adsorption of other proteins or other substances as the immobilized enzyme is used. This may alter the properties of the immobilized enzyme or, if the substance adsorbed is a substrate for the enzyme, the rate will probably decrease depending on the surface mobility of enzyme and substrate.

Adsorption of the enzyme may be necessary to facilitate the covalent reactions described later in this presentation. Stabilization of enzymes temporarily adsorbed onto a matrix has been achieved by cross-linking the protein in a chemical reaction subsequent to its physical adsorption.

Site-specific chemical modification of biomolecules

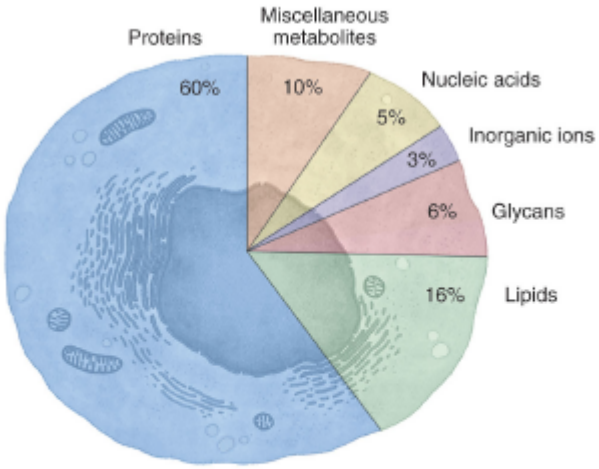


Figure 1 Composition of a typical mammalian cell[11]. Although proteins comprise the largest fraction of a cell's dry mass, it is estimated that more than half are modified with glycans, lipids or other metabolites[113]. Methods for visualizing both proteins and non-proteinaceous biomolecules would enhance our understanding of living systems.

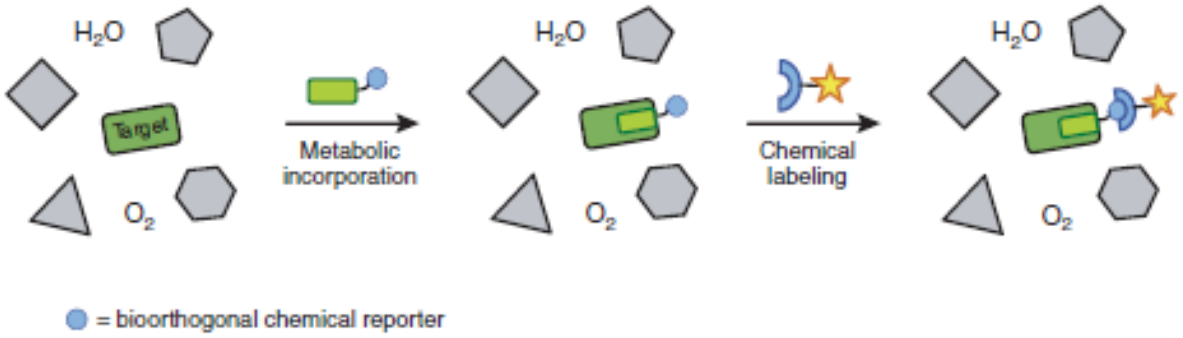
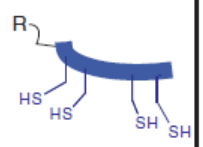
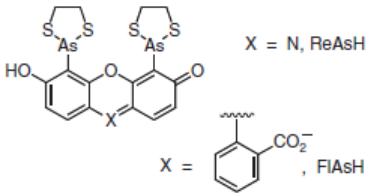
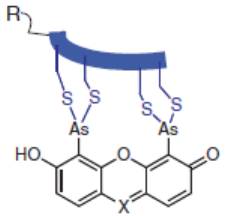
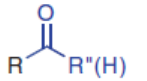
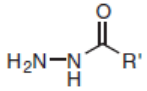
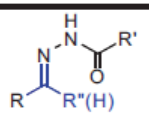
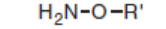
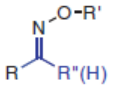
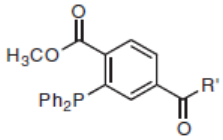
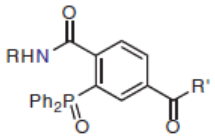
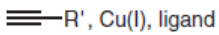
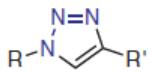
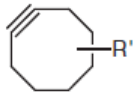
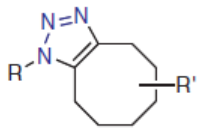
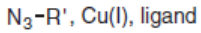
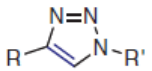


Figure 2 The bioorthogonal chemical reporter strategy. A chemical reporter (blue circle) linked to a substrate (light green box) is introduced into a target biomolecule through cellular metabolism. In a second step, the reporter is covalently tagged with an exogenously delivered probe (blue arc). Both the chemical reporter and exogenous probe must avoid side reactions with nontarget biomolecules (gray shapes).

Table 1 Chemical reporters and bioorthogonal reactions used in living systems.

Chemical reporter	Reactive partner (R´ = probe)	Ligation product	Target[a] (R)
 Tetracysteine motif	 X = N, ReAsH; X = CO_2^-, FlAsH		Protein[18,31]
 Ketone/aldehyde			Protein[19,20]
			Glycan[22]
R–N_3 Azide	Staudinger ligation 		Protein[17,26]
	'Click' chemistry 		Glycan[30,34] Lipid[25]
	Strain-promoted cycloaddition 		
R— Terminal alkyne	'Click' chemistry 		Protein[29]

[a]Only literature examples provided. Other biomolecules could potentially be labeled in a similar manner.

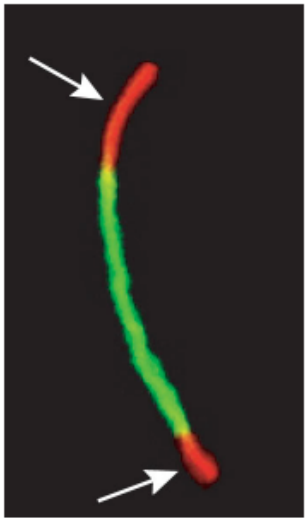


Figure 3 Bioorthogonal chemical reporters and cellular imaging. HeLa cells expressing tetracysteine-fused connexin were treated with FlAsH (green), incubated in medium for 4 hours, then treated with ReAsH (red) and imaged. This two-color pulse-chase labeling experiment demonstrated that newly synthesized connexin is incorporated at the outer edges of existing gap junctions (indicated by white arrows)[37]. Figure reproduced from ref. 37 by permission of the American Association for the Advancement of Science.

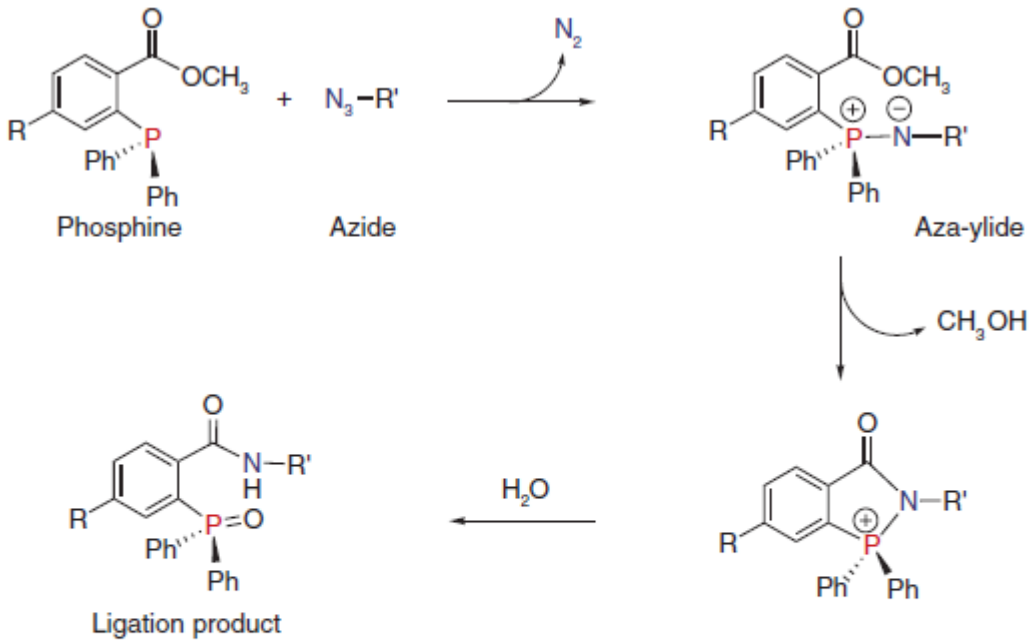


Figure 4 The Staudinger ligation. A triarylphosphine and an azide first react to form an aza-ylide intermediate. The nucleophilic nitrogen atom is trapped in an intramolecular fashion, and the cyclized intermediate hydrolyzes in water to form a stable amide-linked product. In some cases, aryl azides (R' = aryl) may react with phosphines to initially form *O*-alkyl imidates[114].

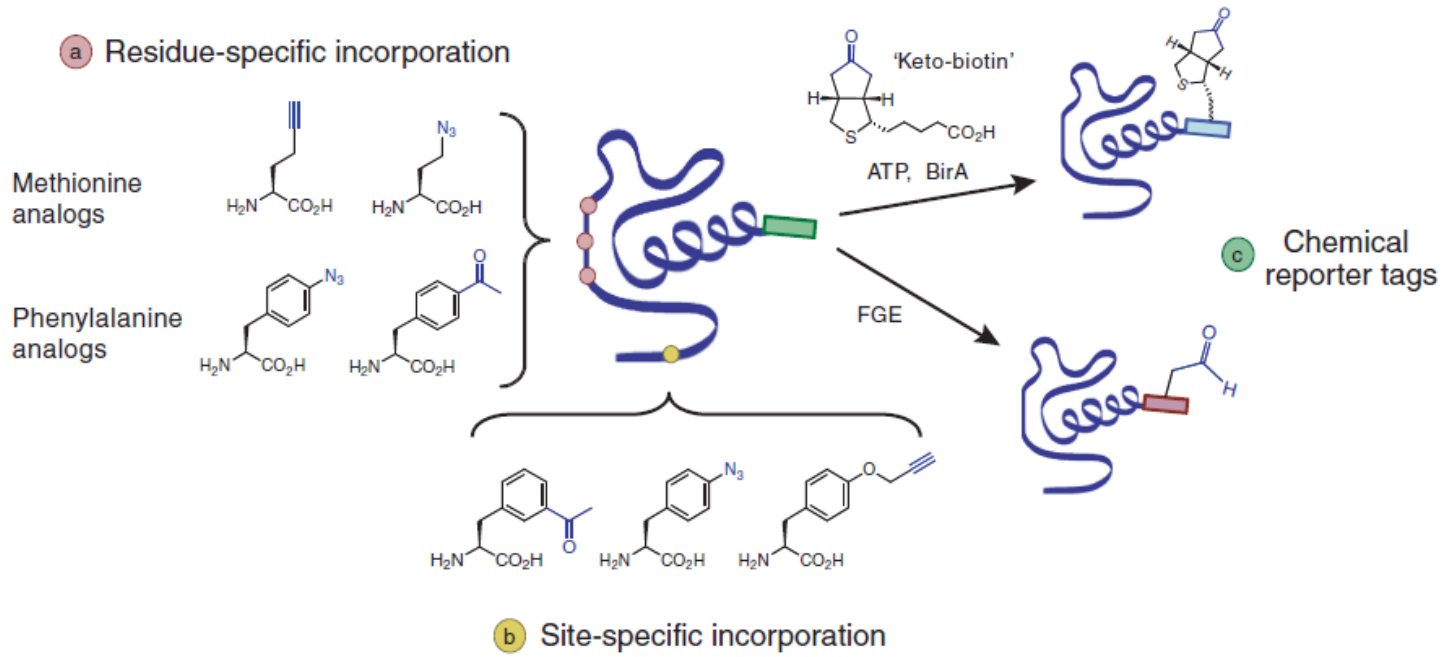


Figure 5 Methods for introducing chemical reporters into proteins. (**a**) Unnatural amino acids bearing ketones, azides and alkynes can be incorporated into target proteins in a residue-specific manner using auxotrophic strains of *E. coli*. (**b**) Amino acids with bioorthogonal side chains can be installed into proteins in a site-specific fashion using nonsense suppression techniques. (**c**) Chemical reporters can be introduced into short peptide sequences using the cell's post-translational machinery. In one example, an analog of biotin ('keto-biotin') is attached to a 15-amino-acid consensus sequence (blue box) by *E. coli* biotin ligase (BirA). Similarly, formylglycine-generating enzyme (FGE) can convert a cysteine residue within a 13-residue consensus sequence (red box) to formylglycine. Both of the these electrophiles can be labeled with hydrazide probes.

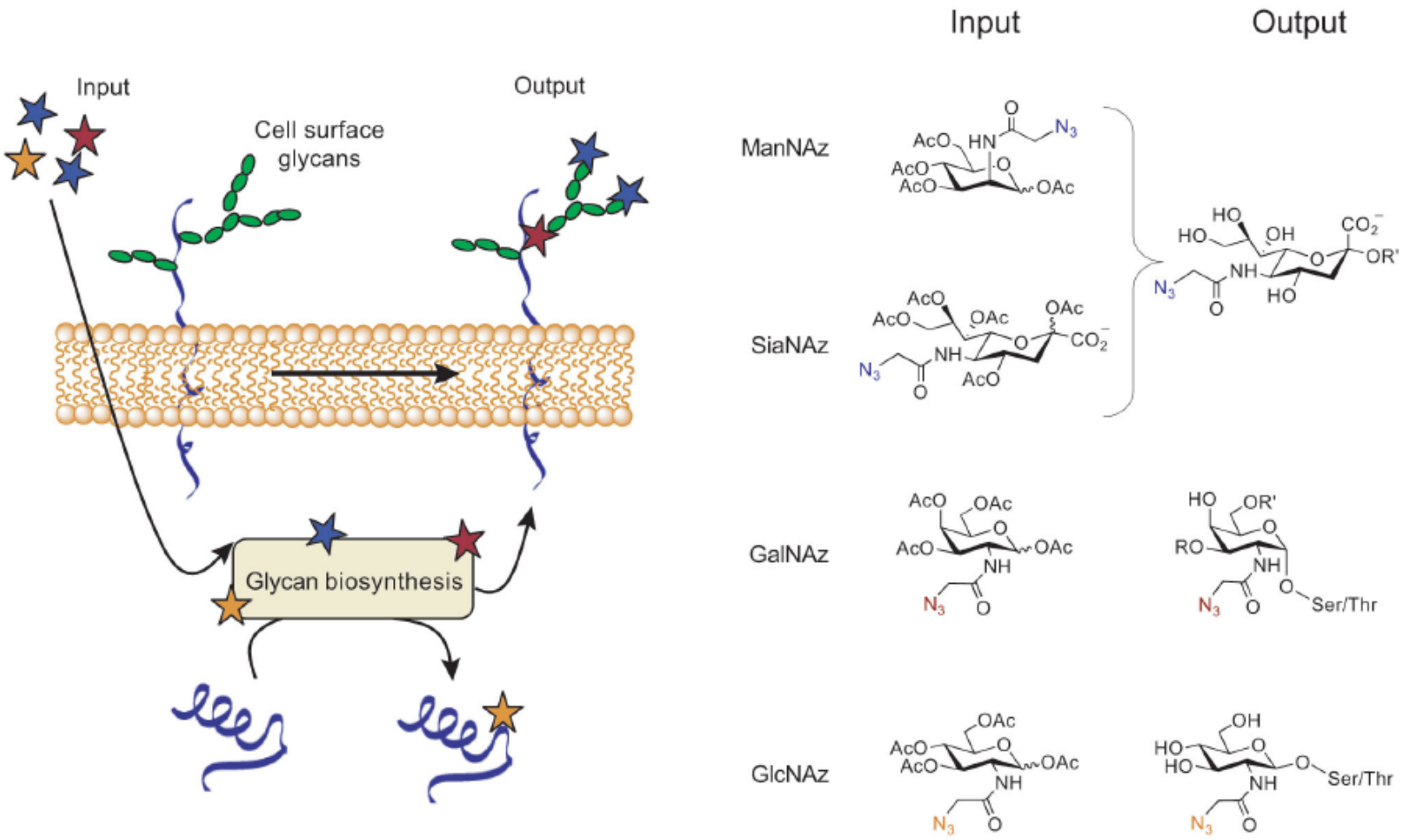
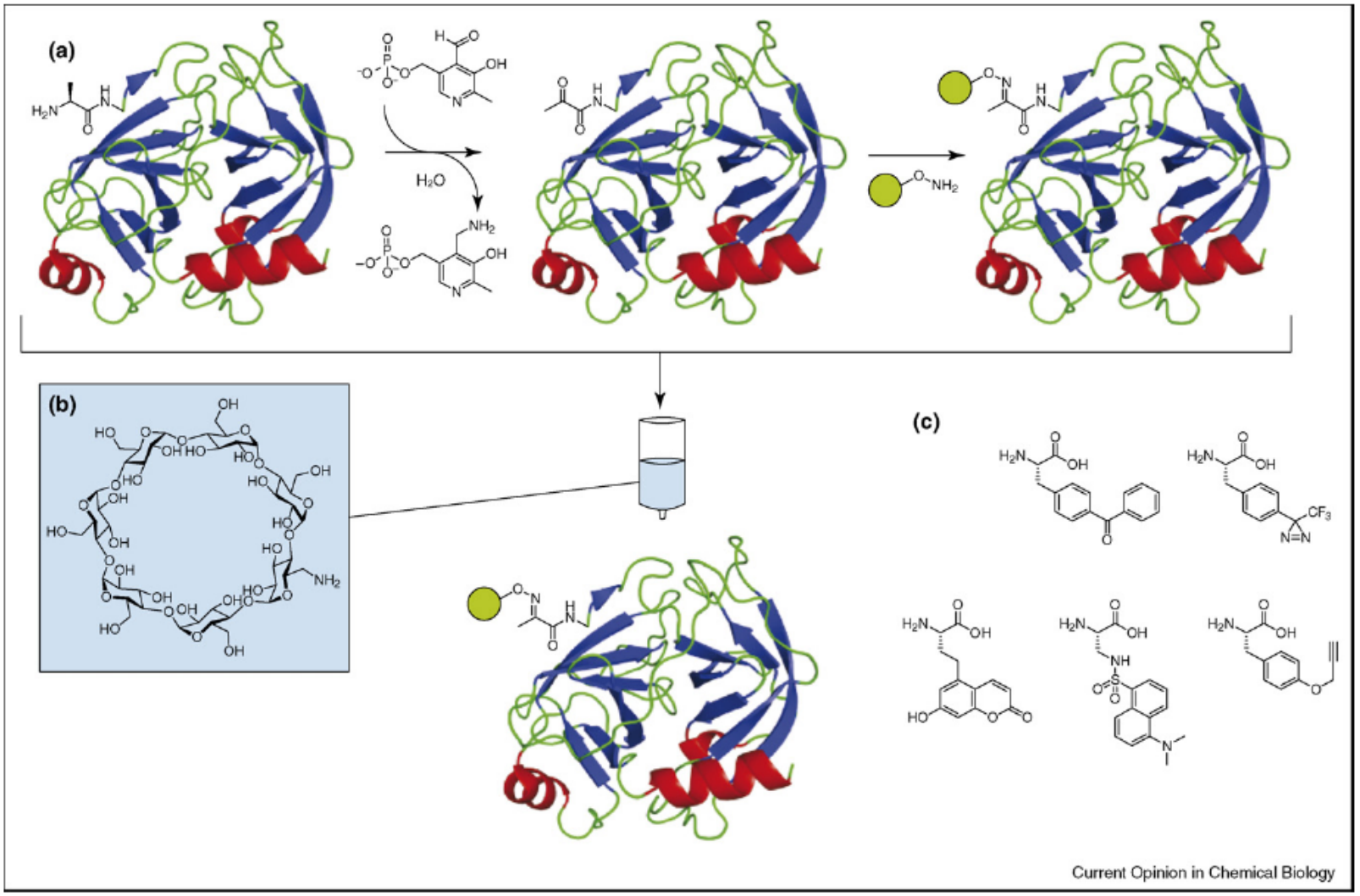
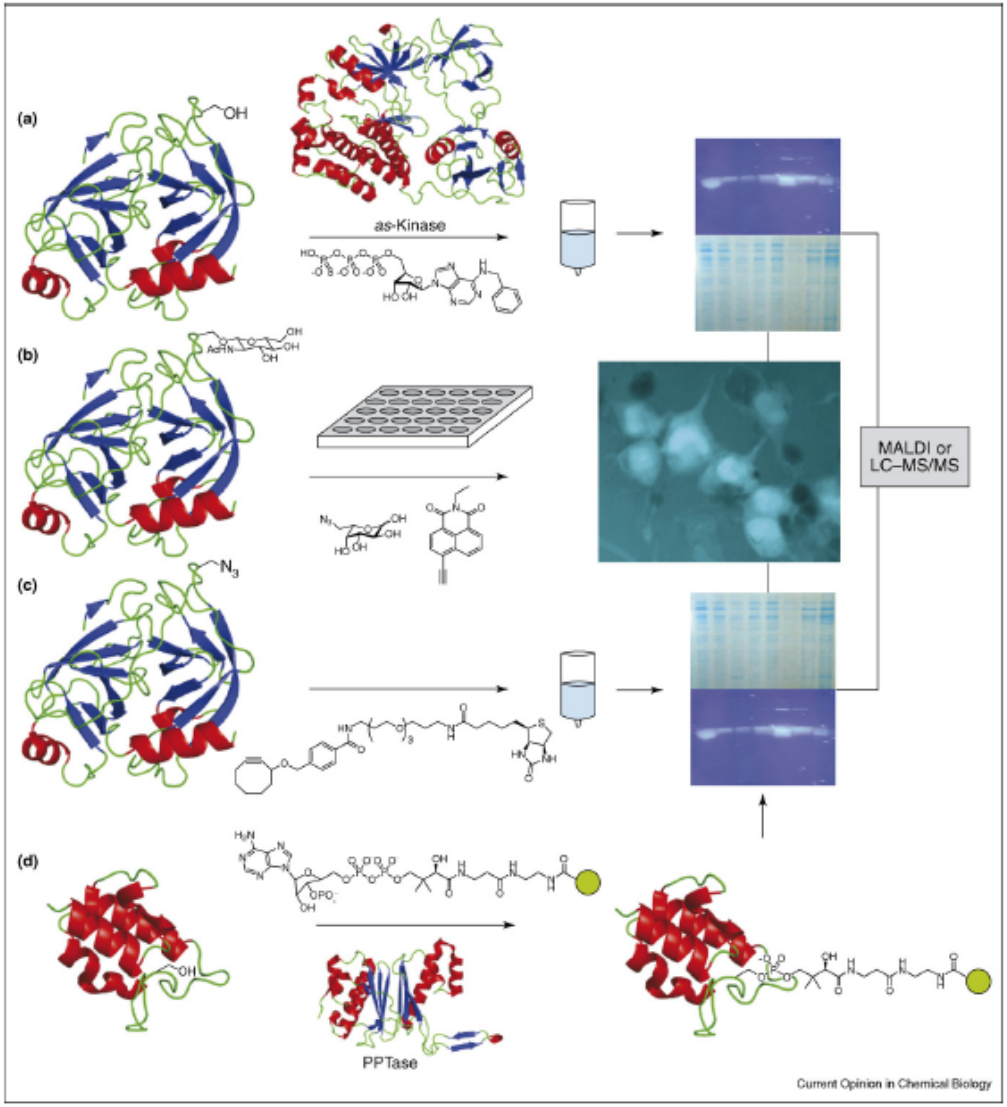


Figure 6 Azides can be incorporated into glycoconjugates using glycan biosynthetic pathways. Azido analogs of ManNAc (ManNAz) and sialic acid (SiaNAz) are metabolized by cells and converted to cell surface azido sialosides. Similarly, an azido analog of GalNAc (GalNAz) can be metabolically introduced at the core position of mucin-type O-linked glycoproteins. An azido analog of GlcNAc (GlcNAz) can be incorporated into cytosolic and nuclear glycoproteins.

Site-specific protein modification



Biochemical methods for site-specific labeling of proteins. **(a)** Proteins containing permissive N-terminal residues (here represented as alanine) can be selectively deaminated by treatment with pyridoxal phosphate to generate a α-keto residue and pyridoxylamine [8]. Subsequent conjugation to an alkoxyamine-appended fluorophore yields a product pool of unlabeled and monolabeled proteins. **(b)** Affinity chromatography of this mixture enables selective isolation of the protein conjugate [9••]. **(c)** Alternatively, methods to incorporate non-natural amino acids enable the insertion of several bioorthogonal chemical functionalities, including photoreactive moieties for crosslinking experiments, intrinsically fluorescent residues for biophysical and imaging applications, or an alkyl-terminating residue for click- and Staudinger-based investigations [10•]. Protein structures were rendered using PyMOL [39]. Trypsinogen (PDB: 1G36) was used as a default representative protein.

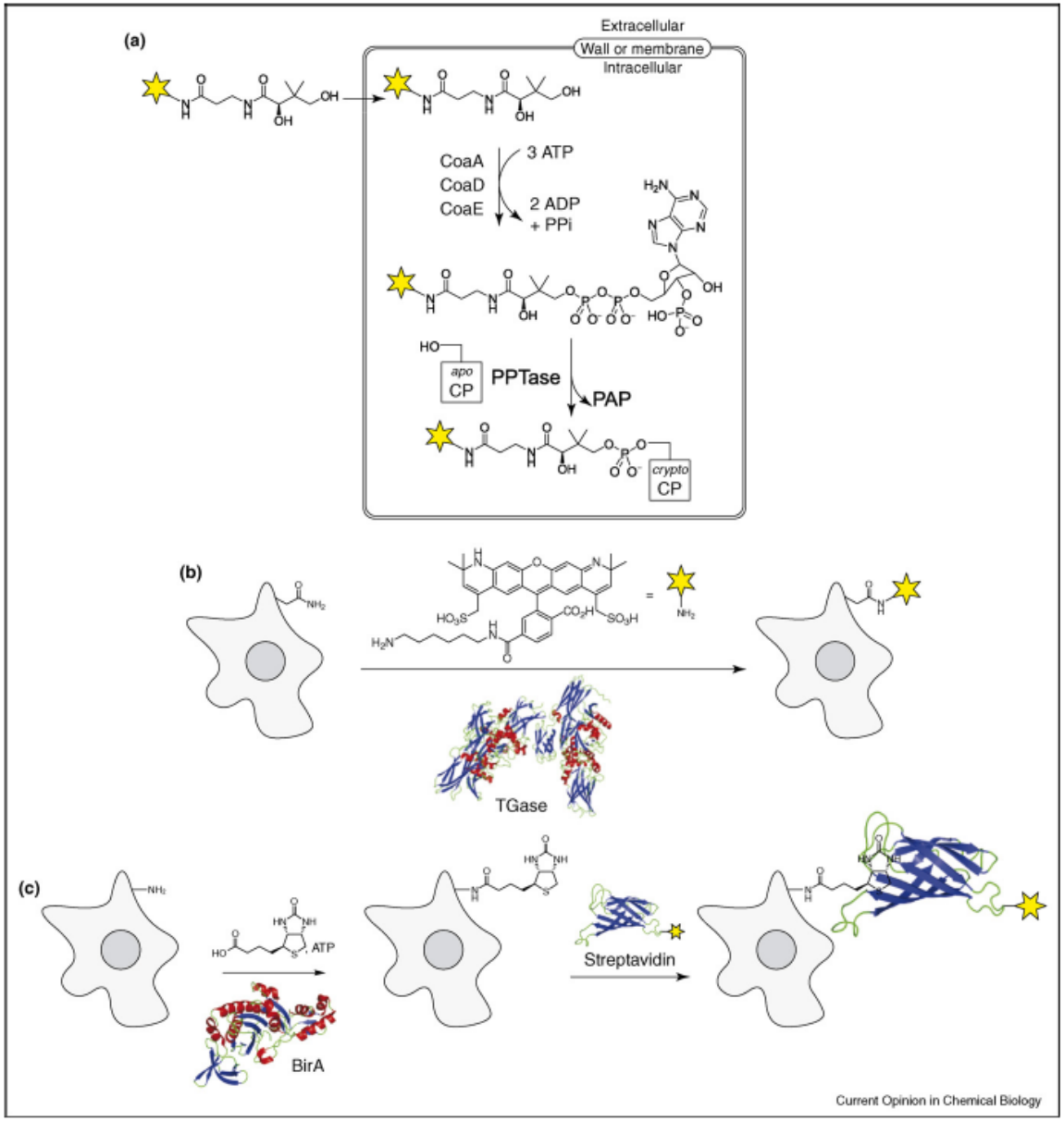


Ex vivo techniques to identify post-translationally modified proteins. **(a)** *Apo*-substrates of an engineered analogue-specific kinase (*as*-kinase) (PDB: 1KSW) can be selectively labeled with N^6-benzyl-ATP-γ-thiophosphate (ATPγS), subsequently derivatized by reactivity of their thiophosphate modification and visualized in gel [14]. **(b)** Glycosylated proteins encoded to be fucosylated within the cell (here represented by an *O*-linked *N*-acetylglucosamine residue) can be modified by metabolic delivery of 6-deoxy-6-azidofucose. Subsequent click modification of fixed cell preparations with alkynyl napthamide probes can be microscopically imaged, visualized in gel or identified by matrix-assisted laser desorption/ionization (MALDI) or LC–MS/MS [19••]. **(c)** Proteins engineered to contain alkyl-azide species can be modified in the absence of catalyst with reporter-appended cyclooctynes for isolation, visualization and identification [21•]. **(d)** *Apo*-carrier proteins (PDB: 1ACP) can be enzymatically phosphopantetheinylated with CoA derivatives by a phosphopantetheinyl transferase (PPTase) (PDB: 1QRO) to be visualized, isolated and identified [24••]. All proteins are followed by the Protein Data Bank (PDB) accession number for the structural data from which the representation was rendered. Trypsinogen (PDB: 1G36) was used as a default representative protein. Protein structures were rendered using PyMOL [39].

Table 1

Site-specific labeling of proteins.

Application	Technique	Labeling mechanism	Fusion size	Limitations	Comments
Biochemical substrate production	PLP-mediated oxidative amination	Reaction with hydrazine or alkoxyamine probe	None	N-terminal residue identity mixed product pools	Affinity chromatography on cyclodextrin-agarose yields pure mono-labeled protein pools
Biochemical substrate production	Non-natural amino acid incorporation	Chemistry is dependent upon the identity of orthogonal functionality in non-biogenic amino acid	None, one residue mutagenesis	Restricted to recombinant cell lines; cellular permeability of non-natural amino acid substrate	Several different chemical functionalities can selected
Kinase substrate pools	Engineered analogue-specific kinase and AT*PγS	AT*P permissive kinase; secondary electrophilic conjugation	None	Preparation of *as*-kinase; proteins must be in the *apo*-state	Culturing transgenic cell lines with *as*-kinase inhibitors produces *apo*-proteins *in vivo*
Fucosyl-residue-containing glycoproteins	Azido-linked fucosyl sugars	Direct incorporation into the glycan; click reaction with alkynyl-appended probes	None		
Polyketide and nonribosomal peptide synthases	Phosphopantetheinylation	Direct labeling of CP domains with CoA analogue and PPTase	None	Proteins must be in the *apo*-state; correct expression time	
Cellular imaging	Phosphopantetheinylation	Enzymatic modification of CP/ybbR fusion	11 amino acids	Cell-surface fusions	CoA derivative precursors are shown to be cell permeable and viable; await evaluation
Cellular imaging	Transglutamination	Enzymatic labeling of Q-tag fusions	7 amino acids	Cell-surface fusions; Ca^{2+} dependent; endogenous amine competition	Might find application to ER labeling where $[Ca^{2+}]$ is permissive
Cellular imaging	Biotinylation	BirA biotinylation of AP-fusions	15 amino acids	Cell-surface fusions; multivalent nature of avidins	Monovalent avidins alleviate multimerization on the cell surface



Enzymatic labeling techniques to image cells. **(a)** Fluorescent pantothenic acid derivatives can be metabolically converted into CoA and attached to carrier proteins by coexpressed phosphopantetheinyl transferase in *E. coli* [33•]. **(b)** Cell-surface proteins fused to glutamine-containing Q-tags can be fluorescently labeled by incubation with amine-terminating fluorophores and transglutaminase (TGase) (PDB: 1L9M) [35]. **(c)** Live cells expressing membrane proteins containing AP fusions can be biotinylated by treatment with biotin, ATP and biotin ligase (BirA) (PDB: 1HXD) [36,37•]. Further labeling with monovalent strepatividin (PDB: 1STP) conjugated to fluorophores allows for imaging without aberrant modulation of receptor function [38]. Protein structures were rendered using PyMOL [39]. All proteins are followed by the Protein Data Bank (PDB) accession number for the structural data from which the representation was rendered.