

Interazioni proteina-proteina

- Giocano un ruolo fondamentale nell'organizzazione strutturale e funzionale della cellula
- Interazioni stabili: complessi proteici
- Interazioni transienti : controllano la maggioranza di processi cellulari

L'interazione proteina- proteina
può essere identificata analizzando l' effetto prodotto

L'interazione proteina-proteina può:

- 1. Modificare le costanti cinetiche di un enzima**
- 2. Indirizzare il substrato verso una determinata via metabolica (substrate channeling)**
- 3. Determinare la formazione di un nuovo sito di binding (F1 ATPasi)**
- 4. Cambiare la specificità di una proteina per un suo substrato
(interazione dei fattori di trascrizione con l' RNA polimerasi indirizza
l' enzima verso diversi promotori)**

**Metodi biochimici : cromatografia di affinità
overlay
crosslinking
Co-immunoprecipitazione**

Metodi biofisici:

- FRET: Foster**
- Resonance**
- Energy**
- Transfer**
- SPR : Surface**
- Plasmon**
- Resonance**

**Metodi genetici : phage display
sistema a doppio ibrido e varianti**

CROMATOGRAFIA DI AFFINITA'

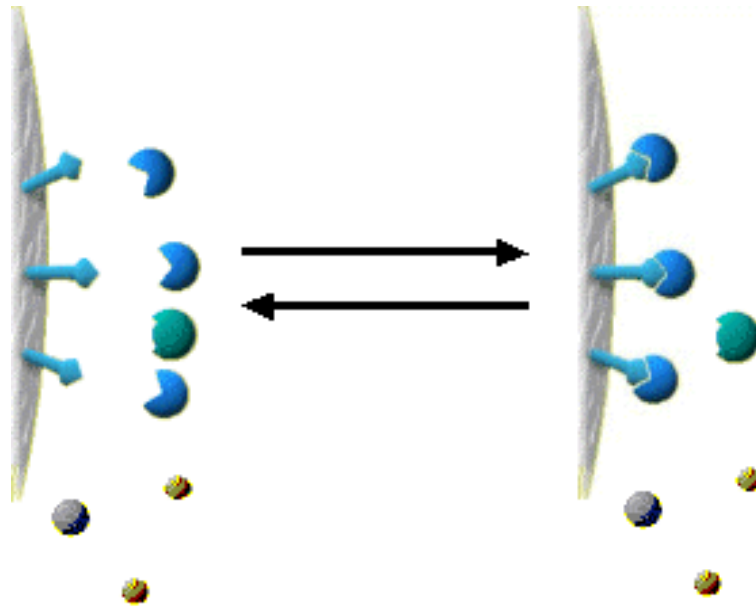
Il metodo si basa sulla formazione reversibile del complesso BPL

- Il legame tra ligando L e “ binding-protein” BP deve essere **reversibile**

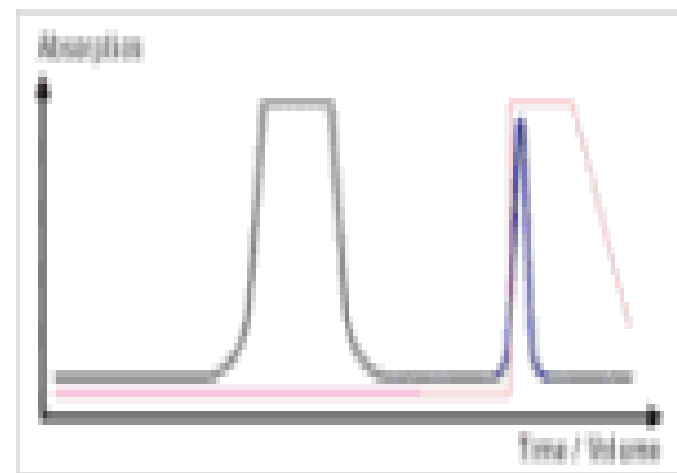
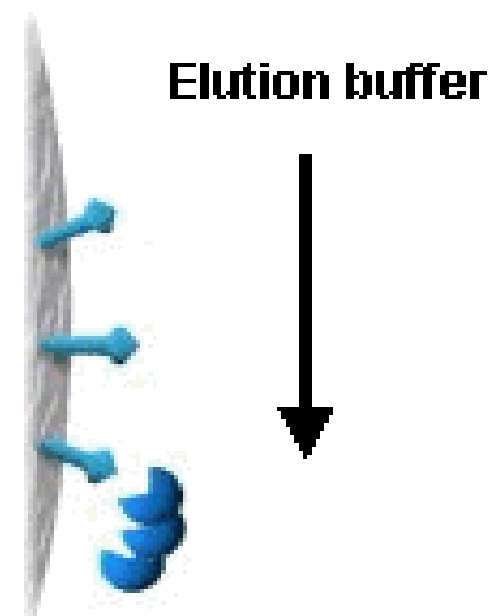
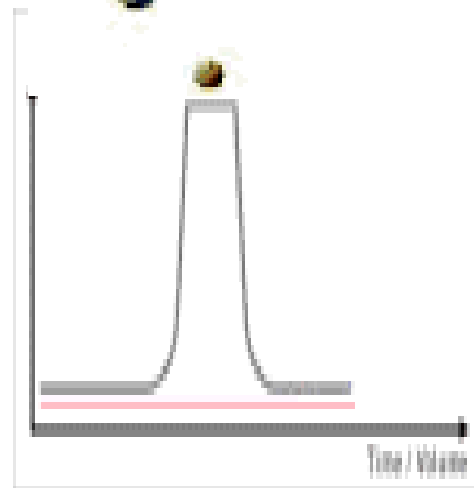
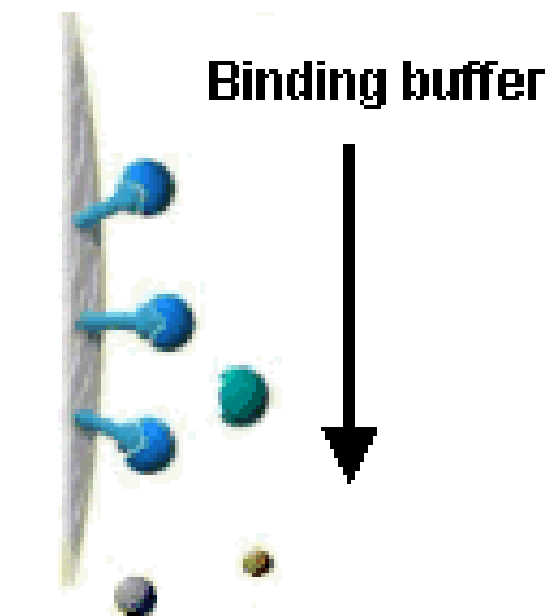
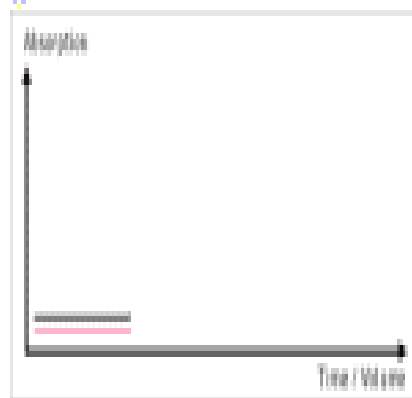
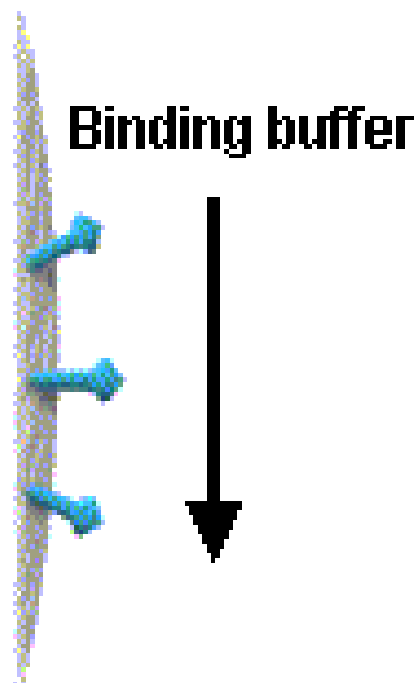


$$k_{+1} [\text{BP}] \cdot [\text{L}] = k_{-1} [\text{BPL}]$$

$$\frac{k_{-1}}{k_{+1}} = K_D \frac{[\text{BP}] \cdot [\text{L}]}{[\text{BPL}]}$$



Complessi con $K_D > 10^{-4}$ M sono difficili da identificare
Complessi con $K_D < 10^{-10}$ M difficilmente si possono eluire in condizioni native



Matrice : una matrice ideale deve avere le seguenti caratteristiche:

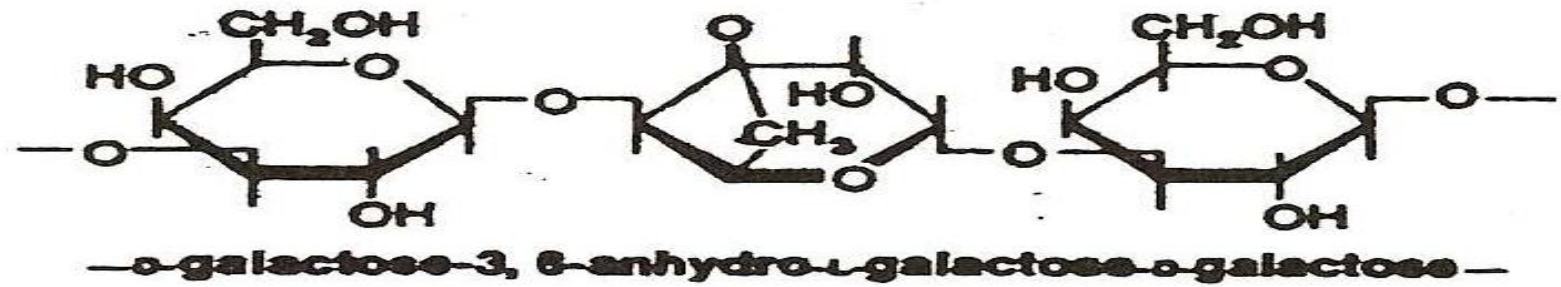
- Contenere gruppi chimici adatti per il legame covalente
- Essere stabile nelle condizioni di legame
- Essere inerte

Possono essere suddivise in due categorie principali:

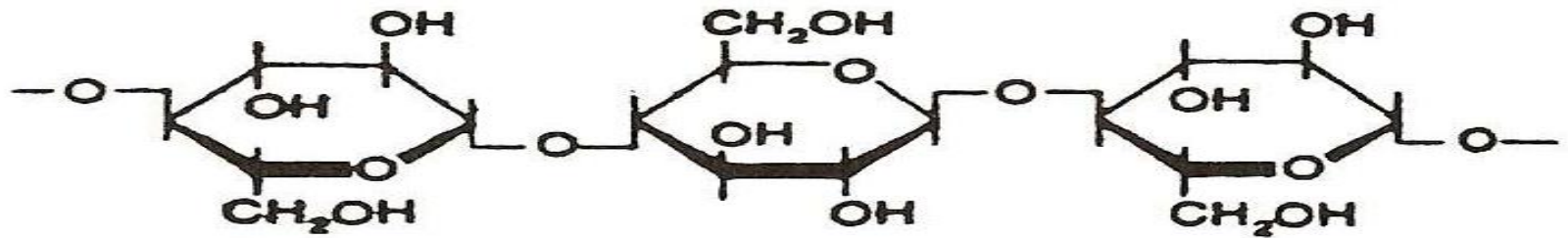
POLIMERI NATURALI: POLISACCARIDI (agarosio, destrano, cellulosa)

POLIMERI SINTETICI (polistirene, poliacrilati))

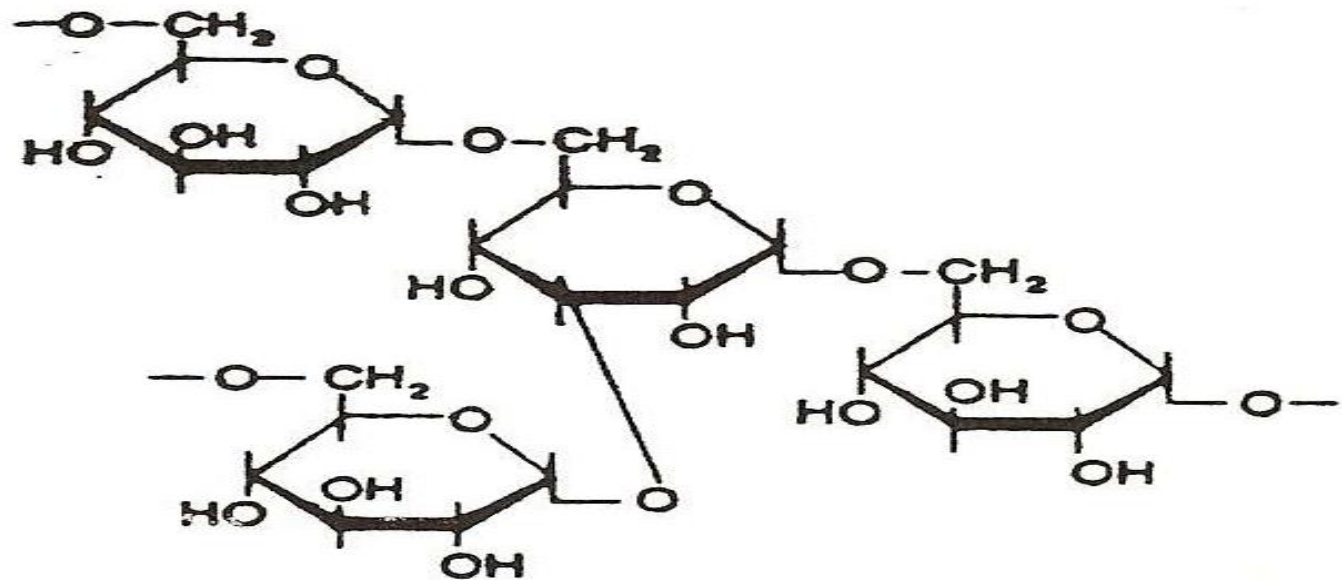
Agarose



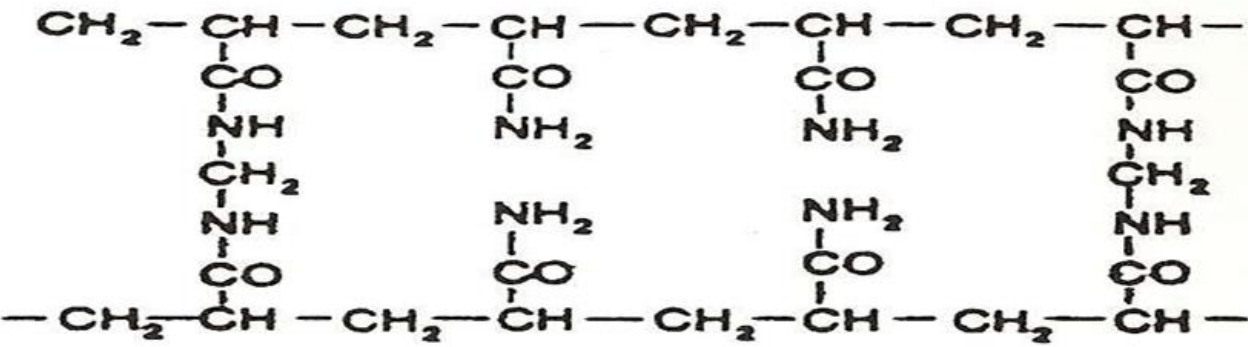
Cellulose



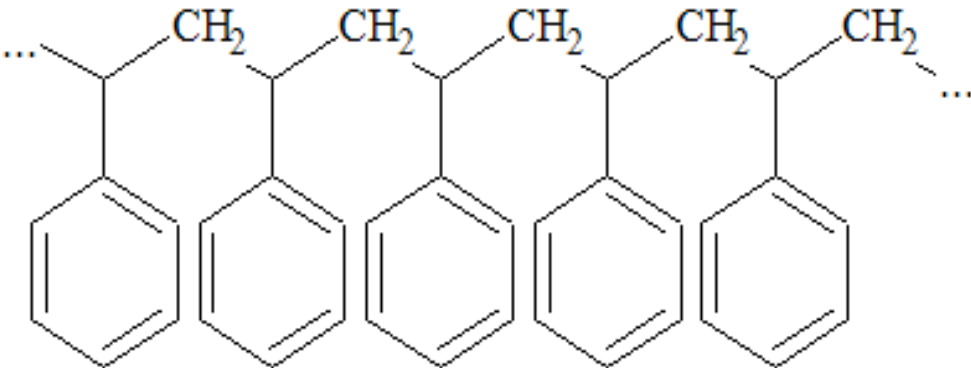
Crosslinked dextran (Sephadex)



Poliacrilamide



Polistirene

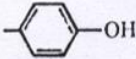
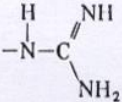
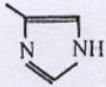
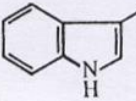


ligandi

- Devono avere uno o più gruppi adatti per il legame covalente non coinvolti con il legame con la binding protein .
- I gruppi più comuni sono $-\text{NH}_2$, COOH , SH , OH

Residui reattivi delle proteine

Table 5.8. Reactive residues of proteins^a

Residue	Originating amino acid
$-\text{NH}_2$	ϵ -Amino of L-lysine and <i>N</i> -terminus amino group
$-\text{SH}$	Thiol of L-cysteine
$-\text{COOH}$	Carboxyl of L-aspartate and L-glutamate and <i>C</i> -terminus carboxyl group
	Phenolic of L-tyrosine
	Guanidino of L-arginine
	Imidazole of L-histidine
$-\text{S}-\text{S}-$	Disulphide of L-cystine
	Indole of L-tryptophan
$\text{CH}_3-\text{S}-$	Thioether of L-methionine
$-\text{CH}_2\text{OH}$	Hydroxyl of L-serine and L-threonine

In genere si interpone al ligando un braccetto spaziatore di dimensione variabile che aumenti l'accessibilità della binding protein al ligando

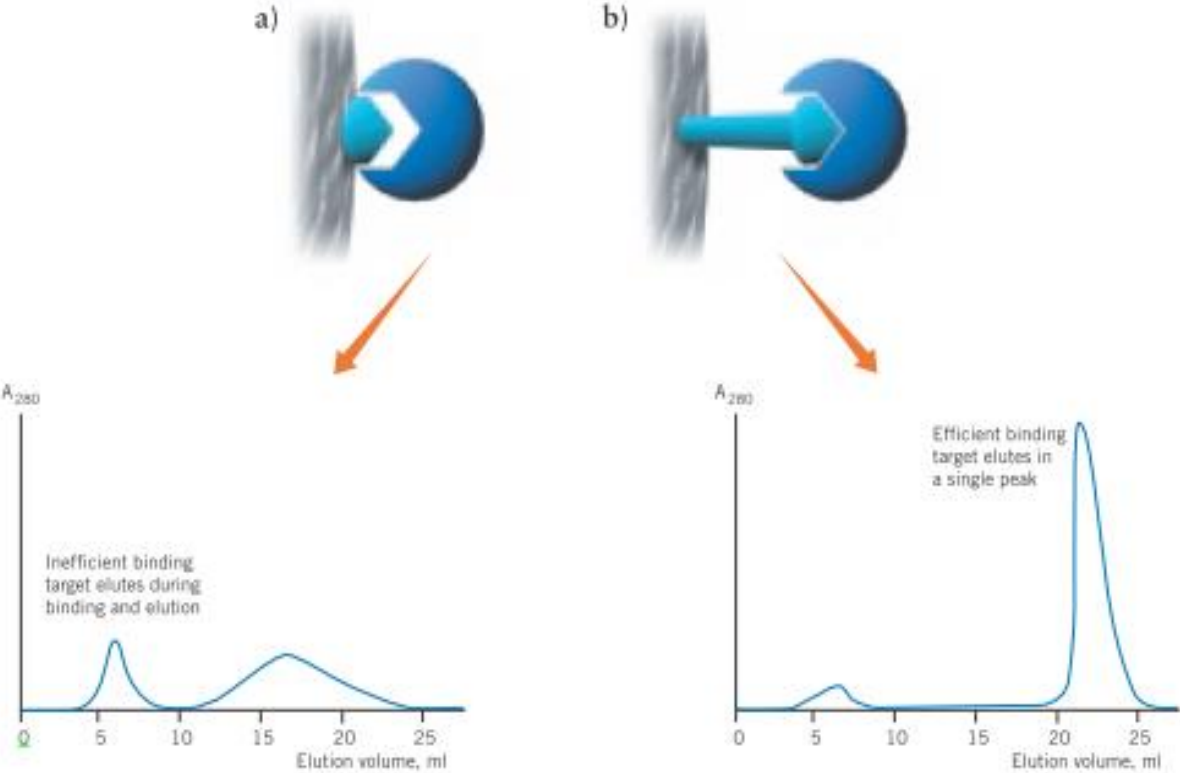
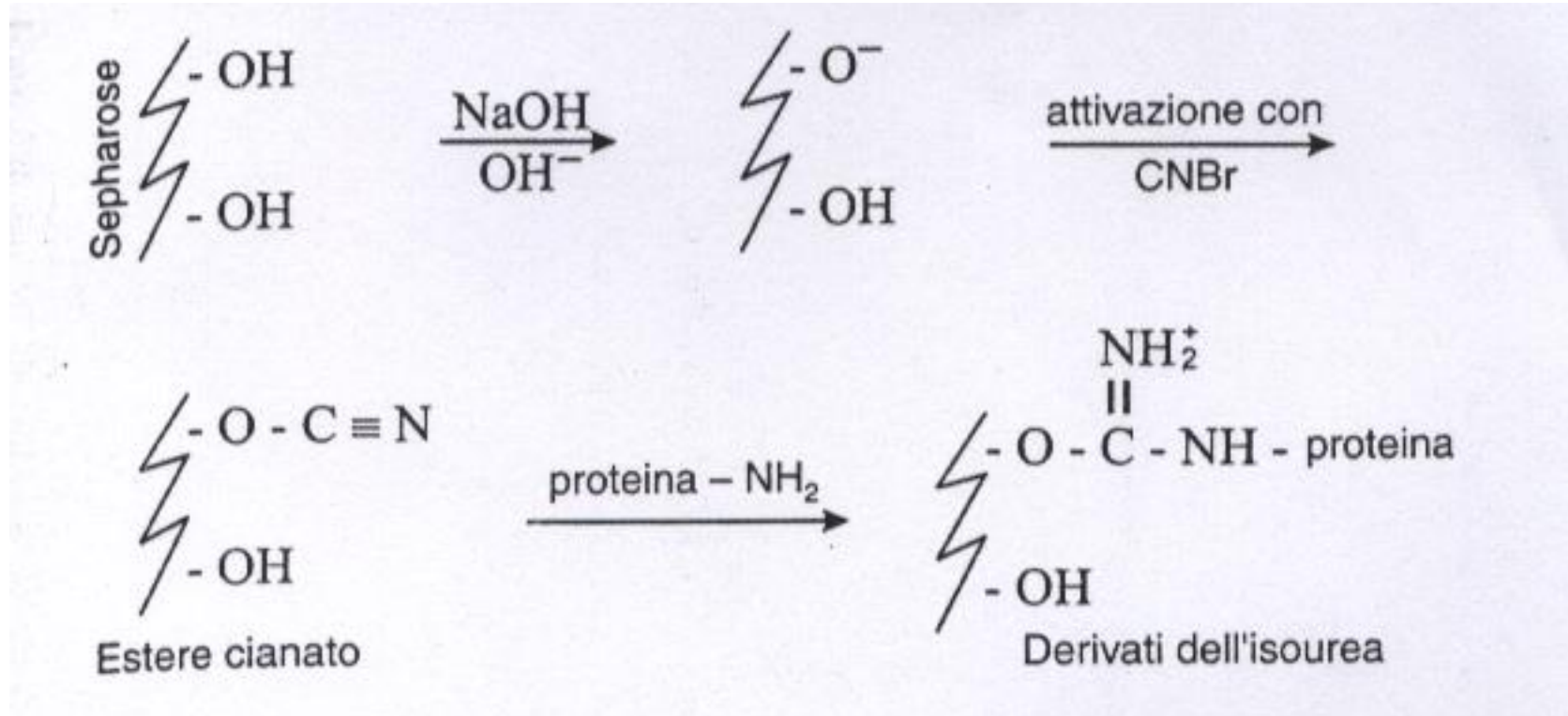


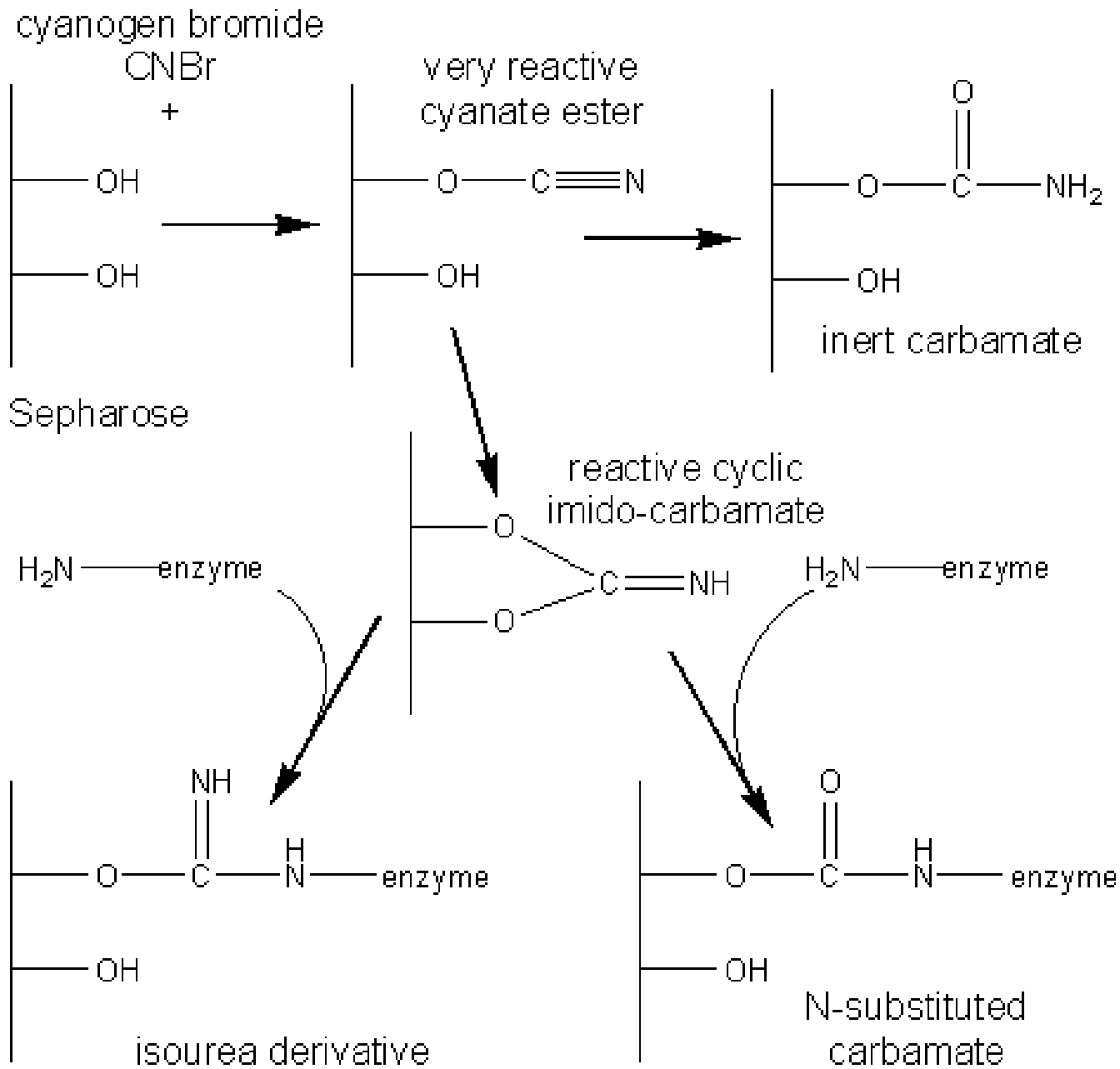
Table 8.

Chemical group on ligand	Length of spacer arm	Structure of spacer arm	Product
Proteins, peptides, amino acids			
amino	10-atom		NHS-activated Sepharose 4 Fast Flow
	None	—	CNBr-activated Sepharose 4B
	10-atom		ECH Sepharose 4B
carboxyl	11-atom		EAH Sepharose 4B
thiol	4-atom		Thiopropyl Sepharose 6B
	10-atom		Activated Thiol Sepharose 4B
	12-atom		Epoxy-activated Sepharose 6B

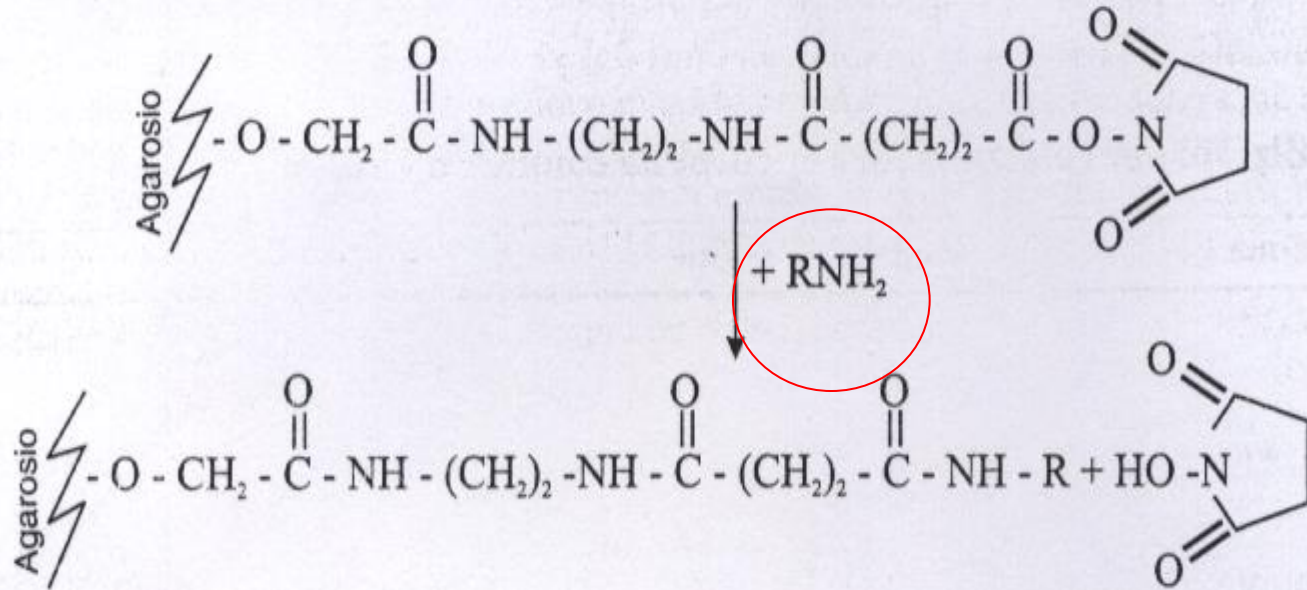
Procedura di accoppiamento mediante bromuro di cianogeno (gruppi amminici)

- Si attiva la resina pH alcalino
- Si risospende la binding-protein
- Si bloccano i rimanenti gruppi attivi
- Si lava

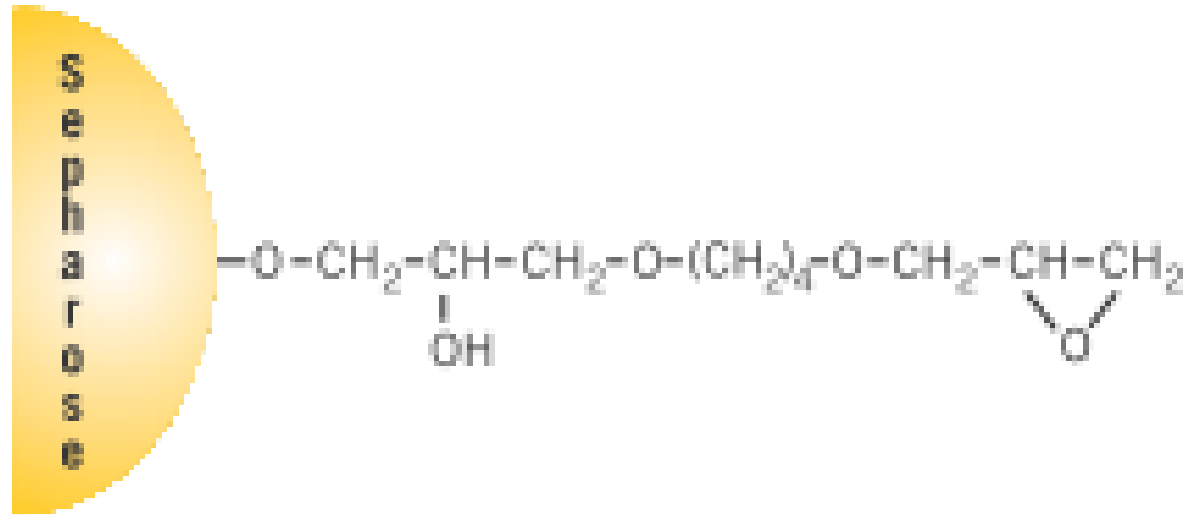




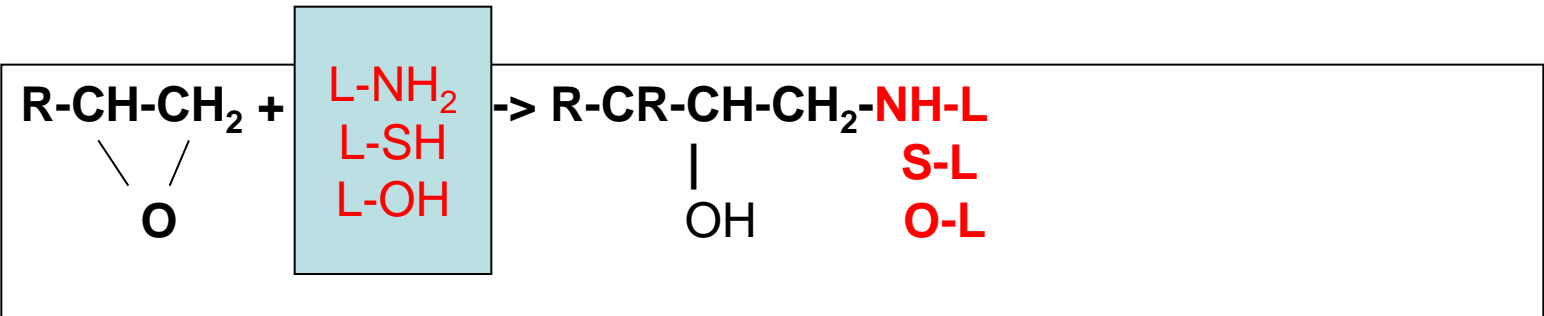
Accoppiamento con esteri della N-idrossisuccinimide (gruppi amminici)

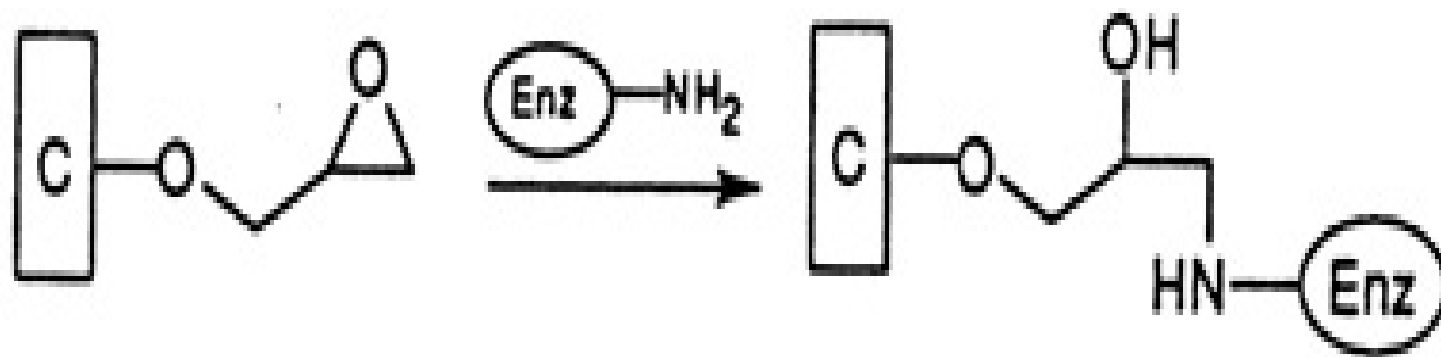


Accoppiamento mediante epossidi pH basico (gruppi NH₂,SH,OH)

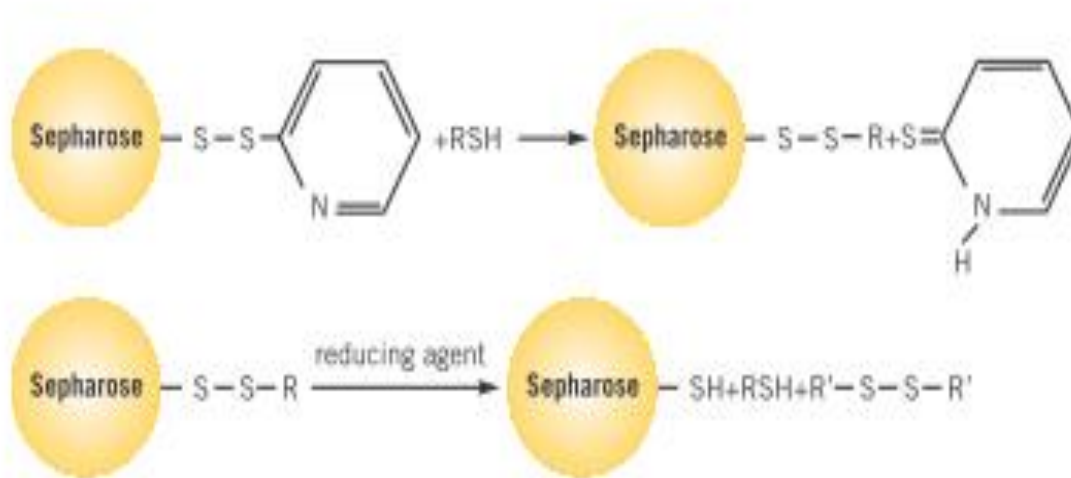


L-NH₂
L-SH
L-OH





Procedimento per legare proteine contenenti gruppi SH



2 piridil-tione

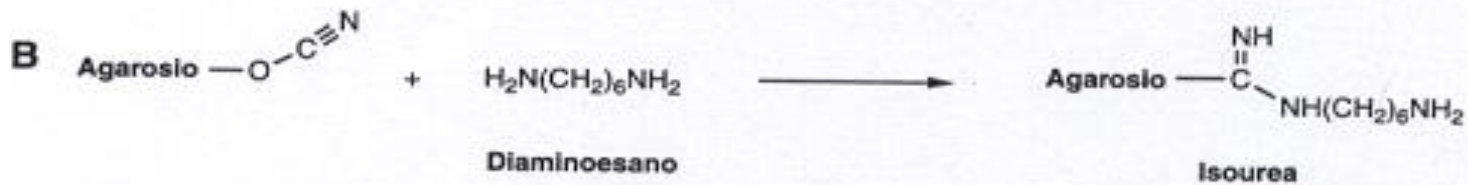
assorbe a 343nm

**L' attacco prevede uno scambio tra gruppi tiolici
formazione di ponti disolfuro tra proteina e resina
liberazione di 2 piridil tione**

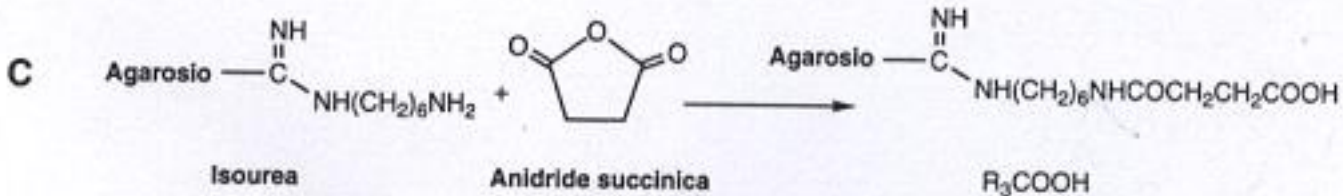
SPAZIATORI

contengono un secondo gruppo funzionale a cui il ligando può essere legato
Esempi sono:

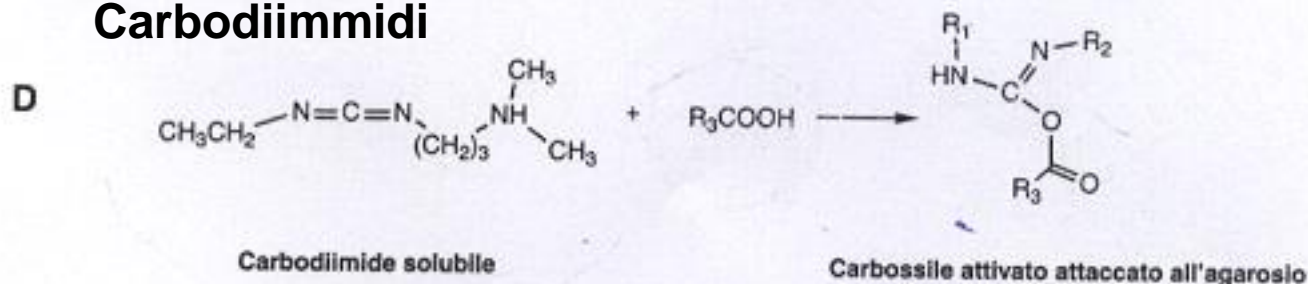
1-6 diamminoesano



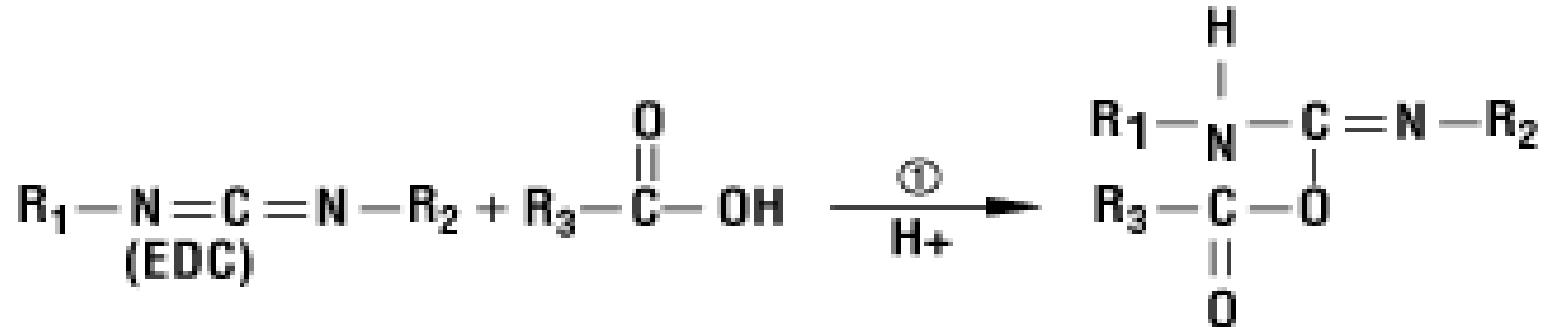
Anidride succinica



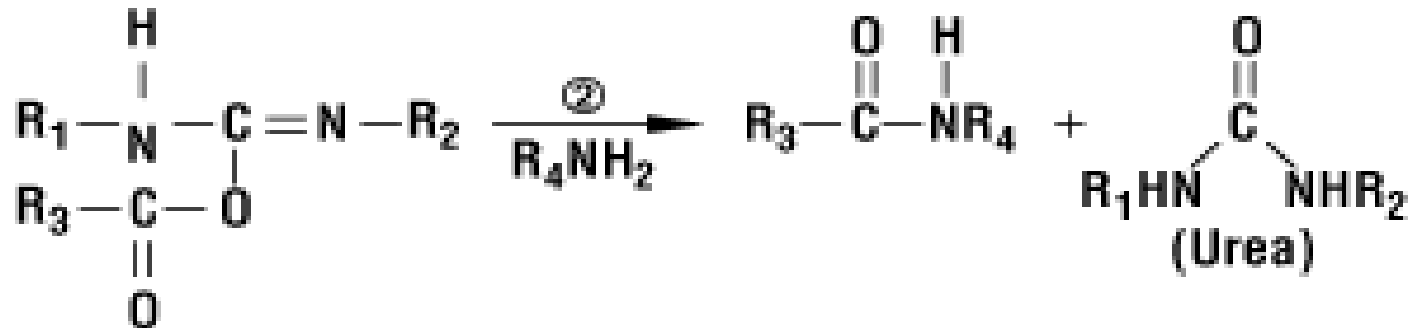
Carbodiimmidi



Reazione di accoppiamento tra gruppi carbossilici e gruppi amminici mediante **carbodiimmidi** (Formazione legame ammidico)



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.



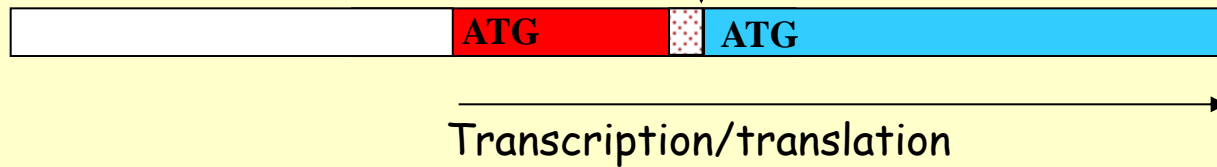
EDC is released as a soluble urea derivative after displacement by the nucleophile, R_4NH_2 .

Produzione di proteine ricombinanti con affinity tag

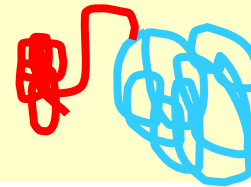
Fusion-protein vector:

Promoters etc.

Cloning site



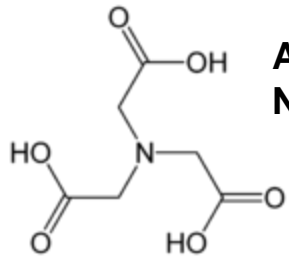
Product is fusion of
Your protein and **Affinity tag**



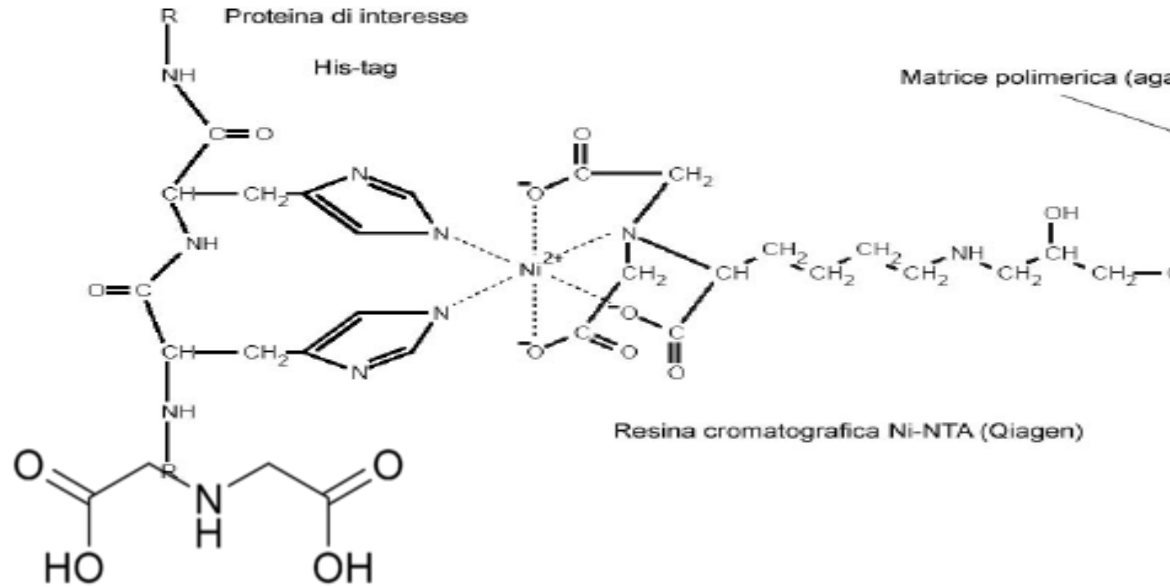
AFFINITY TAG

- **Peptidi o proteine** che possono essere purificate mediante l'interazione **a piccole molecole** immobilizzate sulle resine Ad esempio
- HisTag, glutathione S-transferasi, maltosio binding protein si legano a resine che contengono rispettivamente un metallo chelato, il glutathione ed il maltosio)
- **Peptidi** che possono essere purificati su resine in cui sono immobilizzate le **proteine partners** (calmodulin binding protein) CBP si lega a resine in cui è stata immobilizzata la calmodulina
- **Peptidi** che possono essere purificati su resine in cui sono immobilizzati anticorpi (peptide FLAG etc)

CROMATOGRAFIA DI AFFINITA' SU METALLO CHELATO



**Acido nitriltriacetico
NTA**

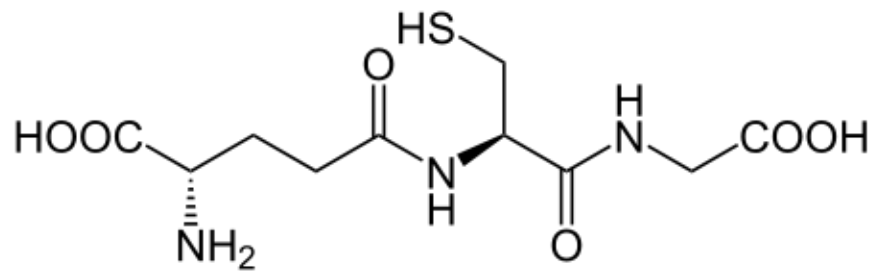


-Tag: serie di sei His

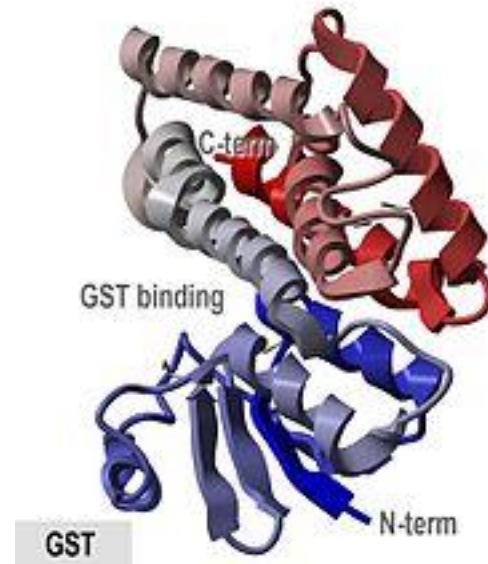
-Il peptide si lega alla resina anche in condizioni denaturanti

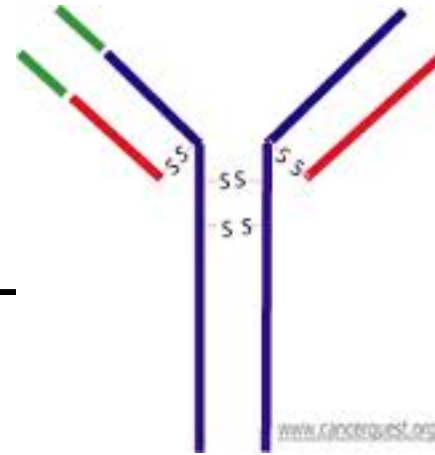
-Eluizione con tampone contenente imidazolo a anche variando il pH

Glutatione S trasferase



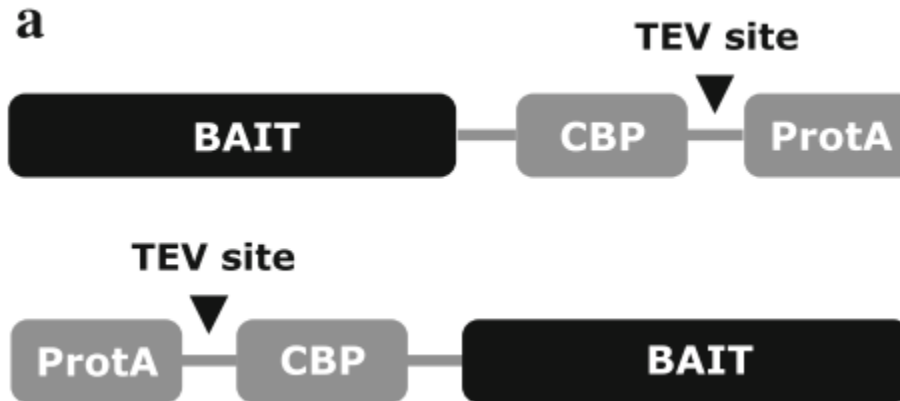
γ -L-glutamyl-L-cisteinilglicina





DYKDDDDK	bynthetic FLAG	N, C terminal	anti-FLAG M1
EQKLISEEDL	human c-Myc	N, C terminal	9E10
MASMTGGQQMG	T7 gene 10	N terminal	T7.Tag Ab
QPELAPEDPED	HSV protein D	C terminal	HSV.Tag Ab
RPKPQQFFGLM	substance P	C terminal	NC1/34
YPYDVPDYA	influenza HA1	N, C terminal	12CA5

TAP TANDEM AFFINITY PURIFICATION tag consiste di due IgG binding domains (**proteina A** di *Staphylococcus aureus*) un peptide che lega la calmodulina (**CBP**) separati da un sito di taglio di per una proteasi TEV



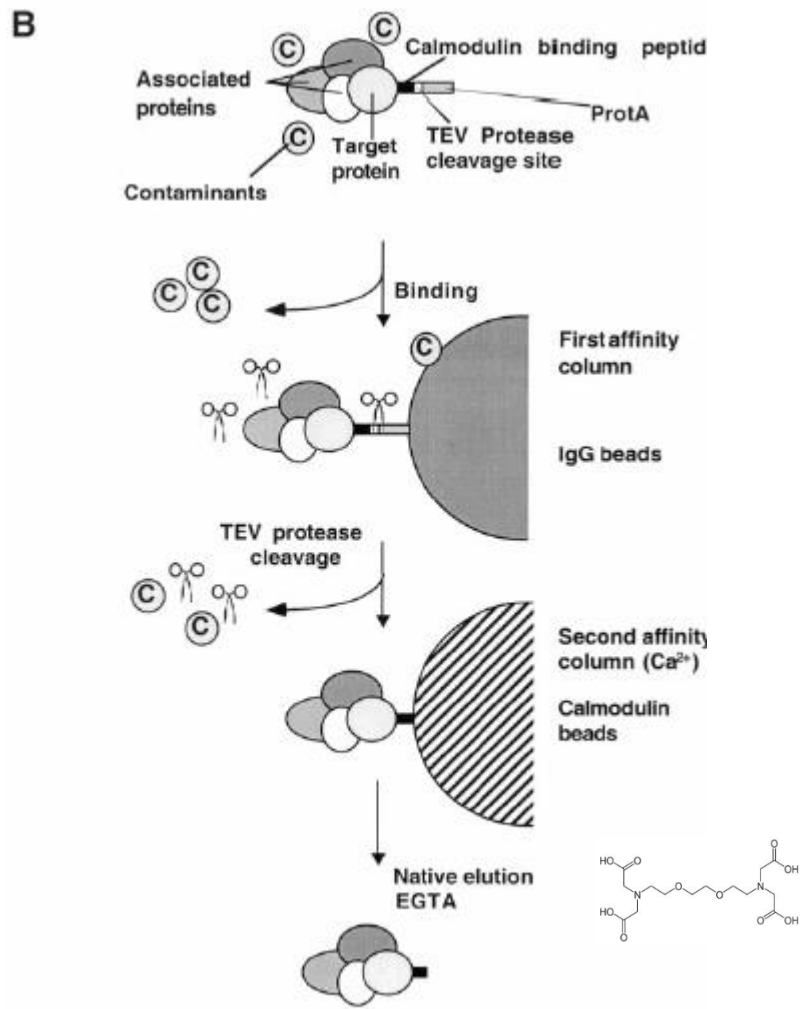


FIG. 1. (A) Schematic representation of the C- and N-terminal TAP tags. (B) Overview of the TAP purification strategy.

Functional organization of the yeast proteome by systematic analysis of protein complexes,

Gavin AC et al.
Nature 415, 141-147 (2002) |

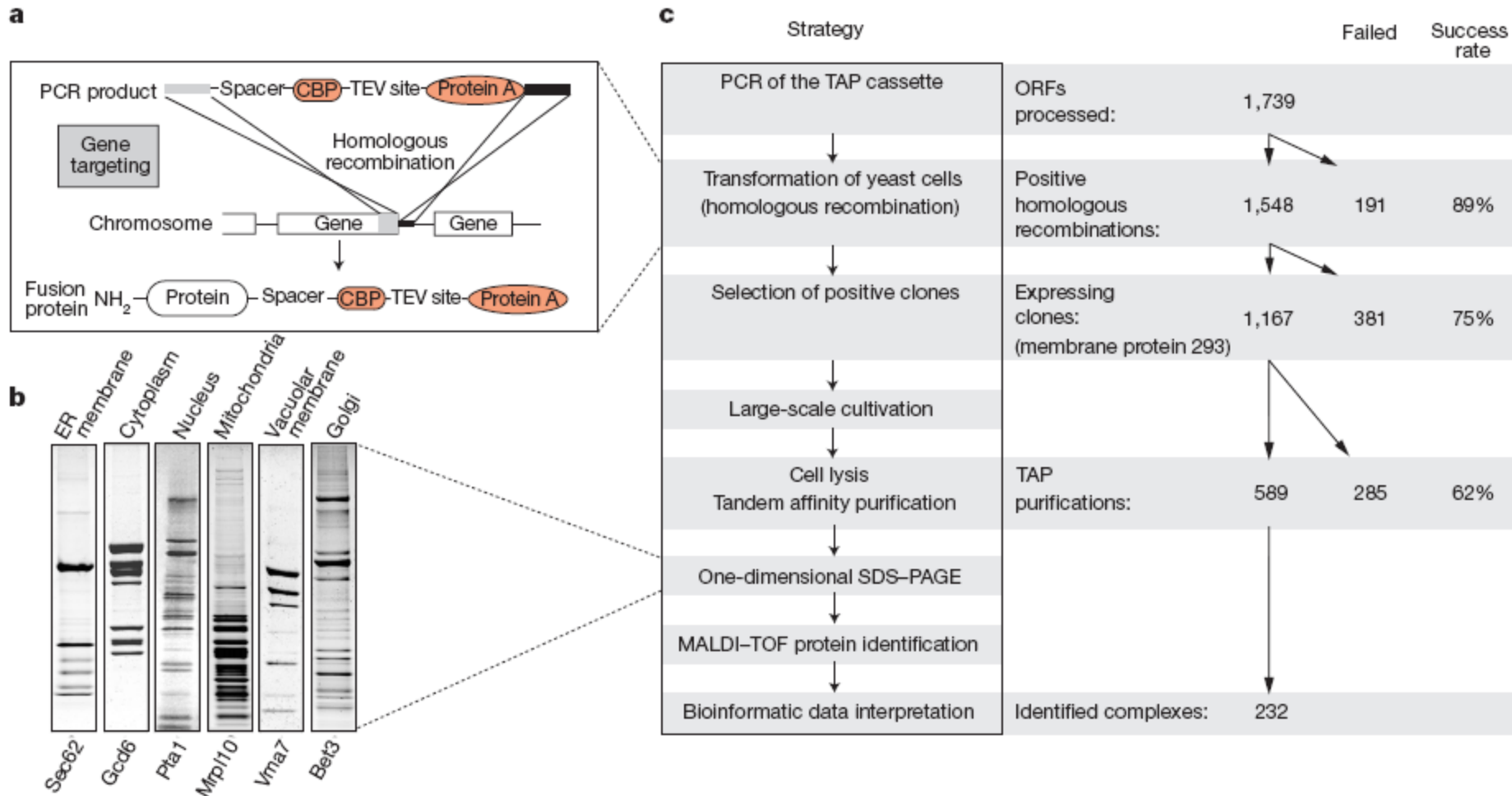


Figure 1 Synopsis of the screen. a. Schematic representation of the gene targeting strategy. b. Protein gel and stained with Coomassie. Tagged proteins are indicated at the top.

Table 1 General features of the TAP tags discussed in the text

TAP tag	Approximate size (kDa)	Recovery (%)	Cleavage site	References
ProtA-CBP	21	20–30 ^a	TEV	Rigaut et al. (1999)
SBP-CBP	8	NA ^b	None ^c	Braman et al. (2007)
FLAG-HA	3	NA	None	Zenser et al. (2008)
3× FLAG-His	3	10–20	None	Yang et al. (2006)
ProtA-ProtC	19	10–20	TEV	Schimanski et al. (2005)
ProtG-SBP	19	5	TEV	Bürcstümmer et al. (2006)
2× FLAG-ProtA	19	5–30	TEV	Tsai and Carstens (2006)
His-2× Strep II	6	16	2× TEV	Giannone et al. (2007)
2× Strep II-FLAG	5	27–48	None	Gloeckner et al. (2007)
SBP-HA	5	30–40	None	Glatter et al. (2009)
SBP-His	8 + 1 ^d	>50	None	Li et al. (2011)
His-biotin	10	NA	None	Tagwerker et al. (2006)
GFP-S/His ^e	36	NA	TEV/HRV3C ^f	Cheeseman and Desai (2005)

¹ The average value in yeast (the corresponding value in higher eukaryotes is usually much lower)

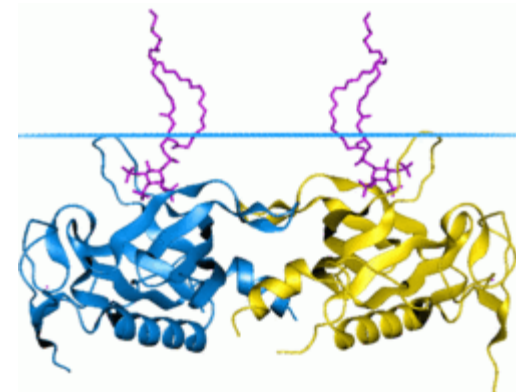
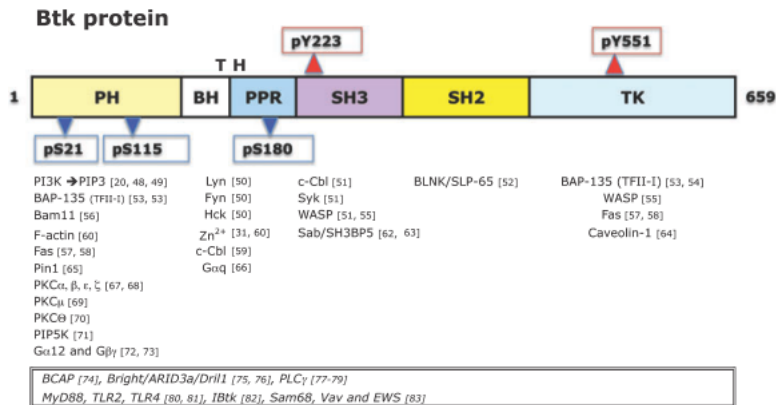
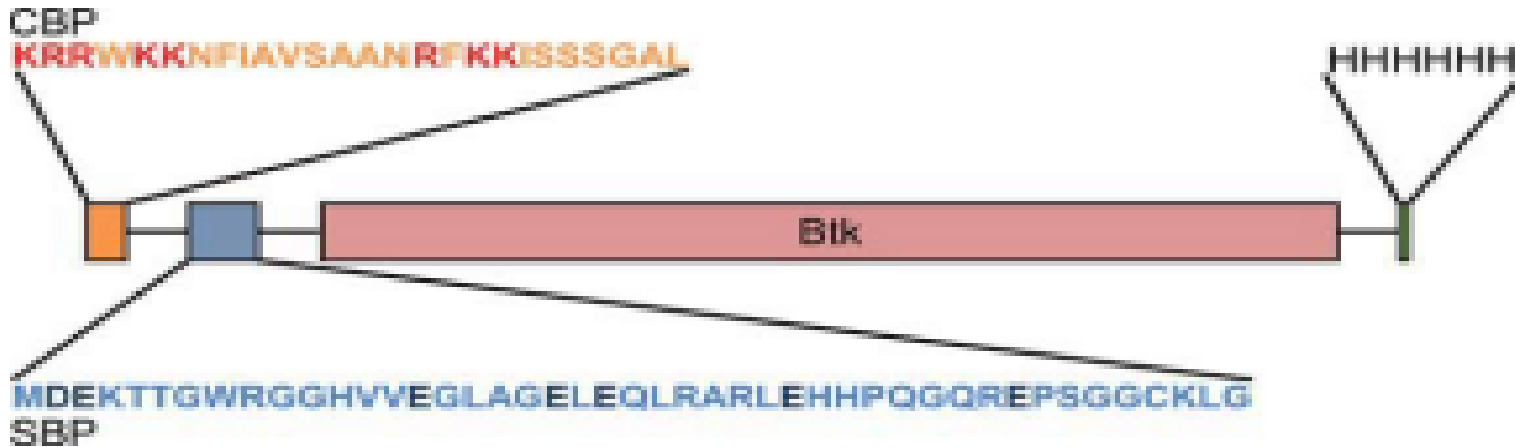
² NA not available

³ Enzymatic cleavage is not required

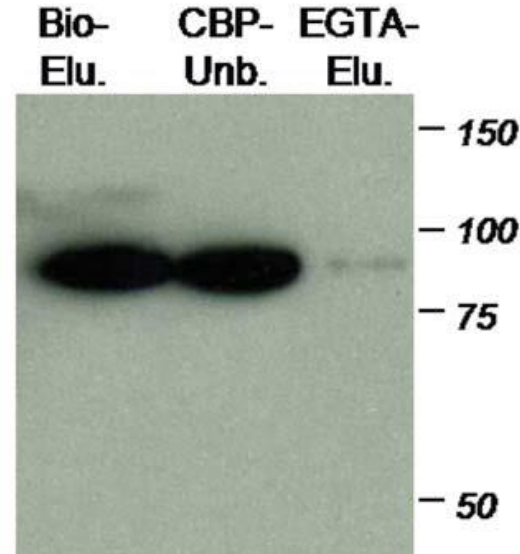
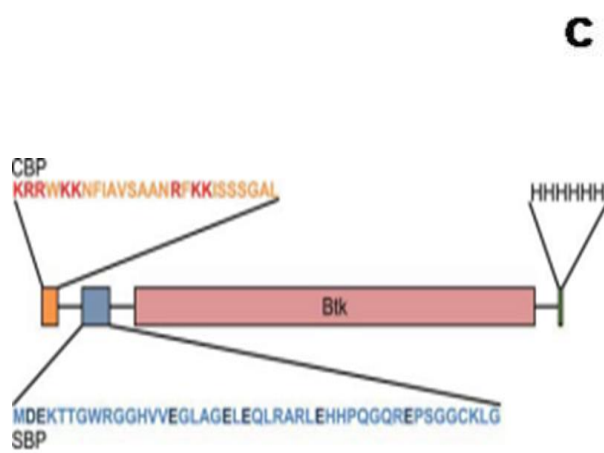
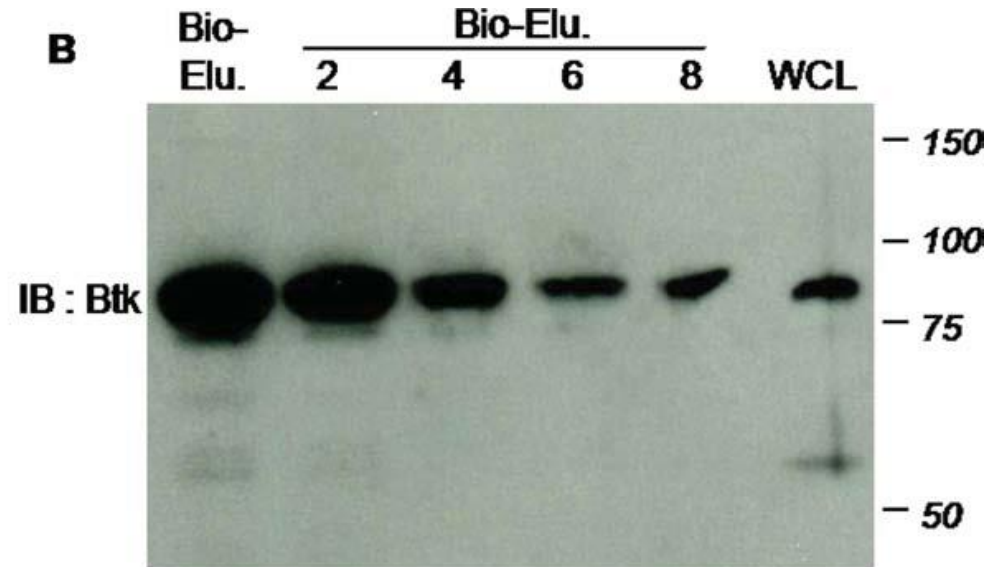
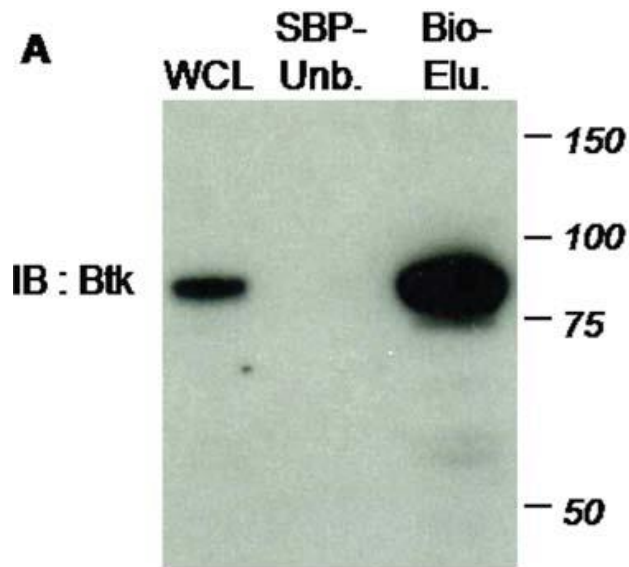
Protein Sci. 2011 Jan;20(1):140-9.

Highly efficient purification of protein complexes from mammalian cells using a novel streptavidin-binding peptide and hexahistidine tandem tag system: application to Bruton's tyrosine kinase.

Li Y, Franklin S, Zhang MJ, Vondriska TM.



Dual streptavidin- and calmodulin-based purification



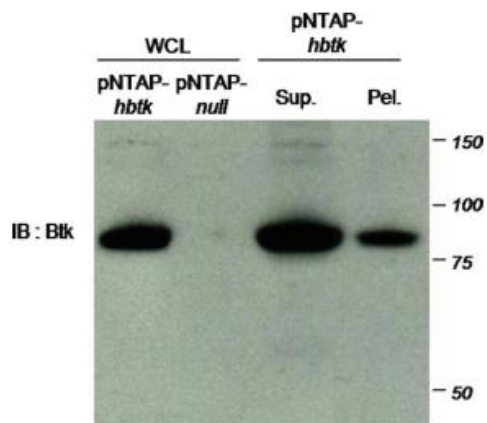
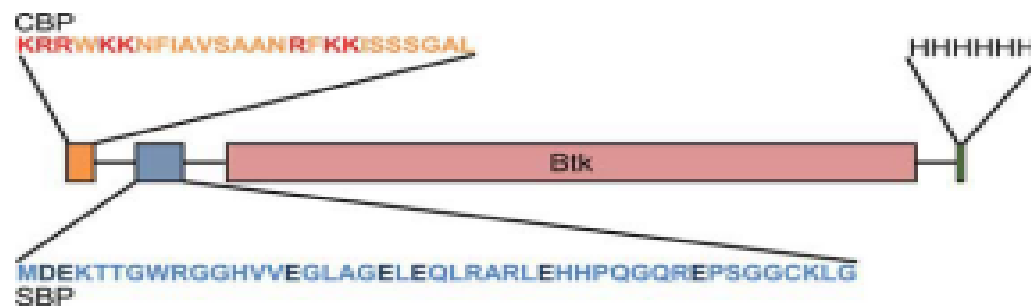
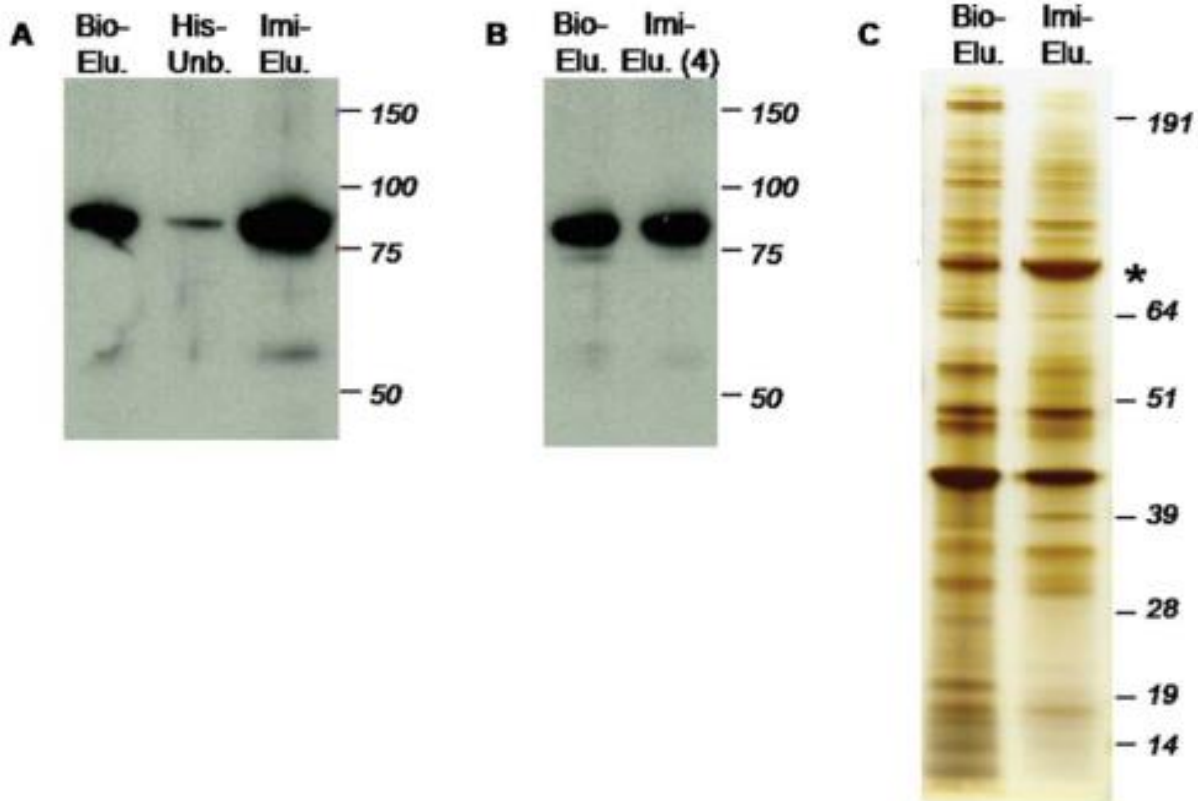


Figure 2. Western blot analysis of human Btk expression and extraction. Whole cell lysate (WCL) of pNTAP-*hBtk*- or empty vector- (pNTAP-null) transfected HEK-293 cells were separated along with supernatant (sup) or pellet (pel) extraction from pNTAP-*hBtk* transfected cells, followed by immunoblotting for Btk. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]



Dual streptavidin- and nickel-based purification



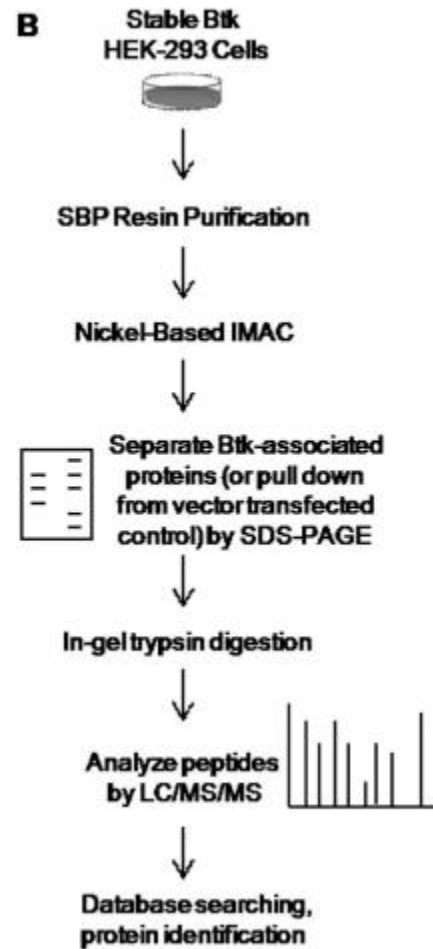
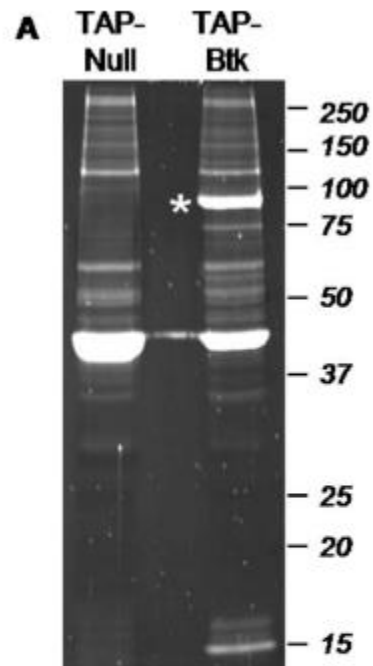


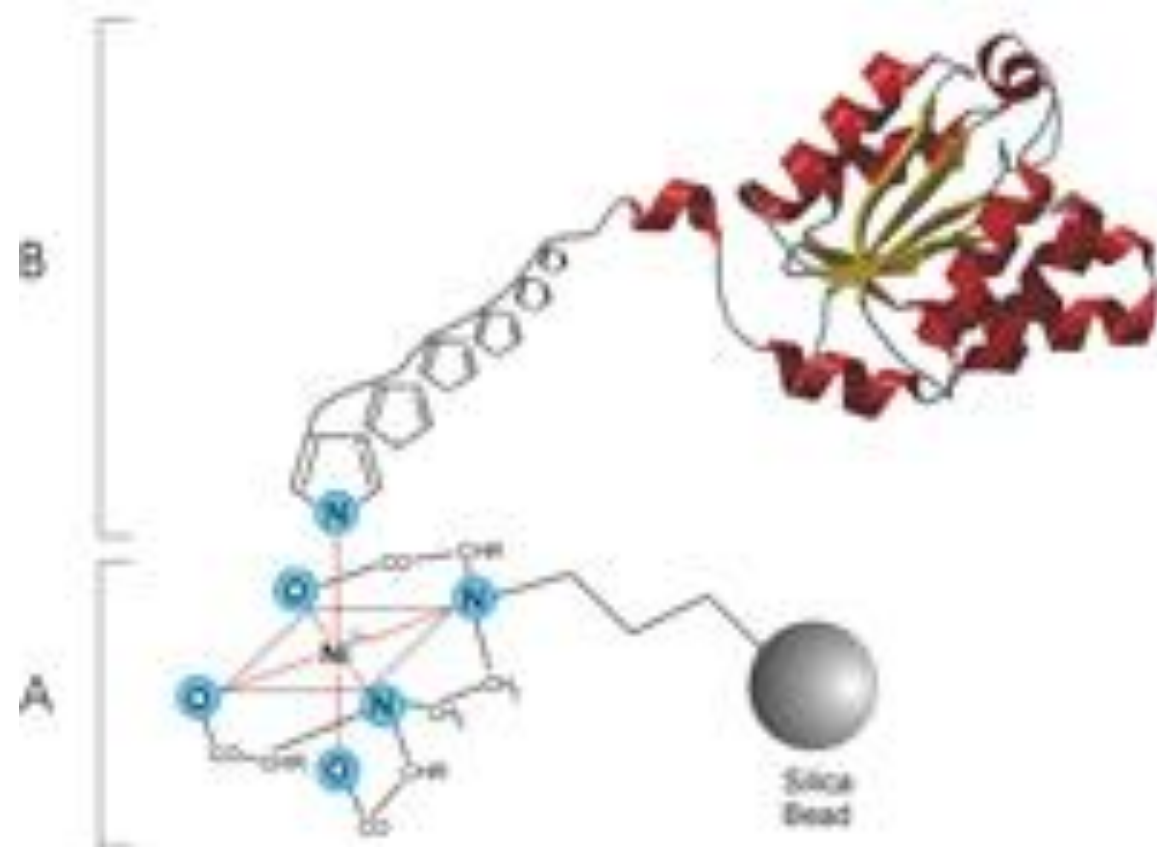
Table II. *Btk Interacting Proteins Identified by TAP and Mass Spectrometry*

UniProt/IPI	Protein Name	MW ^a	Xcorr ^b	Known Function ^c
P23396/IPI00011253	40S ribosomal protein S3	26,671	90.3	Protein translation machinery
Q9Y4W6/IPI00001091	AFG-like protein	88,528	40.2	ATP-dependent protease
P68133/IPI00021428	Alpha skeletal actin	42,023	216.4	Contraction
P12814/IPI00013508	Alpha-actinin	102,993	60.2	Myofilament (actin) anchoring protein
P25705/IPI00440493	ATP synthase alpha subunit	59,713	210.3	ATP production in mitochondria
P06576/IPI00303476	ATP synthase beta subunit	56,524	140.3	ATP production in mitochondria
Q9BYX7/IPI00888712	Beta-actin	41,988	178.3	Cytoskeleton
B4DMH3/IPI00798401	Coronin-1C	49,347	30.3	Cytokinesis, cell motility
O00571/IPI00215637	DDX3X, ATP-dependent RNA helicase	73,198	30.2	Nucleotide-binding, RNA processing
Q16643/IPI00003406	Drebrin	71,381	30.3	Cell migration
Q05639/IPI00014424	Elongation factor 1 alpha	50,438	30.2	Promotes t-RNA binding to ribosome
P21333/IPI00302592	Filamin-A	279,841	30.2	Anchors cytoskeleton to membrane
P04406/IPI00219018	Glyceraldehyde-3-phosphate dehydrogenase	36,030	40.2	Glycolytic enzyme
Q86YZ3/IPI00398625	Hornerin	282,226	40.3	Epidermis formation
P13647/IPI00009867	Keratin, type II	62,340	250.3	Cytoskeletal structure
Q9H1R3/IPI00221127	Myosin light chain kinase 2	64,644	40.2	Regulates contraction of striated muscle
P28331/IPI00604664	NADH-ubiquinone oxidoreductase	80,945	50.3	Mitochondrial respiratory chain
P23246/IPI00010740	SFPQ, splicing factor	76,101	110.2	Nucleotide-binding, spliceosome formation
P02549/IPI00220741	Spectrin, alpha chain	279,998	40.2	Cytoskeletal structure
P40939/IPI00031522	Trifunctional enzyme, alpha subunit	82,947	70.3	Fatty acid metabolism
P68366/IPI00007750	Tubulin, alpha 4A chain	49,892	30.2	Major constituent of microtubules
P68371/IPI00007752	Tubulin, beta 2C chain	49,799	38.3	Major constituent of microtubules
Q12792/IPI00183508	Twinfilin	43,890	40.3	Actin binding, polymerization

^a Molecular weight, as calculated from amino acid sequence.

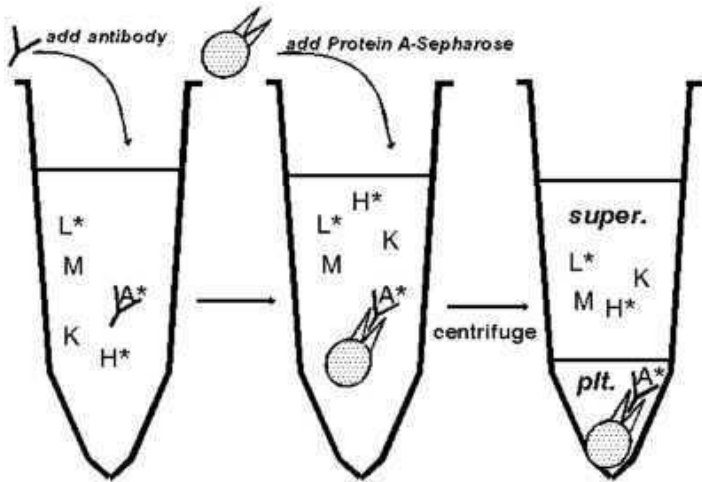
^b As determined by SEQUEST from highest single run in which protein was identified.

^c Determined from UniProt annotation.



IMMUNOPRECIPITAZIONE

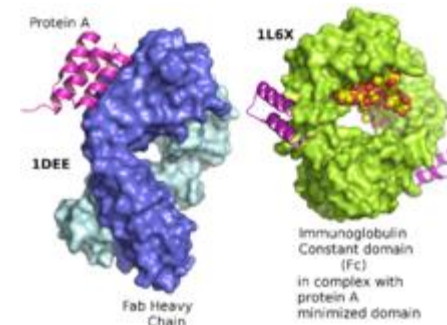
E' un metodo per purificare un antigene proteico utilizzando un anticorpo



- Si fa reagire un anticorpo (monoclonale o policlonale) con un target specifico
- Si aggiunge la proteina A o proteina G immobilizzata su sefarosio
- Si “precipita” l’immunocomplesso per centrifugazione. Ogni proteina non “precipitata” dal supporto- proteina A o G viene lavata via
- Si analizza l’immunocomplesso precipitato

CO-IMMUNO PRECIPITAZIONE

La reazione di immunoprecipitazione può far “precipitare” oltre all’antigene altre proteine che interagiscono con l’antigene



Valutazione co-immunoprecipitazione

I componenti dell' immunocomplesso sono eluiti e analizzati su SDS-PAGE seguita da Western blot per verificare l' identità dell' antigene

- Confermare che le proteine co-precipitate si ottengano solo con l' anticorpo contro la proteina esca**
- Verificare che l' anticorpo non riconosca direttamente le altre proteina coprecipitate**
- Determinare che l' interazione avvenga nella cellula e non sia una conseguenza della lisi**

Affinity blot, Overlay o Far Western blotting

La tecnica prevede la

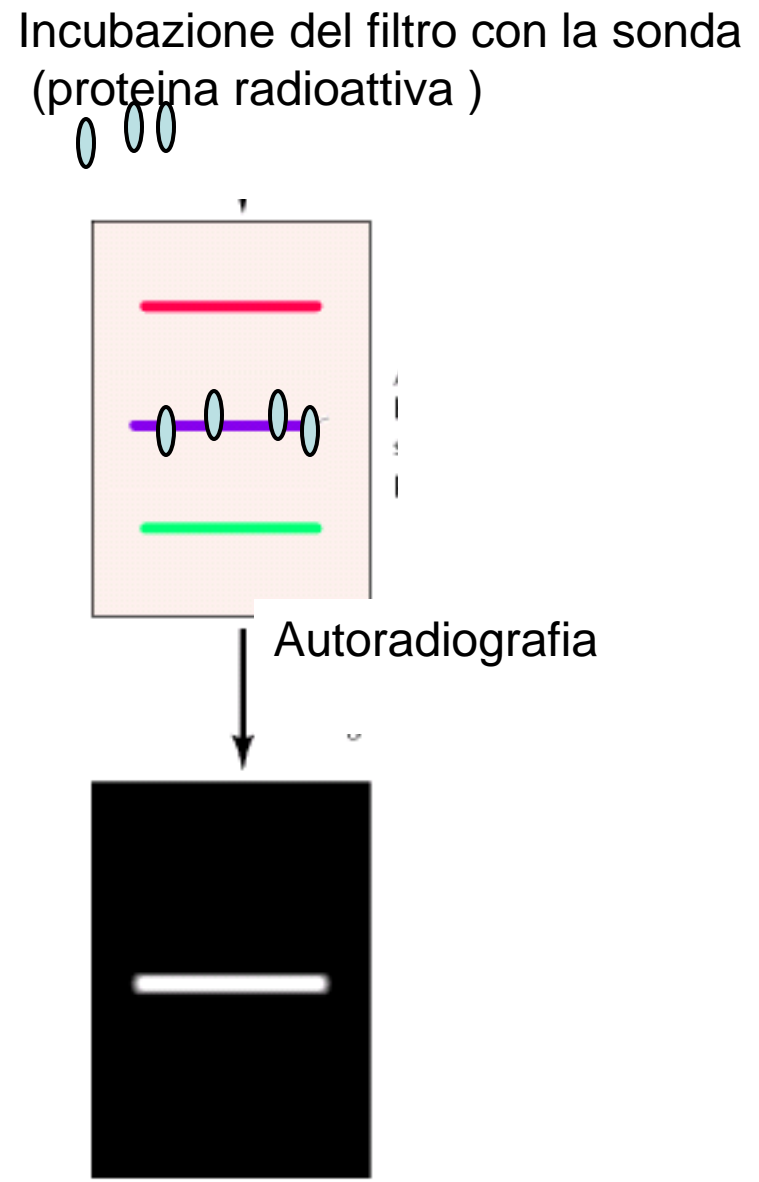
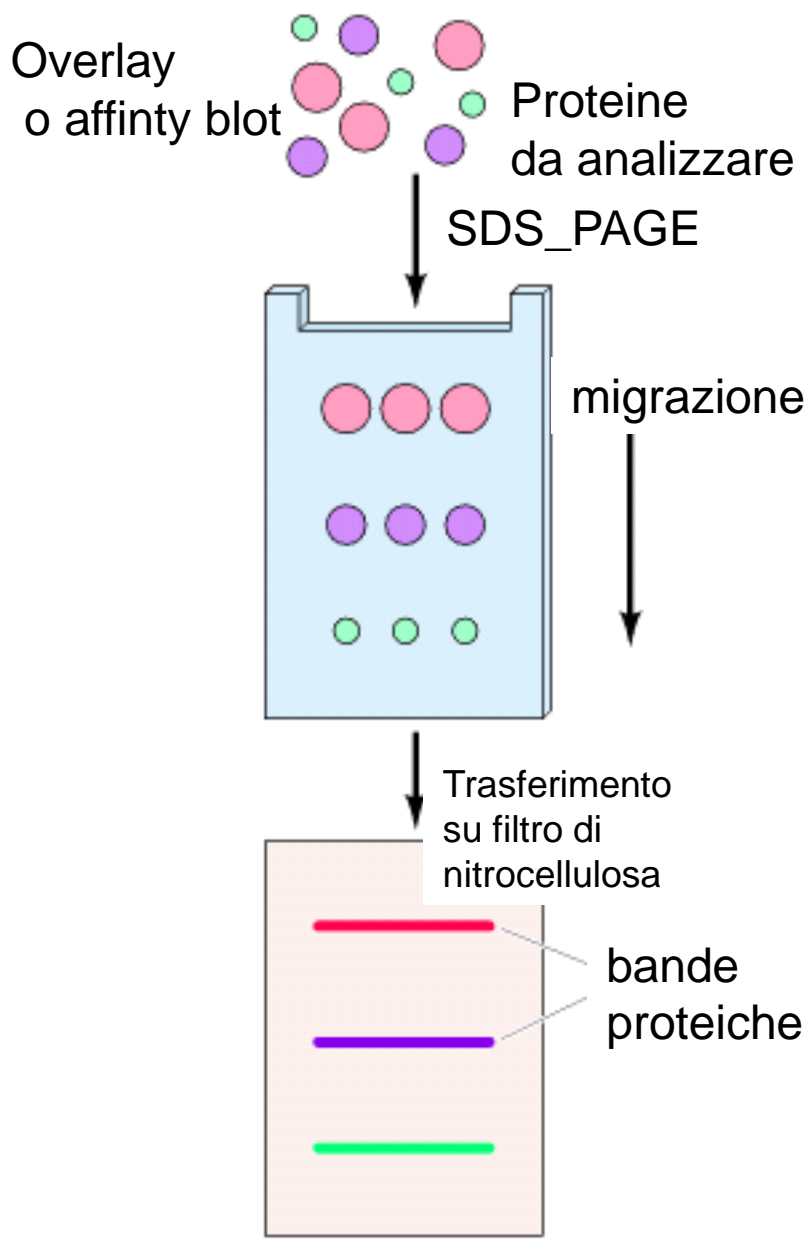
- separazione delle proteine da analizzare in SDS-PAGE
- blotting su nitrocellulosa o PVDF
- ' incubazione con la sonda di interesse (proteina di cui si vuol studiare l' interazione).

La sonda proteica può essere :

- Radioattiva
- Biotinilata, fluorescente
- una proteina di fusione per la quale sono disponibili anticorpi

Limiti:

SDS-PAGE avviene in condizioni riducenti e denaturanti



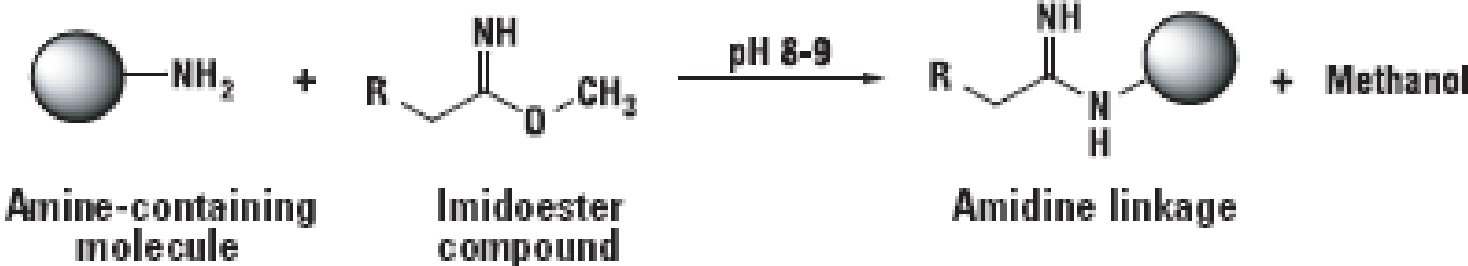
CROSSLINKING

- **I crosslinkers permettono di legare covalentemente due proteine che interagiscono**
- **Sono molecole che contengono due gruppi funzionali reattivi uguali (omobifunzionali) o diversi (eterobifunzionali) separati da un braccio spaziatore**

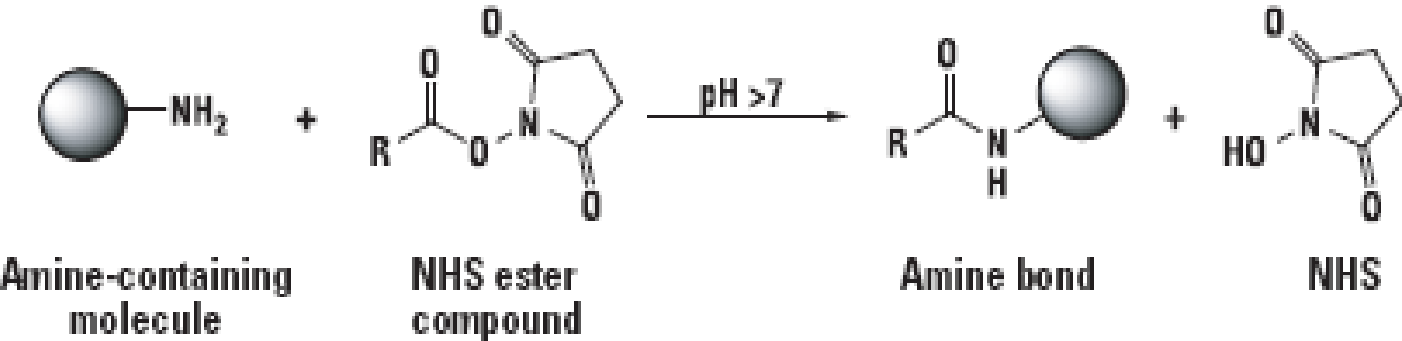
Schemi di reazione dei crosslinker per i gruppi amminici

Immidoesteri

Sono instabili a pH neutro



Esteri dell' N-idrossi-succinimide



Schemi di reazione dei crosslinker per i gruppi sulfidrilici

**Maleimmidi
a pH neutro formano
legami tioetere stabili**

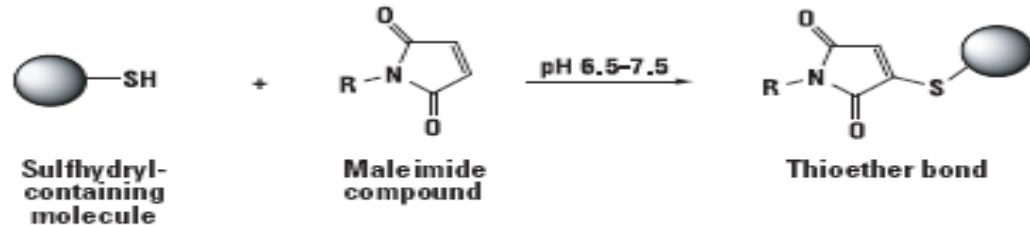


Figure 8. Maleimide reaction scheme.

Alogenuri alchilici

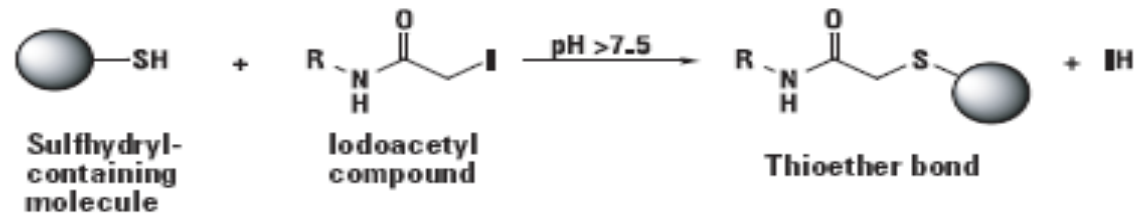


Figure 9. Active halogen reaction scheme.

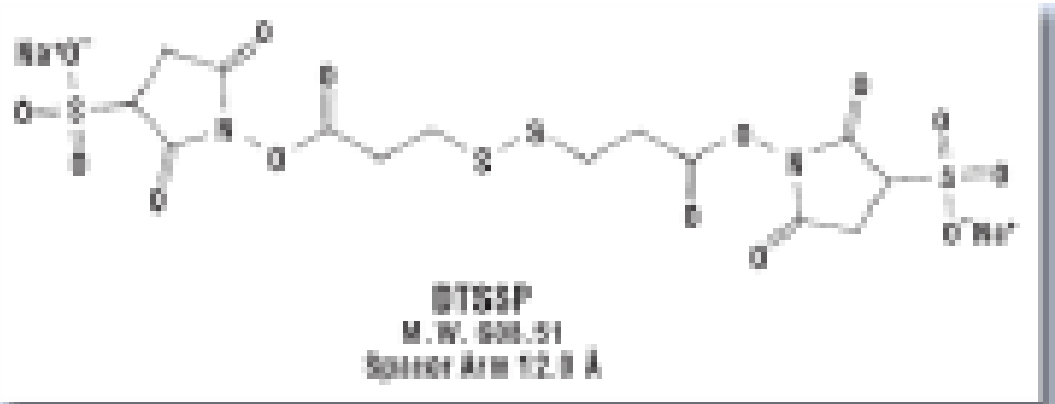
**Piridil-disolfuri
Formano ponti disolfuro.
Il 2-piridil-tione assorbe a
343 nm.**



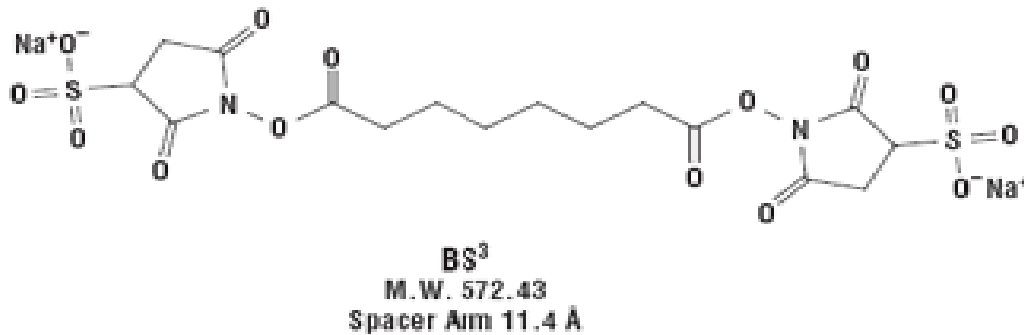
Crosslinker omobifunzionali

DTSSP 3,3'-

Dithiobis(sulfosuccinimidylpropionate)



BS3 Bis(Sulfosuccinimidyl)suberate



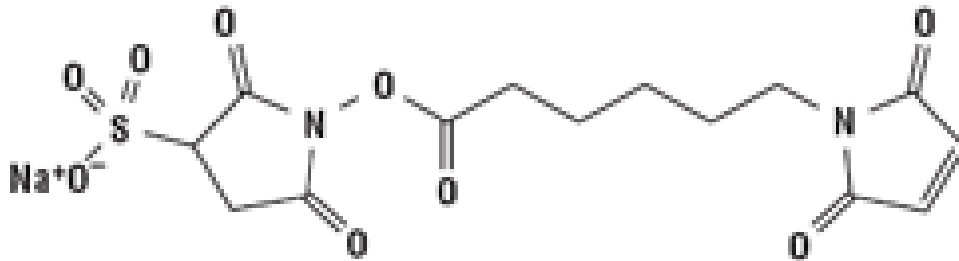
Crosslinker eterobifunzionali

Sulfo-LC-SPDP (Sulfosuccinimidyl 6-(3'-[2-pyridyldithio]-propionamido)hexanoate)



Sulfo-LC-SPDP
M.W. 527.57
Spacer Arm 15.6 Å

Sulfo-EMCS ([N-e-Maleimidocaproyloxy] sulfosuccinimide ester)



Sulfo-EMCS
M.W. 410.33
Spacer Arm 9.4 Å

Aril azidi

In presenza luce UV formano il nitrene una specie fortemente reattiva

Vantaggi

I crosslinker contenenti azidi sono chimicamente inerti finché non vengono sottoposti ad una breve esposizione a raggi UV. Gli intermedi reattivi hanno una vita molto breve e reagiscono indiscriminatamente per formare legami covalenti.

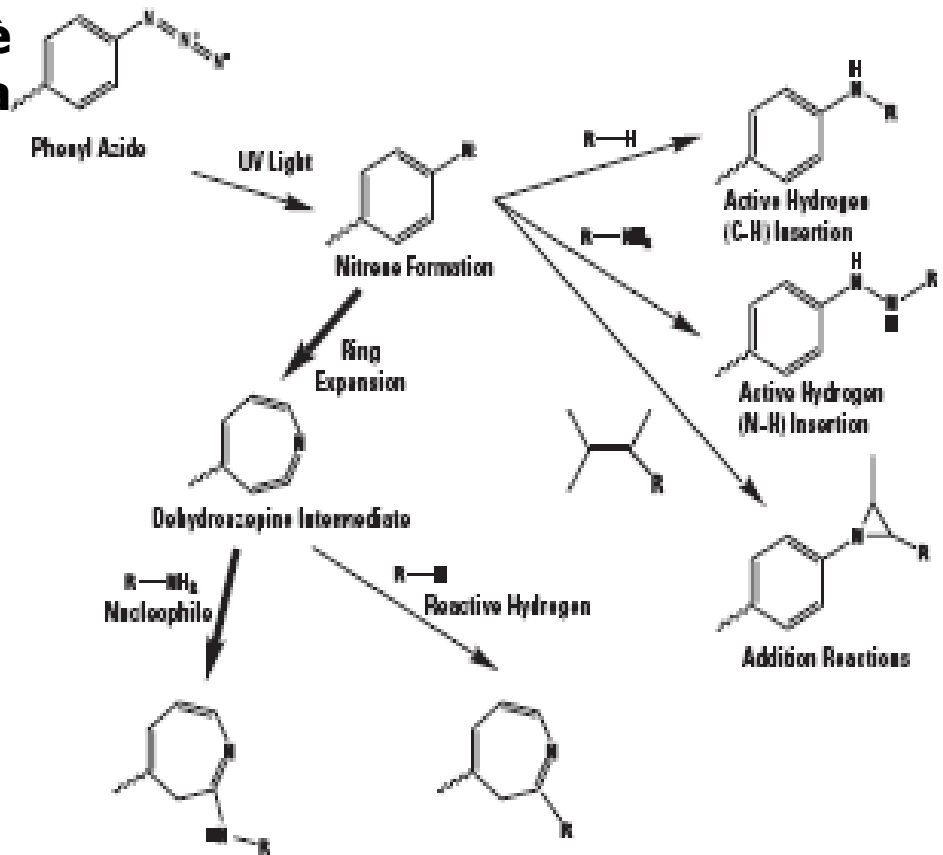


Figure 14. Possible reaction pathways of aryl azide crosslinkers.

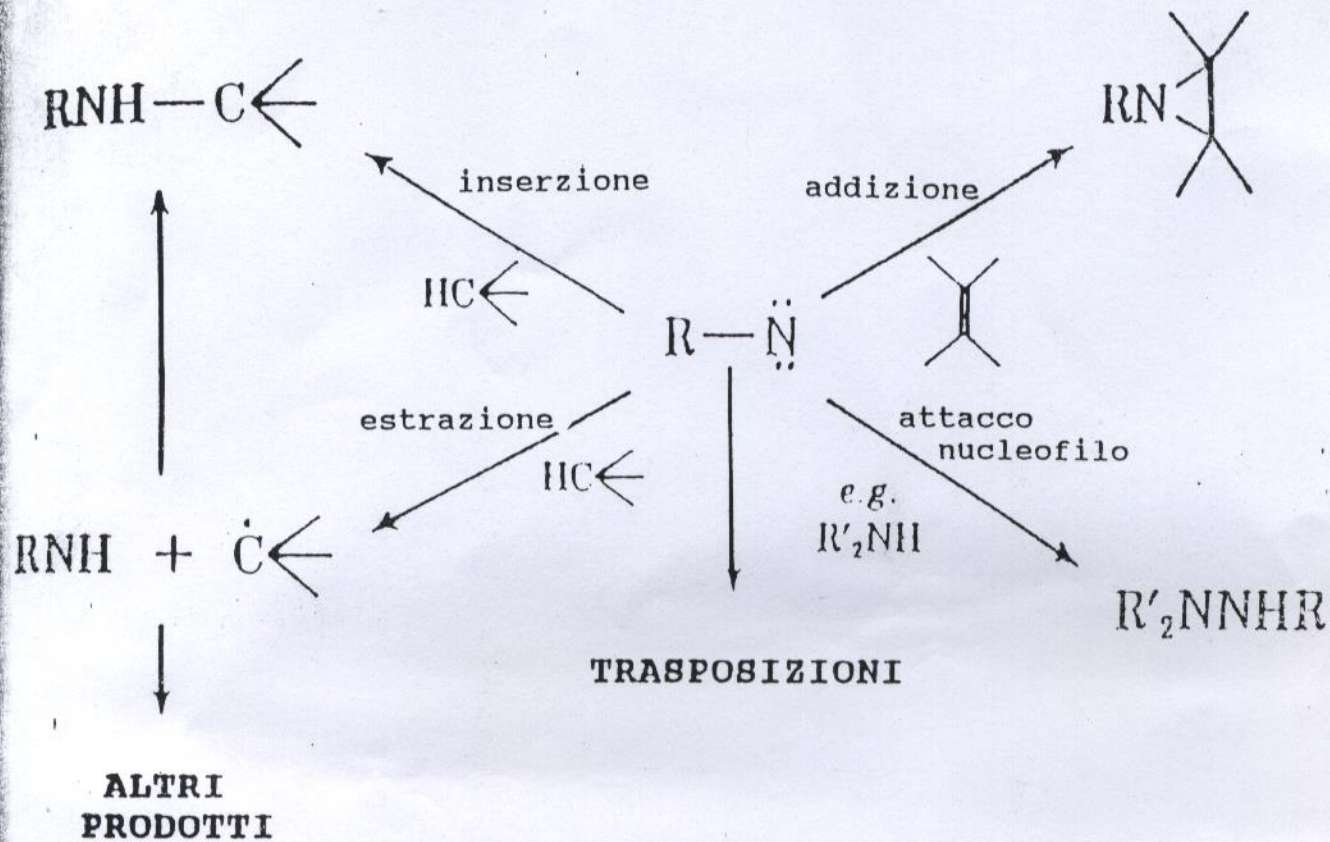
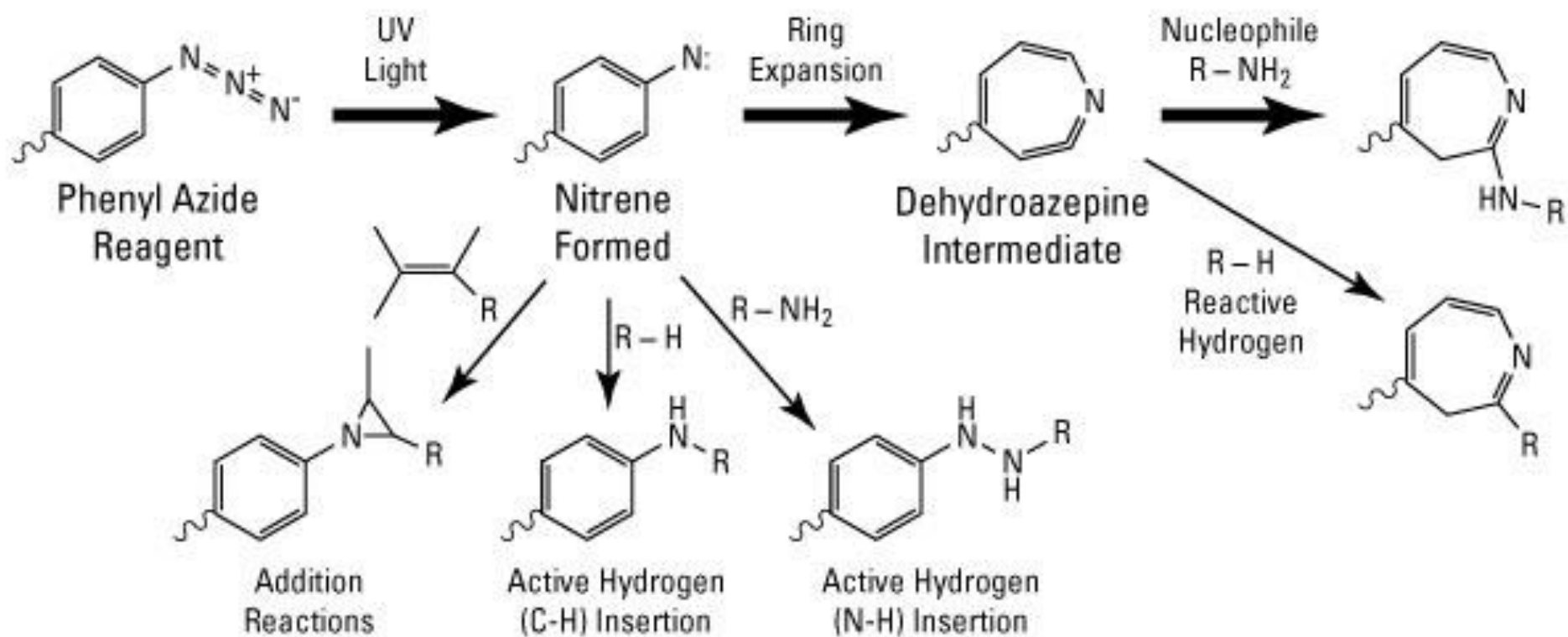
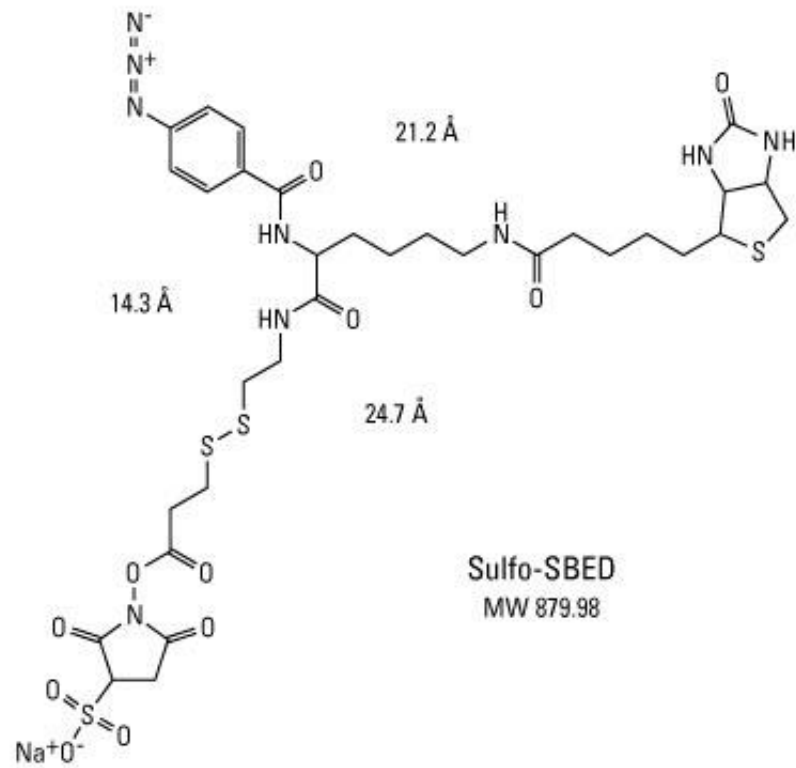


Fig. 4 Possibili reazioni del nitrene



- Possibili reazioni del nitrene
- Inserzione su legami C-H
- Estrazione di un atomo di idrogeno dal carbonio seguita da accoppiamento radicalico
- Addizione
- Attacco nucleofilo



Criteri di scelta dei crosslinkers

- **Specificità chimica**
- **Lunghezza del braccio spaziatore**
- **Solubilità in acqua e permeabilità alle membrane**
- **Gruppi reattivi uguali (omobifunzionali) o differenti(eterobifunzionali)**
- **Gruppi termoreattivi o fotoreattivi**
- **Crosslink reversibile o irreversibile**
- **Possibilità di marcatura radioattiva**