



Labeling of biomolecules for PET imaging

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Antibodies:

- Immunization, vaccination and antisera
- Somatic rearrangements in B cells
- Monoclonal antibody strategy
- Antibody fragments
- Antibodies in imaging
- Other proteins, peptides and aptamers relevant for imaging
- Reactive groups and label attachment strategies
- Labeling via recombinant and metabolic approaches
- Pretargeting

PET Radioisotopes:

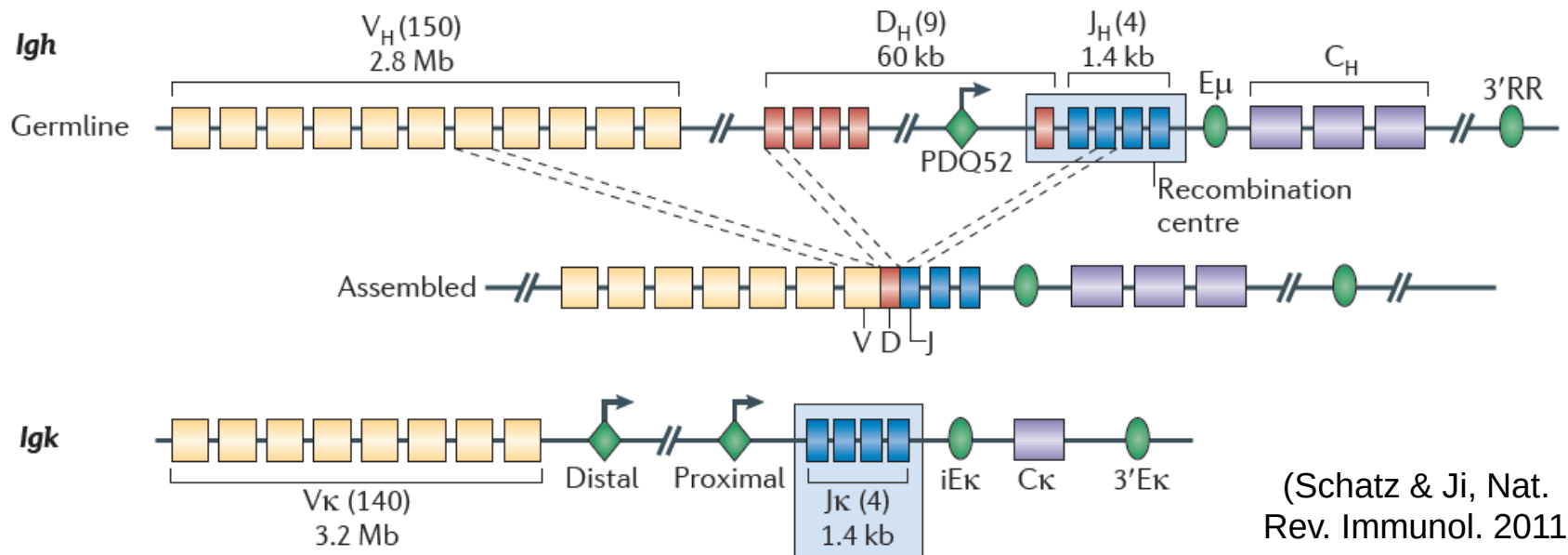
- Complex chemistry – oligodentate ligands
- Attachment of cations to biomolecules
- Strategies for ^{68}Ga , ^{64}Cu , ^{89}Zr and ^{140}Nd

Antibody labeling workflow

- Variolation (18th century): Extract from survivors' smallpox lesions (attenuated virus) can be used to immunize healthy persons (risk of severe infection)
- Vaccination (1796, Edward Jenner):
 - Observation with dairy workers: Cowpox infection is mild in humans, might lead to immunity against smallpox
 - Experimental vaccination AND CHALLENGE of a 8yo boy
- Sera from immunized animals contains factors capable of neutralizing tetanus and diphtheria toxins (Emil von Behring, Nobel Prize 1901)
- Sera from immunized animals can be used to detect the antigens
 - Major breakthrough in biochemistry
- Immunization:
 - Against proteins, peptides, glycans, small molecules
 - Antigen alone may not be sufficient to elicit immune response
 - Monomeric antigens have to be crosslinked (for B cell receptor signalling)
 - Non-protein antigens should be linked to a protein (for T cell epitopes)
 - Adjuvants stimulate the immune cells to produce antibodies
 - Freund's adjuvant: Antigen emulsion in mineral oil, mixed with *Mycobacterium tuberculosis*



- Adaptive immunity: B and T lymphocytes first described in bursa / thymus
- Somatic recombination and hypermutation between gene segments leads to enormous variability of B cell receptors during B cell development (similarly: T cell receptors in T cells).



- Transcription (splicing variants), translation, posttranslational modifications
- Target recognition (and stimulation by T_H cells) leads to differentiation to plasma cells and production of antibodies.

- Vaccination of mice, immortalization and cloning of antibody-producing cells allows generation of antibodies of predefined specificity in unlimited amounts.
- Genes can then be cloned, engineered (e.g. humanized) and heterologously expressed e.g. in CHO or insect cells.



The Nobel Prize in Physiology or Medicine 1984

Niels K. Jerne, Georges J.F. Köhler, César Milstein



Niels K. Jerne



Georges J.F. Köhler

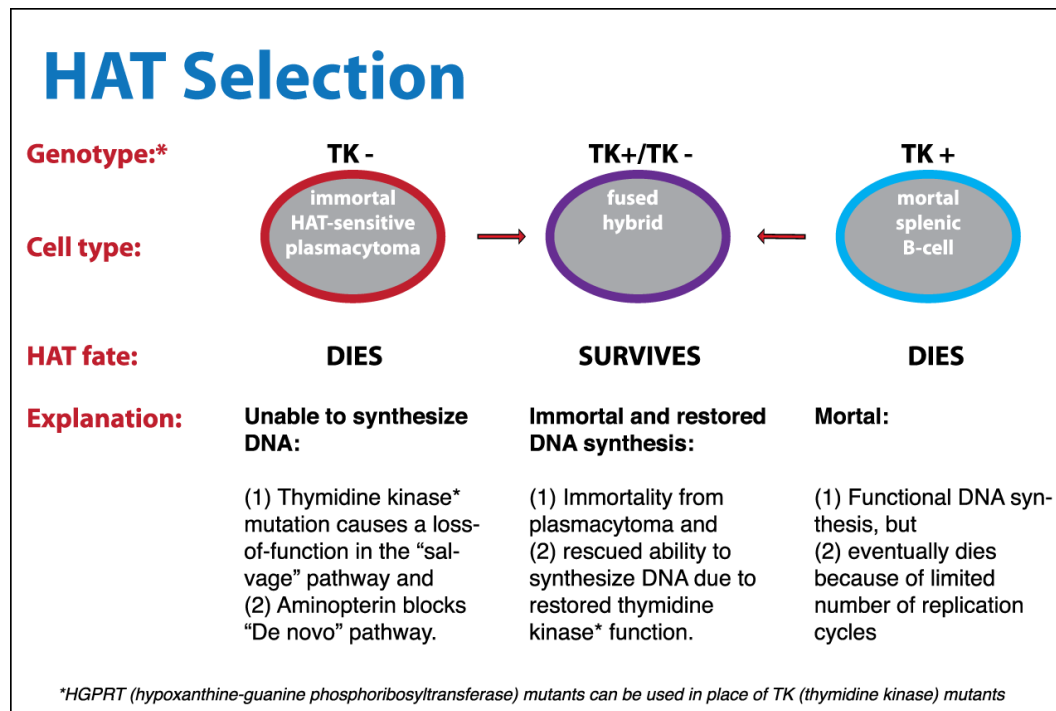


César Milstein

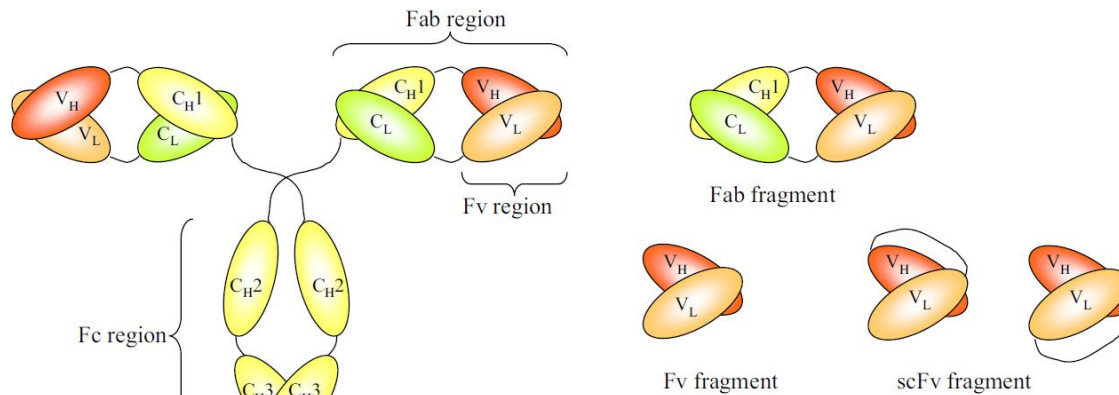
The Nobel Prize in Physiology or Medicine 1984 was awarded jointly to Niels K. Jerne, Georges J.F. Köhler and César Milstein *"for theories concerning the specificity in development and control of the immune system and the discovery of the principle for production of monoclonal antibodies"*.



- After immunization, the spleen is disrupted and cells are washed out.
- Splenic B cells (mortal) are fused with myeloma (=plasmacytoma) cells (immortal but HGPRT⁻ or TK⁻).
- When cultured in medium containing aminopterin (folate antagonist, blocks DNA *de novo* synthesis), hypoxanthine and thymidine (enabling salvage pathway requiring HGPRT and TK), only fused cell clones grow.
- Clones and subclones are screened for secretion of suitable antibodies.



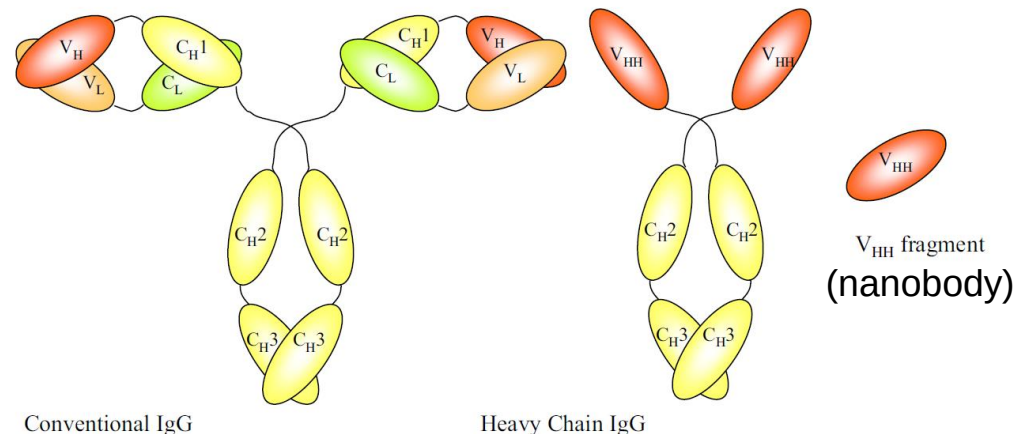
Mammal antibodies



Conventional IgG

Depending on the constant part: IgA, IgD, IgE, IgG, IgM with respective subtypes...

Camel and lama antibodies (in addition):



Conventional IgG

Heavy Chain IgG

V_{HH} fragment
(nanobody)

Joosten et al., *Microbial Cell Factories* 2003, 2:1



Sukhanova et al. Protocol Exchange 2012
doi:10.1038/protex.2012.042

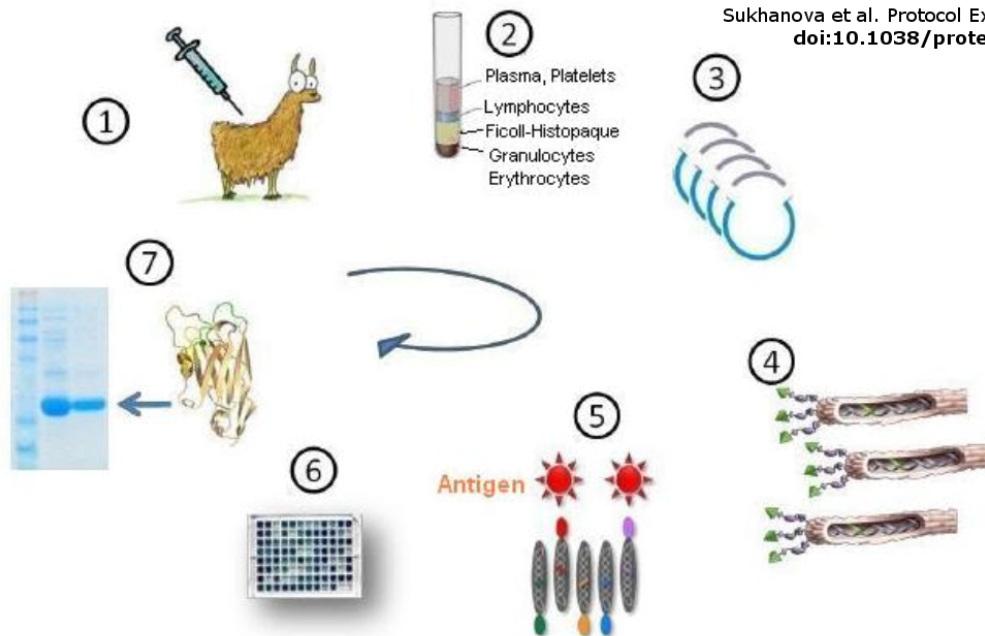
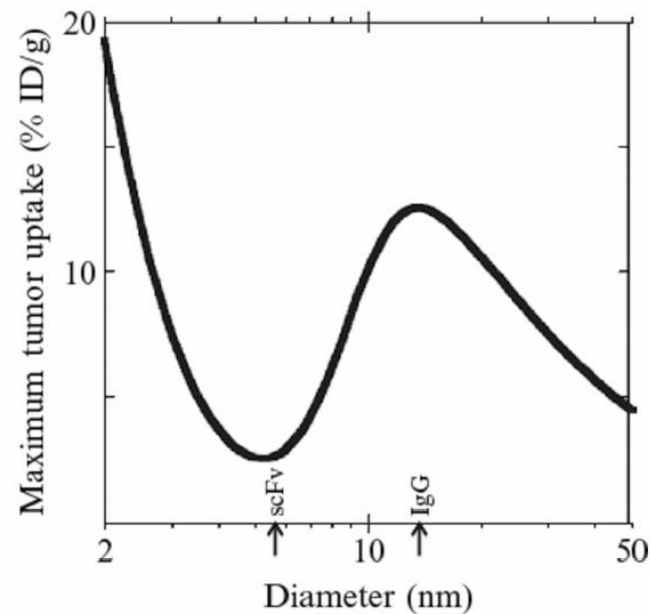
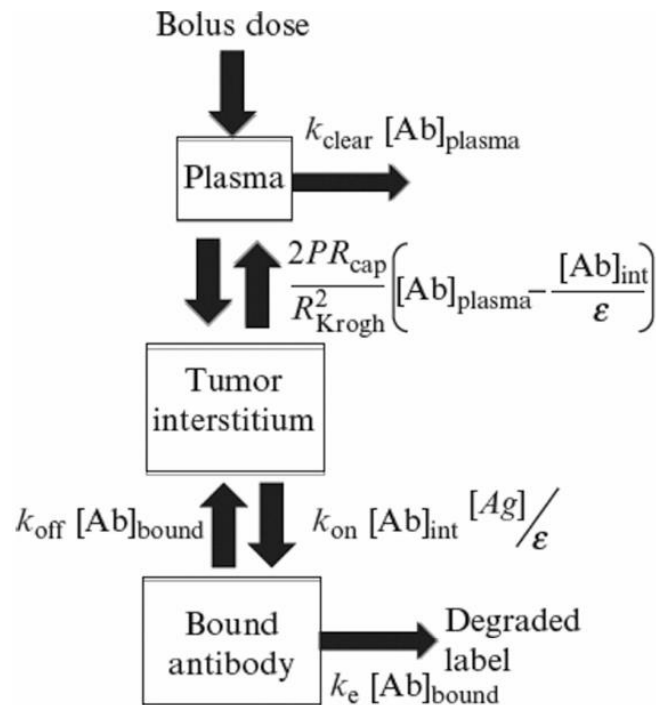


Figure 5. Schematic overview describing the selection of single-domain antibodies.

- (1) Llamas are immunized with the antigen of interest.
- (2) Peripheral blood mononuclear cells are recovered from blood using density gradient centrifugation (ficoll).
- (3) A pool of cDNA coding for sdAb genes is amplified by RT-PCR from a total RNA preparation and cloned into a phagemid vector.
- (4) A helper phage is used to produce a library of phage particles carrying sdAbs on their surface by fusion to the minor coat protein p3.
- (5) This phage-sdAb library is enriched with binders by incubation with immobilized antigen, washing and elution.
- (6) A monoclonal screening assay allows the identification of the binders.
- (7) Soluble sdAbs corresponding to positive phage-sdAbs are produced, purified from *E. coli* and analyzed by SDS-PAGE.

- Smaller fragments:
 - Faster diffusion and imaging
 - Faster excretion, lower serum half time, lower tissue uptake

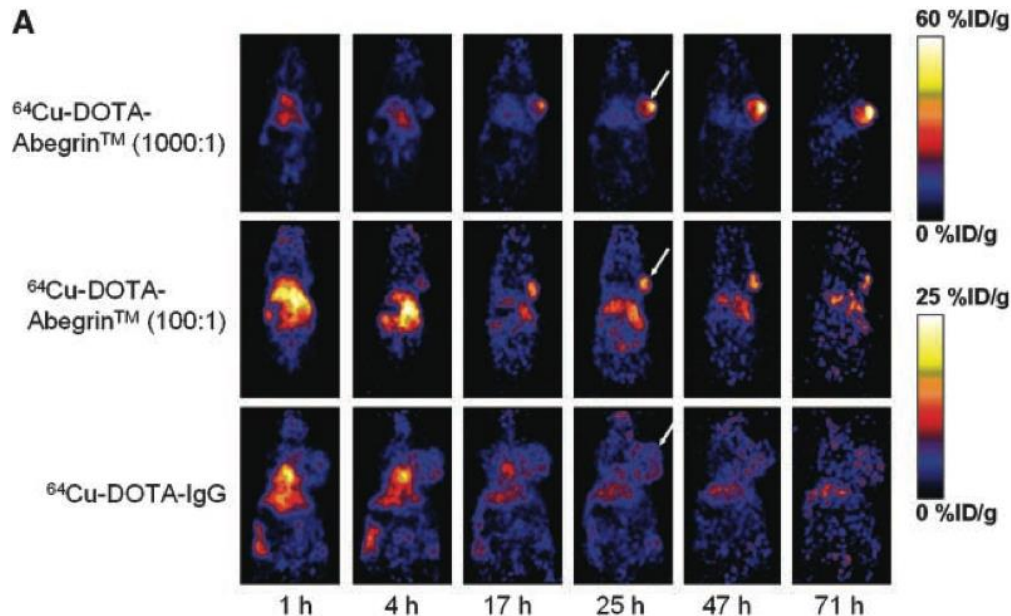
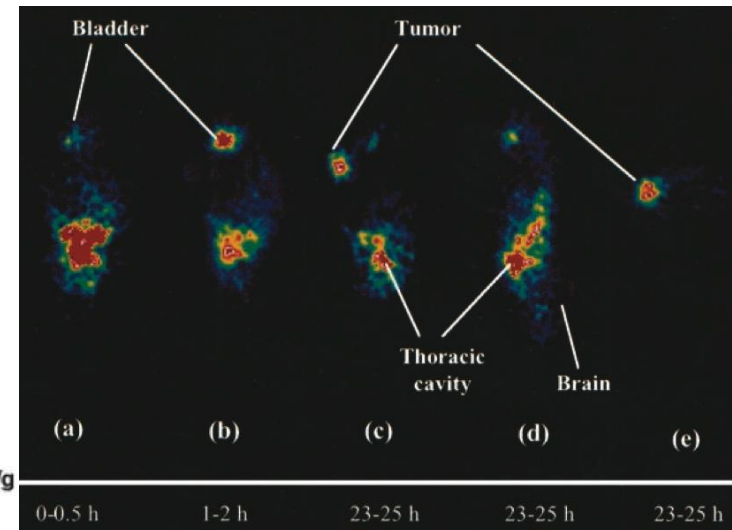


Dane Wittrup, *Methods Enzymol.* 2012 ; 503: 255–268.



Angiogenesis

- anti-VEGF
(Vascular Endothelial Growth Factor)
 ^{124}I -labeled monoclonal antibody
(Collingridge *et al.*, Cancer Res. 2002)



- anti-Integrin $\alpha_v\beta_3$
(Cai *et al.*, Cancer Res. 2006)



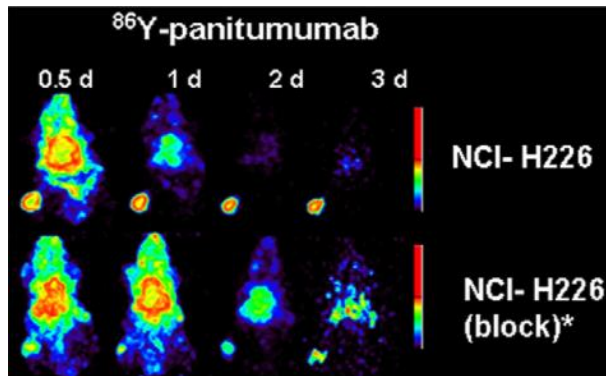
Immunology/Inflammation

- anti-CD20 in Rheumatoid Arthritis
 ^{99m}Tc -labeled rituximab
(Malviya *et al.*, Mol. Imaging Biol. 2012)

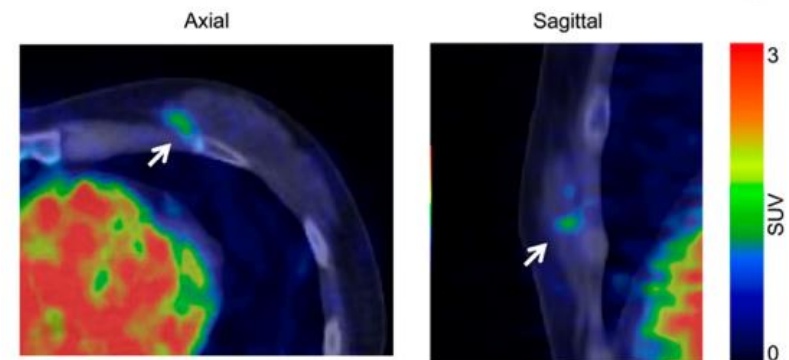


Hematology/Oncology

- anti-CD20 (Non-Hodgkin Lymphoma)
- anti-EGFR family (HER1, HER2/neu, ...)



Xenograft model
(Nayak *et al.*, PLoS ONE 2011)



^{64}Cu -labeled trastuzumab, breast cancer
(Tamura *et al.*, J Nucl Med 2013)

Advantages:

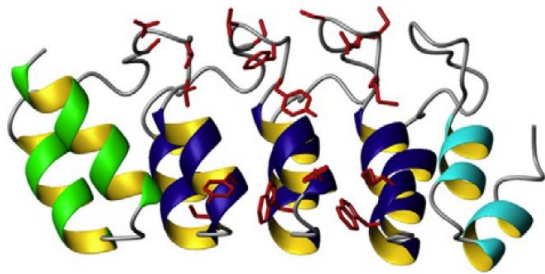
- Universal - possible for many proteins
- Easy - Often therapeutic antibodies available
- Specific - Selectivity between homologous proteins

Pitfalls:

- Only for extracellular proteins
- Large proteins - diffusion limits
- Species specific (Xenograft or transgenic models)

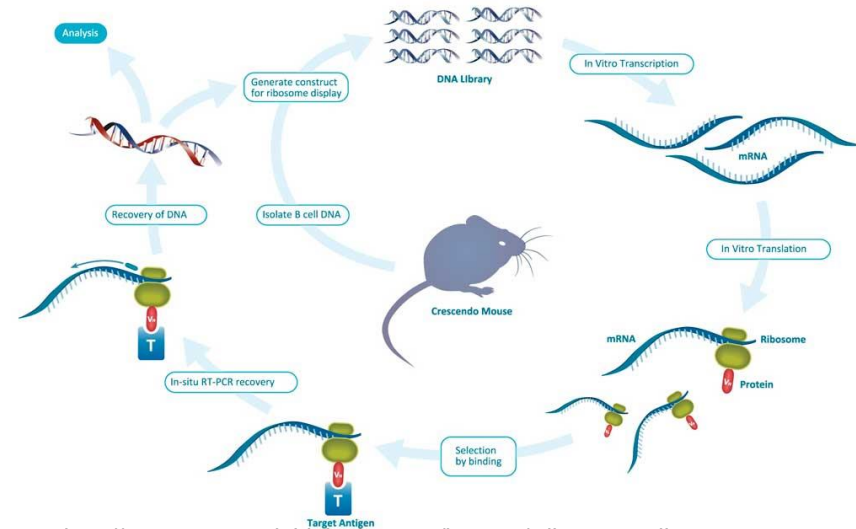
- Proteins

- DARPinS (Designed Ankyrin Repeat Proteins, 14 kDa): Affinity-based selection from libraries by ribosome display (DNA→RNA→protein)
- Chemokines... for receptor imaging
- Lectins for carbohydrate detection



Methods in Enzymology, Volume 503

ISSN 0076-6879, DOI: 10.1016/B978-0-12-396962-0.00005-7

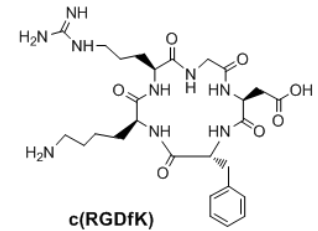


http://www.crescendobiologics.com/images/Ribosome_diagram_v2.JPG

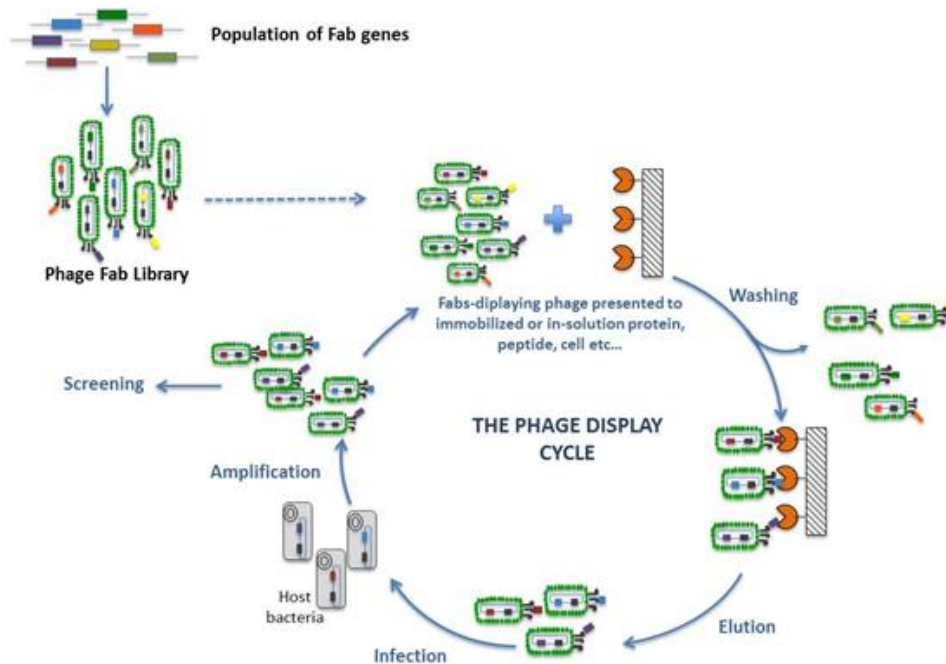
Lectin	Mitogenic Activity	Blood Group Specificity	Preferred Sugar Specificity	Inhibitor or Eluting Sugar	Special Application
Peanut	No	T antigen (M, N)	Galβ3GalNAc	Gal	Detection of relocalization of Tag antigen in large bowel carcinoma
<i>Phaseolus vulgaris</i> Erythroagglutinin (PHA-E)	Yes	A(-SA)	Galβ4GlcNAcβ2Manα6 (GlcNAcβ4) (GlcNAcβ4Manα3) Manβ4	bovine thyroglobulin, acetic acid	Binding to central nervous system cells (HNK-1 antigen)
<i>Phaseolus vulgaris</i> Agglutinin (PHA-E+L)	Yes	A(-SA)	See PHA-E/PHA-L	bovine thyroglobulin, acetic acid	Binding to central nervous system cells (HNK-1 antigen)
<i>Phaseolus vulgaris</i> Leucoagglutinin (PHA-L)	Yes	–	Galβ4GlcNAcβ6(GlcNAcβ2Manα3)Manα3	bovine thyroglobulin, acetic acid	Anterograde neuronal tracing; metastatic tumor marker; lymphocyte mitogen for lymphokine production

<http://www.vectorlabs.com/data/protocols/K4-K7.pdf>

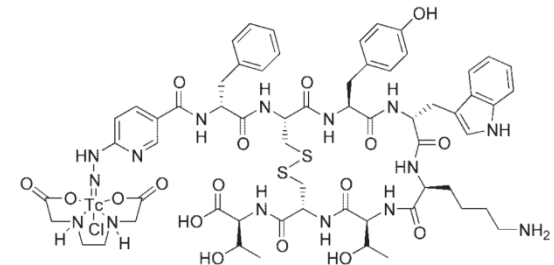
- Peptides
 - Natural peptides
 - Derived from proteins
 - Ligands identified from libraries by phage display etc.



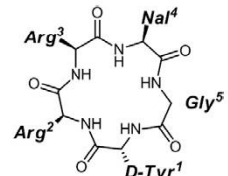
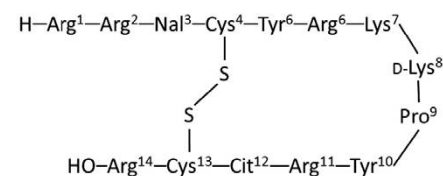
Zhou et al, *Theranostics* (2011)
Integrin $\alpha_v\beta_3$ binding (angiogenesis)
Loop from fibronectin



<http://www.fjb.pt/phadis.html>



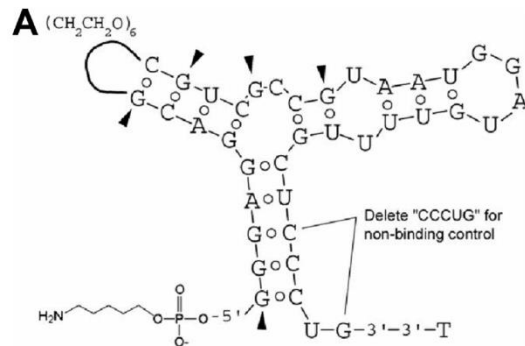
Storch et al., *J Nucl Med.* 2005;46:1561-1569.
Somatostatin analoga based on octreotide
TOC=Edotreotide=Phe¹Tyr³-octreotide (Thr-COOH reduced to alcohol)
TATE=Tyr³-octreotate (D-Phe¹, COOH)



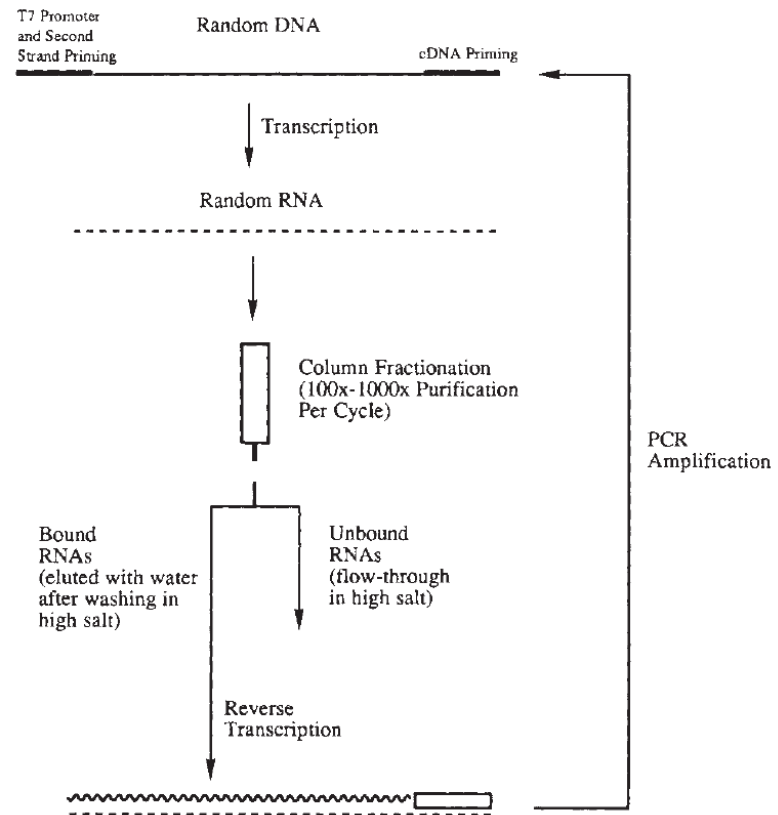
T140 (1) FC131 (2)
Y. Yoshikawa et al./Bioorg. Med. Chem. Lett. 22 (2012) 2146–2150

CXCR4 ligands (natural ligand SDF1 α)
FC131 derived from horseshoe crab peptides

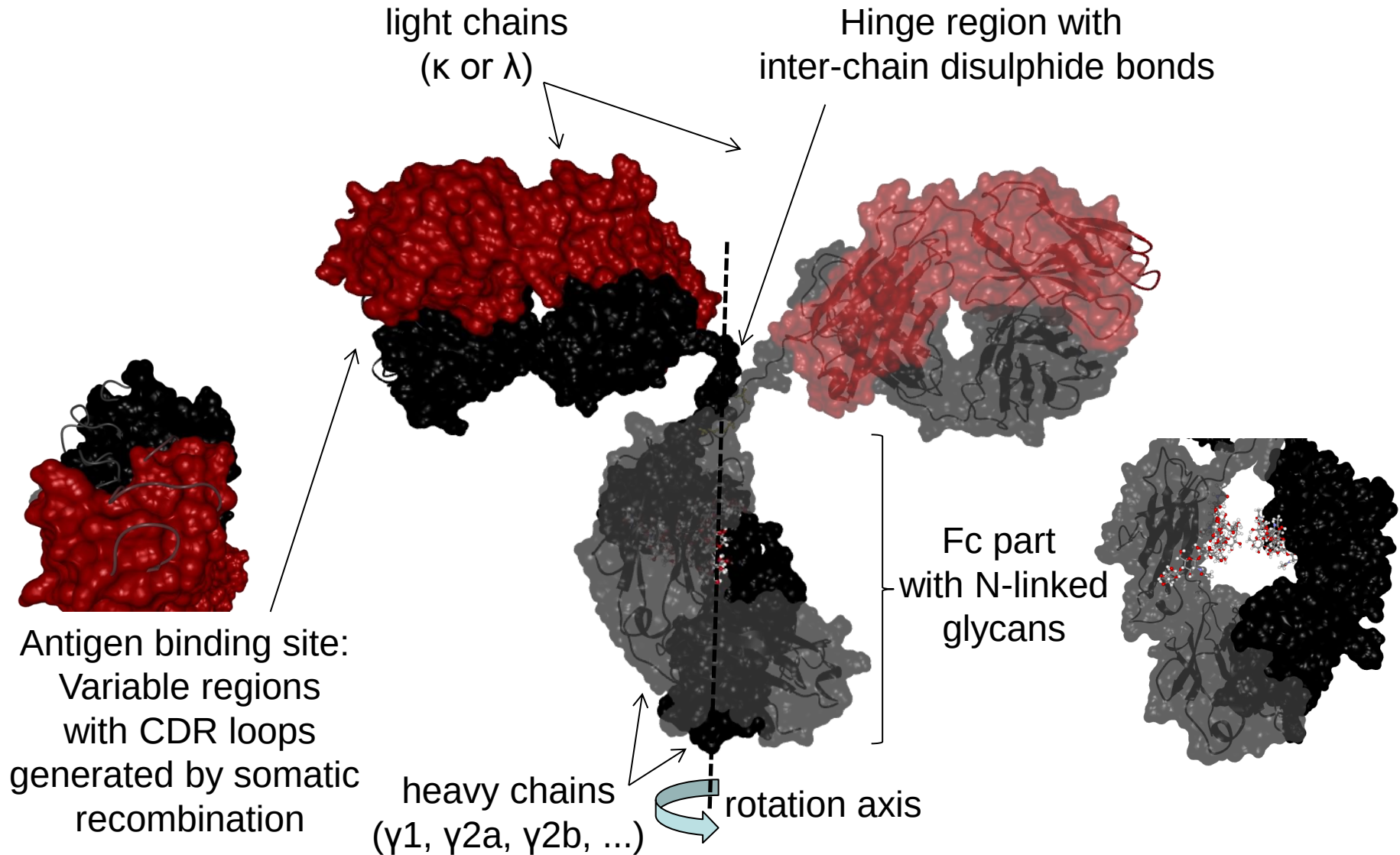
- DNA or RNA Aptamers
 - Selection from libraries by „Systematic Evolution of Ligands by EXponential enrichment” (SELEX)



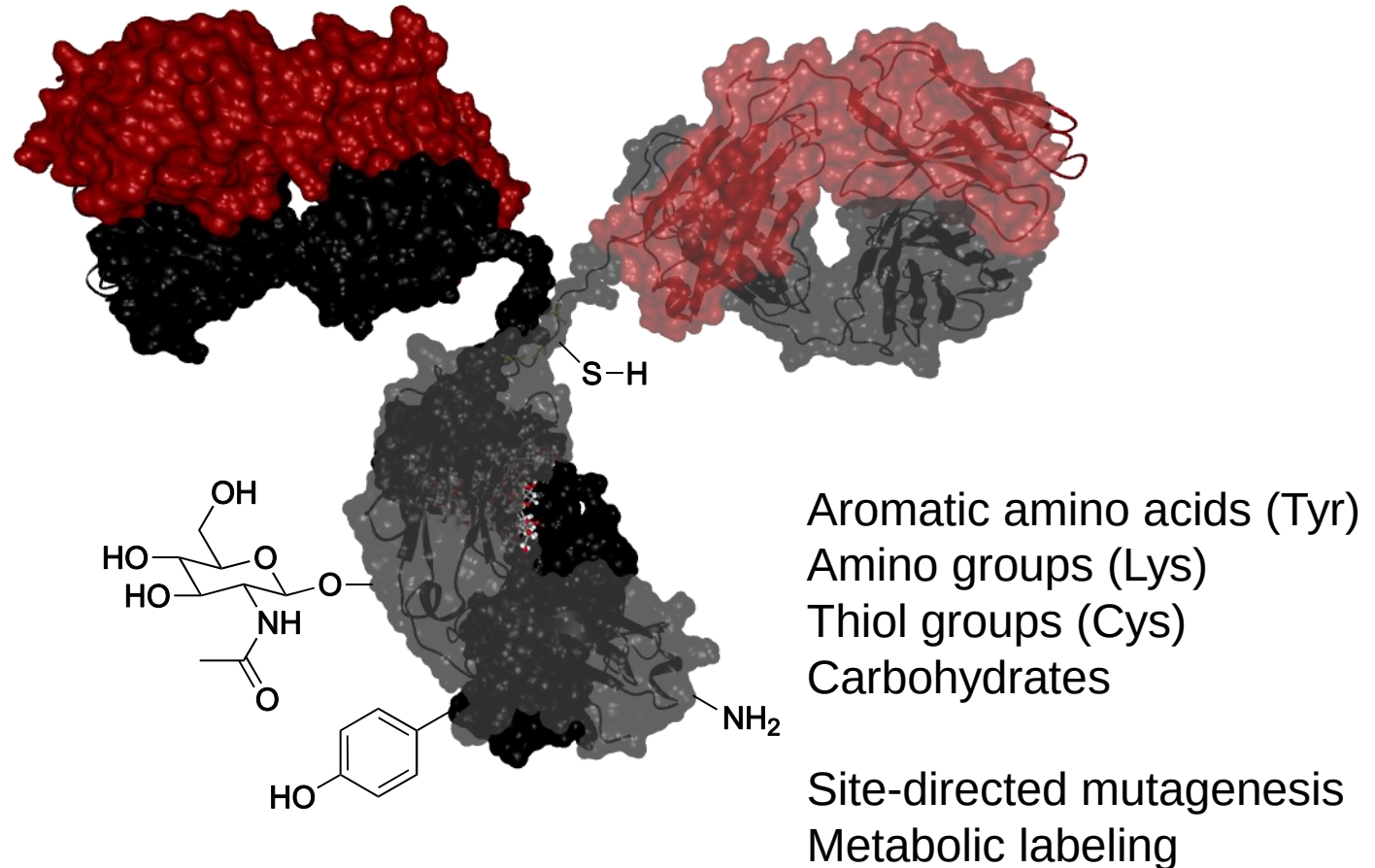
Hong et al., *Curr Med Chem.*
2011 September 1; 18(27):
4195–4205.



Ellington et al. *Nature* **346**, 818 - 822 (30 August 1990)

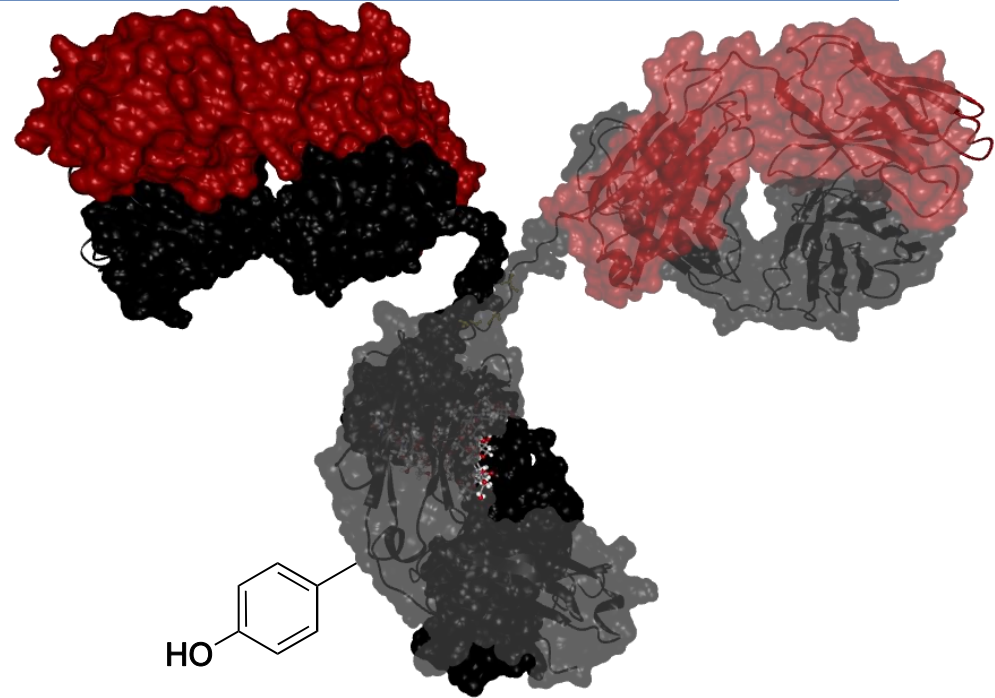


Important:
Retention of Immunoreactivity

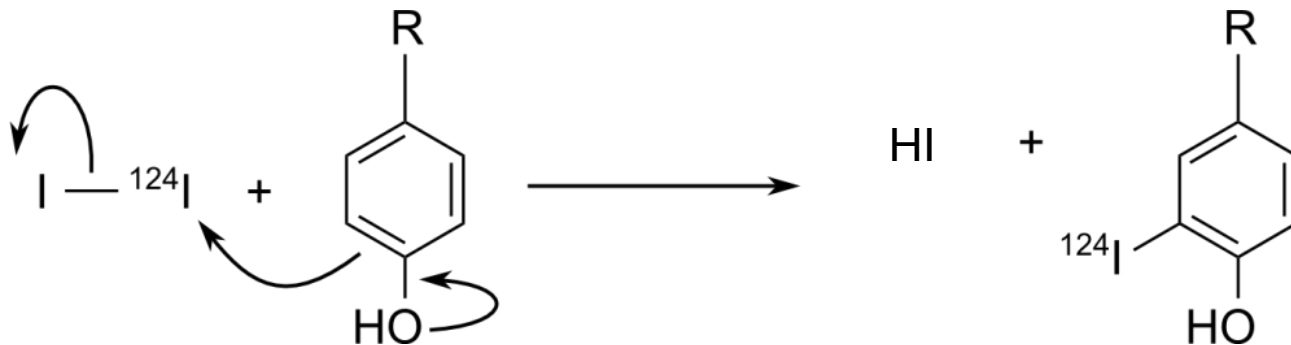




...with halogens:

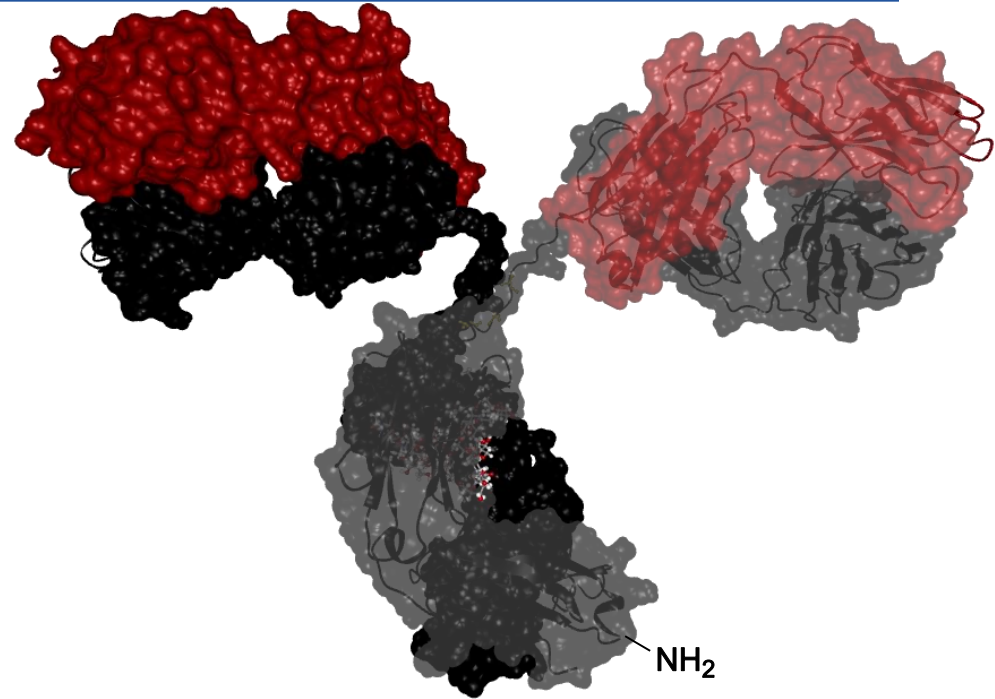


Electrophilic aromatic substitution:

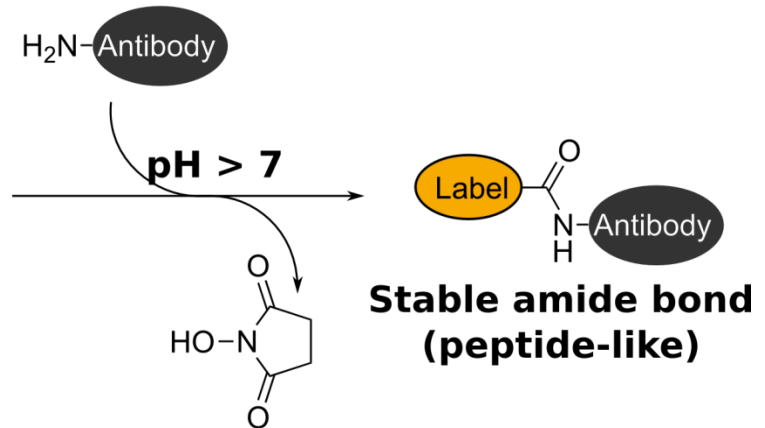
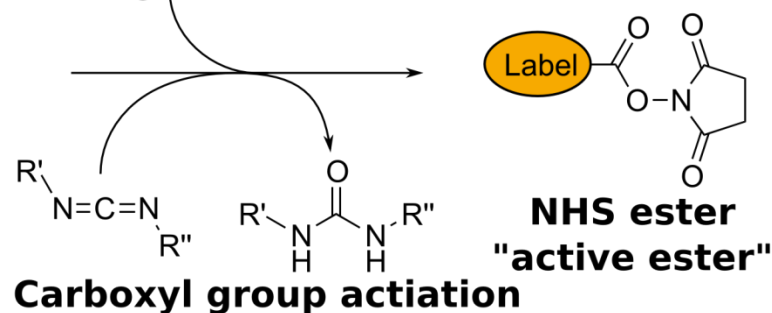
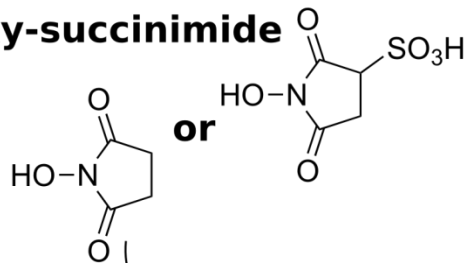


Labeling of Amino Groups...

...with carboxylic acids:



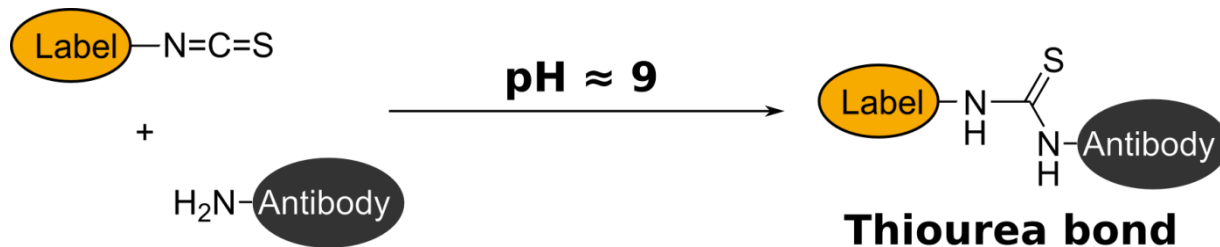
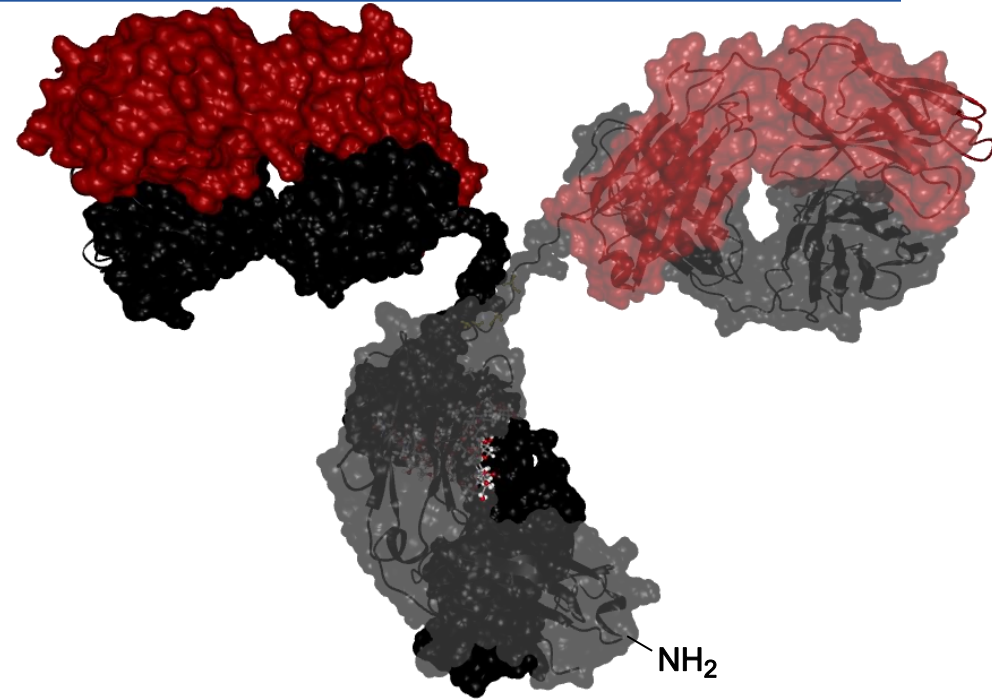
N-Hydroxy-succinimide



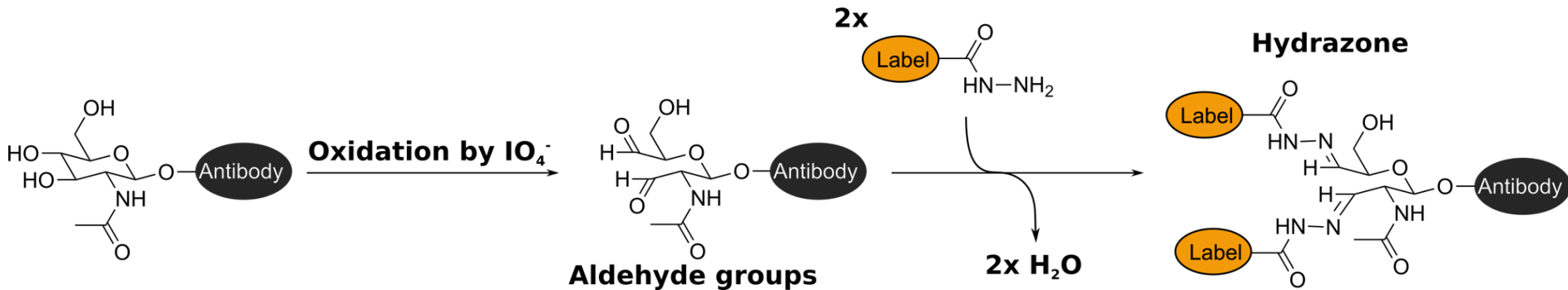
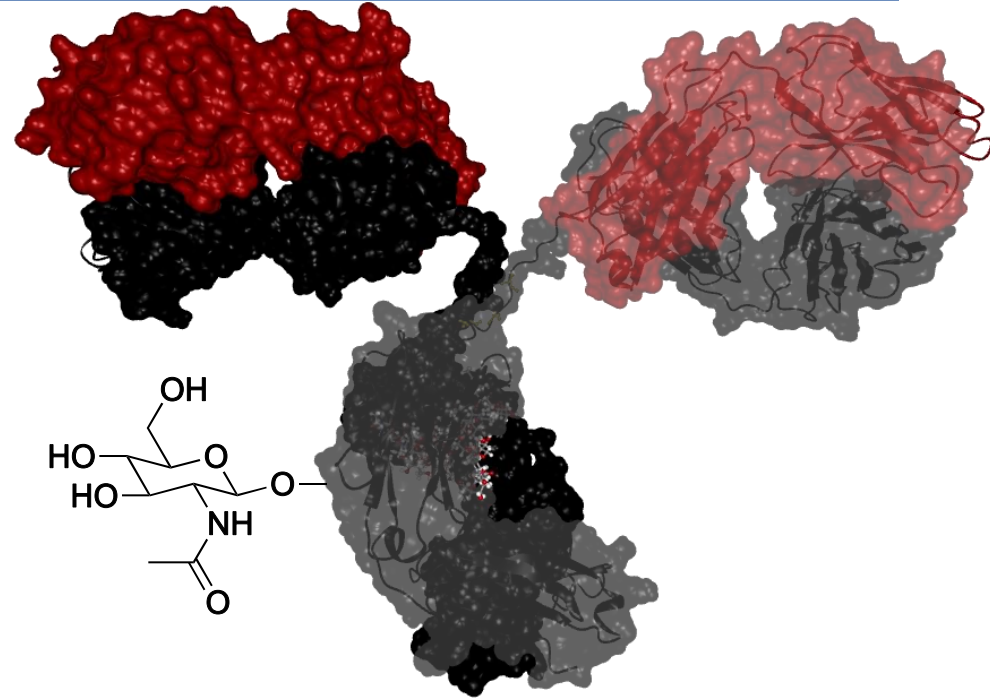
Labeling of Amino Groups...



...with isothiocyanates:



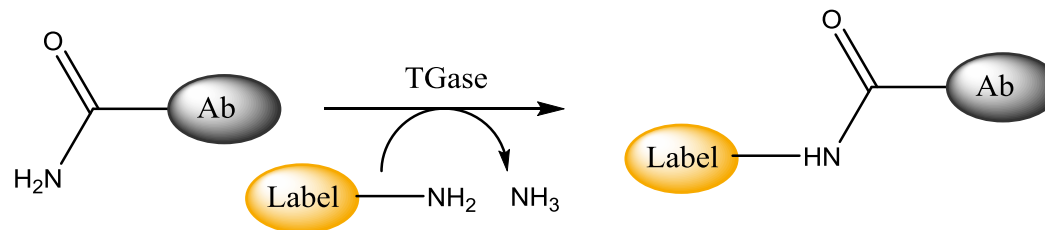
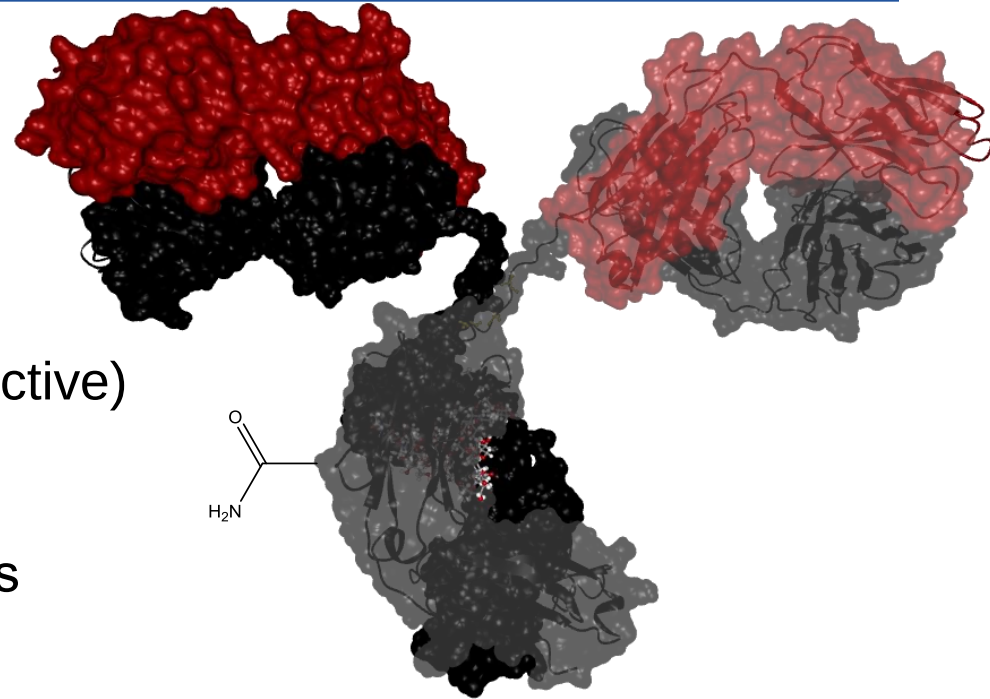
...with hydrazides:





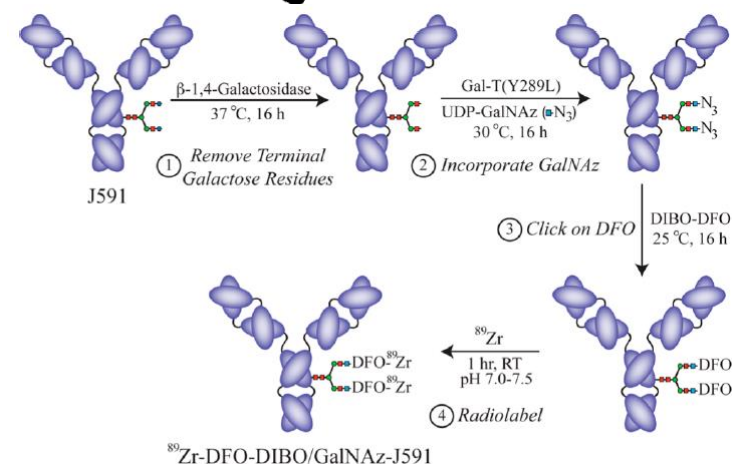
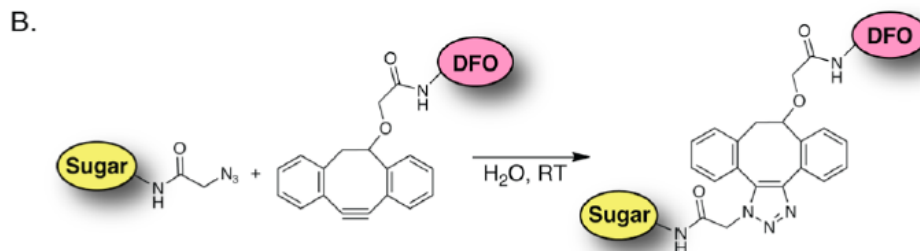
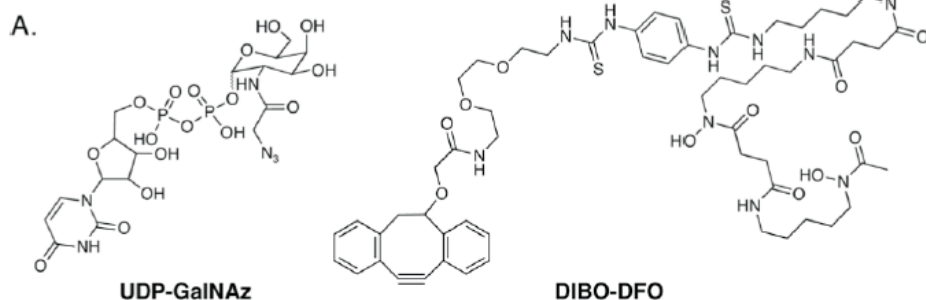
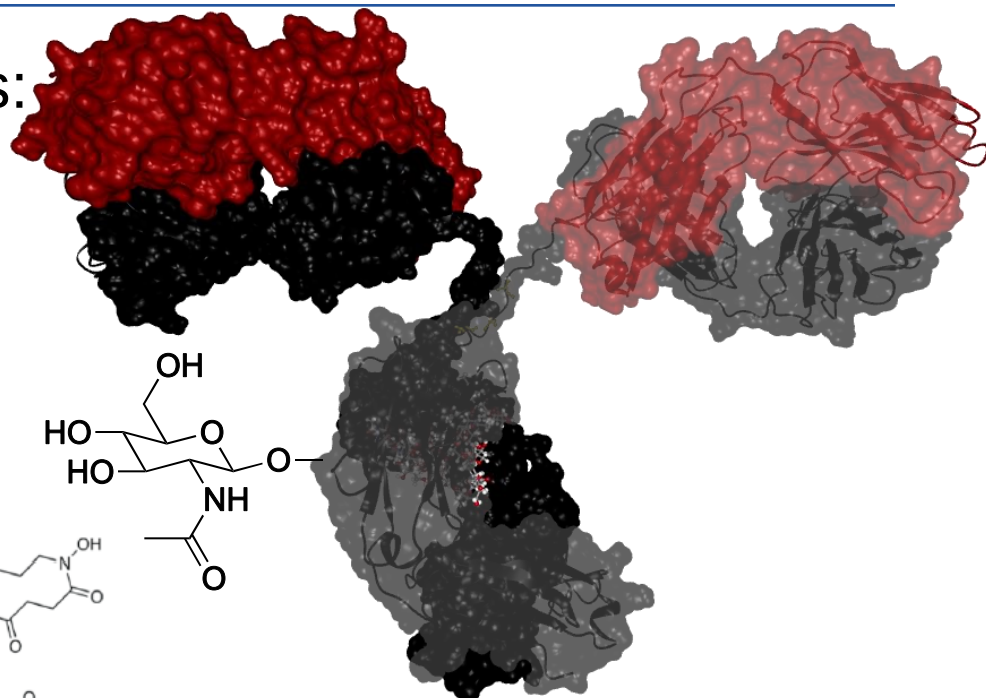
...using transglutaminase:

- Only at accessible sites (site-selective)
- Enzyme needed
- Reaction rather slow
- Enzyme prefers „small“ substrates



...using glycosyl transferases:

- Removal of terminal sugars
- Introduction of GalNAz
- Subsequent „click“ chemistry





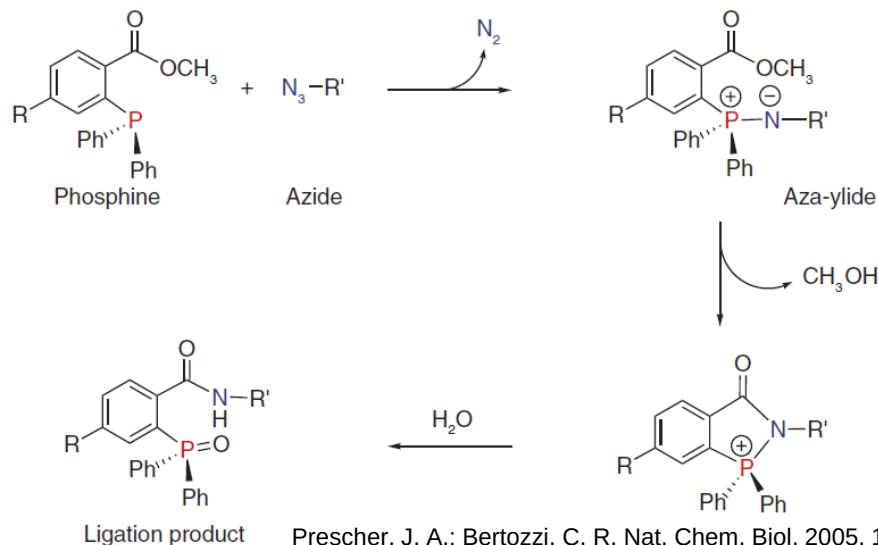
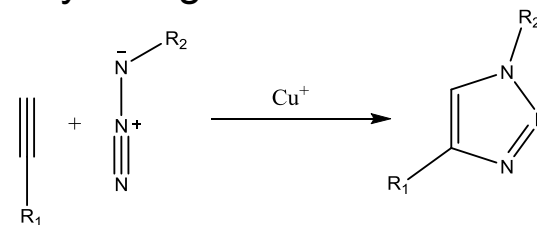
Enzymatic or chemical introduction of a reactive but bioorthogonal moiety and conjugation of this moiety with the actual label

„Click chemistry“:

- First described by Sharpless in 1999
- Chemical reactions that are **selective**, **rapid**, and **highly efficient**
- Cu(I)-catalyzed azide–alkyne cycloaddition (CuAAC), described by Huisgen.
 - highly **bioorthogonal**

• Staudinger ligation

- Bioorthogonal but air sensitive. $0.0020 \text{ M}^{-1} \text{ s}^{-1}$

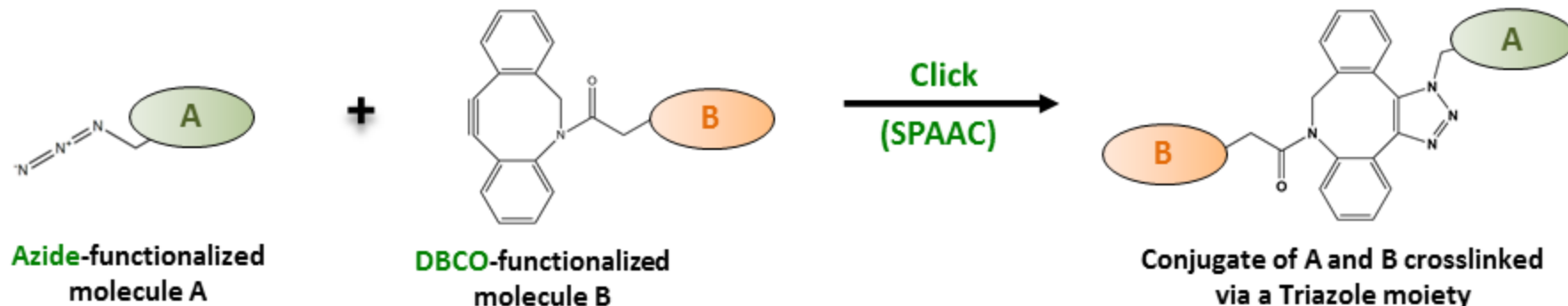


Prescher, J. A.; Bertozzi, C. R. Nat. Chem. Biol. 2005, 1, 13.

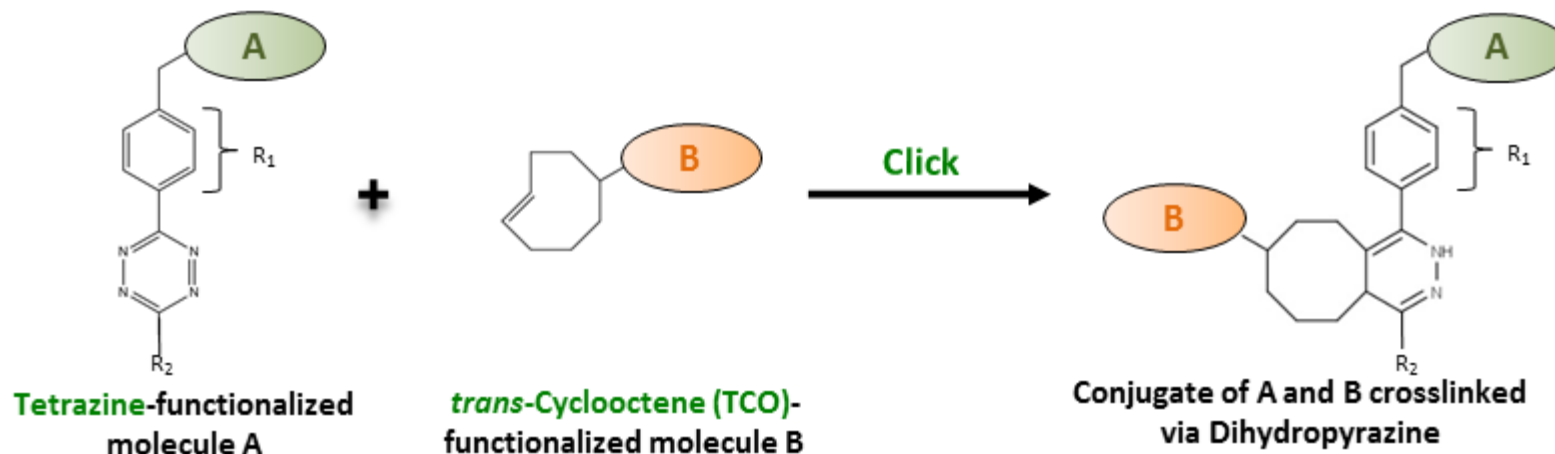
Pretargeted labeling (*in vitro*)



- Catalyst-free, strain-promoted azide-alkyne cycloaddition ($0.45\text{--}0.9\text{ M}^{-1}\text{ s}^{-1}$)

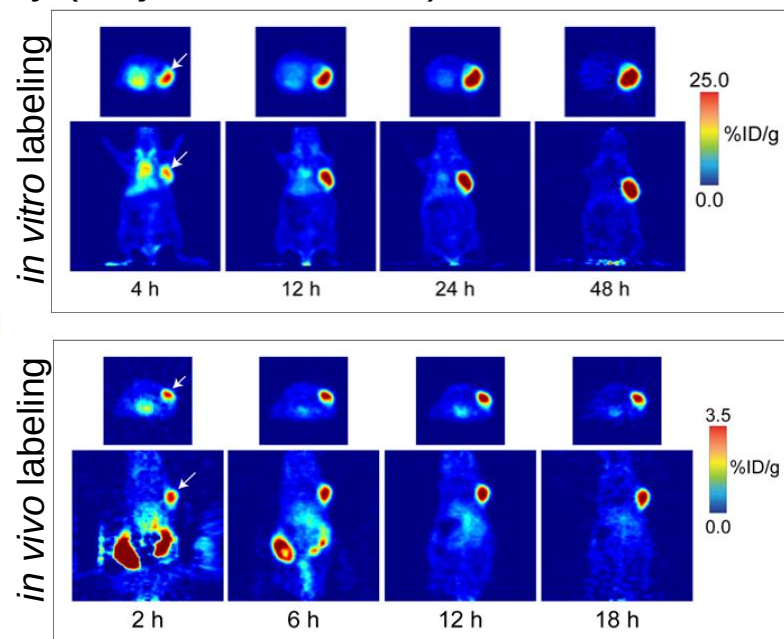
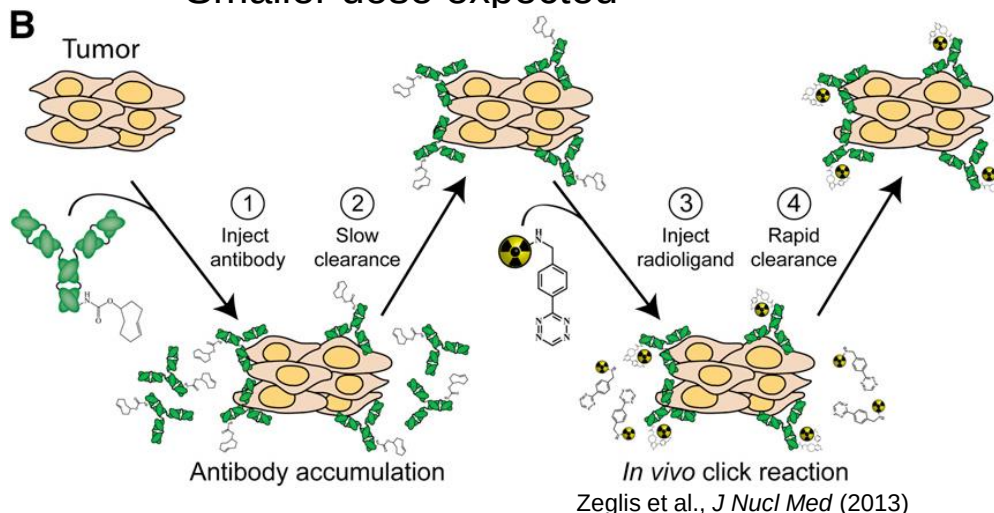
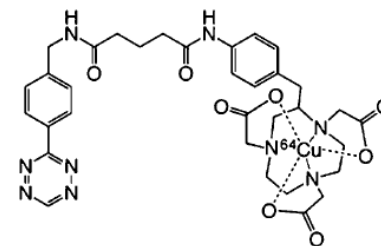


- Reverse electron demand Diels-Alder reaction ($5 - 290000\text{ M}^{-1}\text{ s}^{-1}$ depending on tetrazine and alkene)



http://www.jenabioscience.com/images/741d0cd7d0/20140306_Click_Chemistry_Background_information_pdf_creator.pdf

- Antibody half time can be problematic:
 - Good imaging only after >24 h of uptake time (slow clearance)
 - Requires „long“-lived isotopes
 - Leads to rather high doses
- Pretargeting might be a solution:
 - Injection of „cold“ alkyne-/alkene pretargeted antibody
 - Uptake time without radioactivity
 - Injection of radioisotope targeting the antibody (may be short-lived)
 - Click reaction happens *in vivo*
 - Rapid background clearance and imaging
 - Smaller dose expected

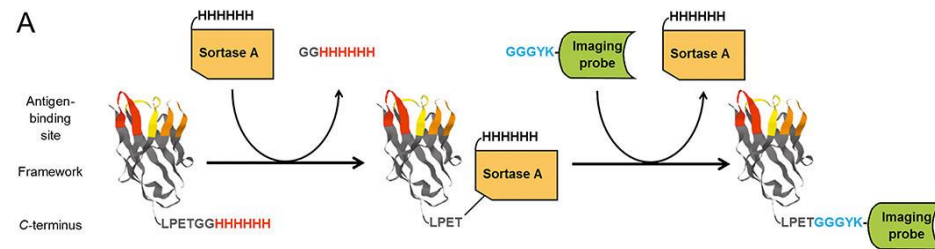




- Metabolic incorporation of Azido-labeled carbohydrates in cell culture
 - Staudinger ligation
 - Copper-free “click chemistry”

- Recombinant tagging:

- With cysteine, inteins or split-inteins
 - Targeting with thiol-reactive reagents
 - Native chemical ligation via thioester
- Enzymatic labeling of sequence tags
 - Tub tag (tubulin tyrosine ligase)
 - Sortase
- Fusion with enzymatic tags
 - SNAP tag
 - Halo tag



Massa et al. 2016

- and many more...

Isotopes for Antibody Imaging



Radionuclide		$t_{1/2}$		
^{15}O		122	seconds	
^{13}N		9.9	minutes	
^{11}C		20	minutes	
^{68}Ga		68.3	minutes	short-living
^{18}F		110	minutes	
^{64}Cu		12.7	hours	intermediate-living
^{86}Y		14.7	hours	
^{76}Br		16.2	hours	long-living
^{89}Zr		78.4	hours	
$^{140}\text{Nd} \rightarrow ^{140}\text{Pr}$		80.8	hours	
^{124}I		100.3	hours	

metals



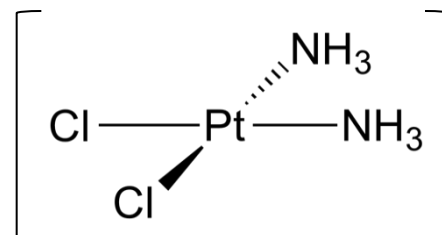
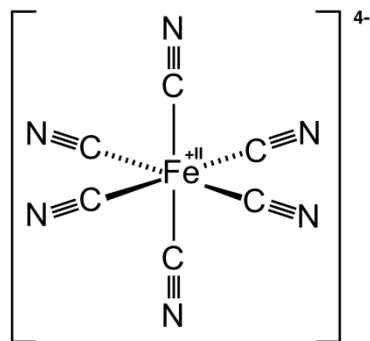
- Metals have low ionization energies
 - Tend to form cations in aqueous/biological systems



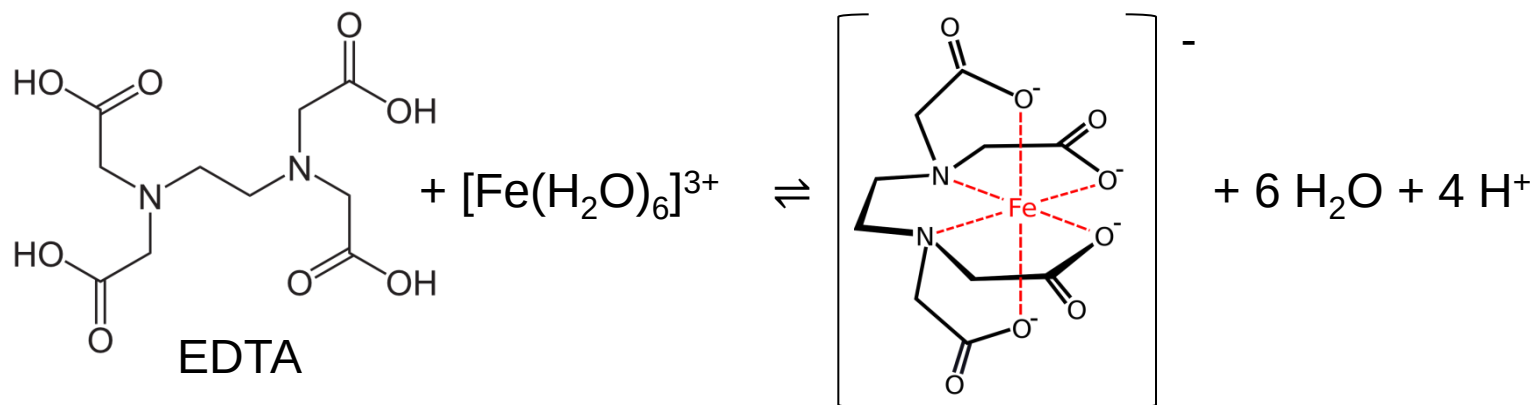
- Direct conjugation by covalent bonds not possible
- Conjugation via coordination complexes



- Coordination complex: ligands with lone e⁻ pairs assemble around central ion and use unoccupied orbitals:

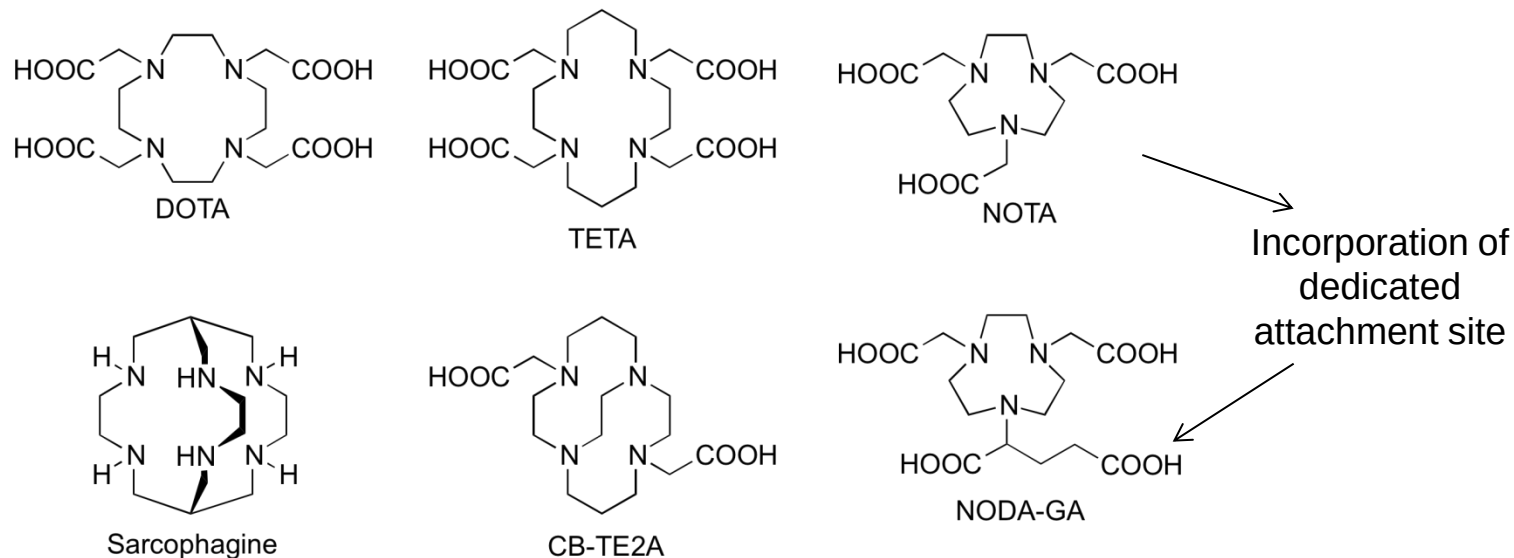


- Multidentate ligands form chelate complexes (stable!):

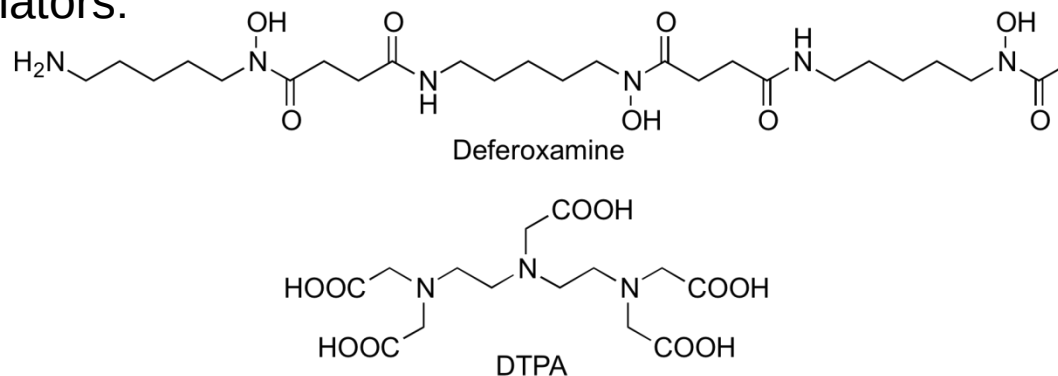




Macrocyclic chelators:



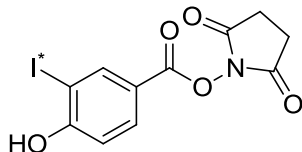
Non-cyclic chelators:



Prosthetic groups and chelators for introduction of radioisotopes:

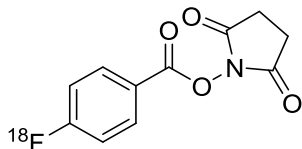
I:

electrophilic iodination (I_2)



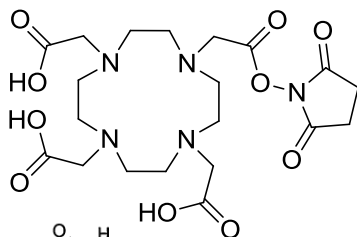
Bolton-Hunter-reagent

F:



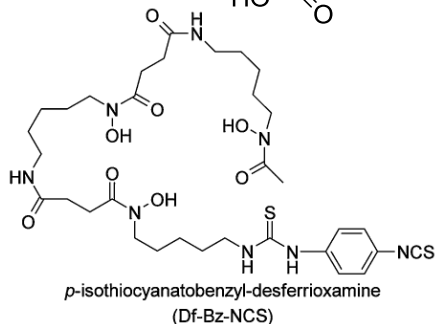
Succinimidofluorobenzoic acid (SFB)

Cu, Ga, Nd:



DOTA-NHS-ester
(suitable for ^{64}Cu , ^{68}Ga , ^{140}Nd)

Zr, In:



DFO-Bz-NCS
(for ^{89}Zr or ^{111}In labeling)

Ga-68:

- $T_{1/2} = 68$ min with 89 % β^+ emission
- Production from Ge-68 generator, in Tübingen ~twice a day:
 - $^{69}\text{Ga}(p,2n)^{68}\text{Ge}$ (cyclotron, off-site)
 - $^{68}\text{Ge}(\beta, \gamma)^{68}\text{Ga}$ ($T_{1/2} = 270.8$ days)
 - Germanium is adsorbed on a solid phase, generated Ga is eluted.
 - Milking similar to $^{99\text{m}}\text{Tc}$ generators (moly cows)
 - Generator (~40000 €) works for ~6 months

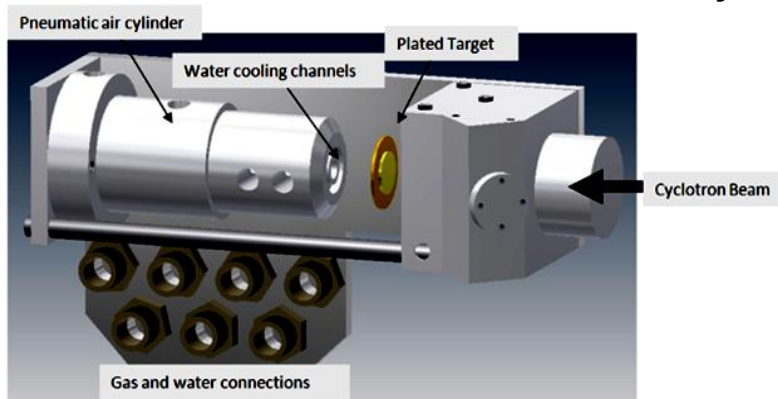
	Cyclotron Co Ltd.	Eckert&Ziegler IPL	I.D.B. Holland B.V.	Isotope Technologies Garching
Origin	Russia	USA	South Africa	Germany
Resin	Titanium-dioxide	Titanium-dioxide	Tin-dioxide	Organic Material
Eluent	0,1M HCl	0,1M HCl	0,6M HCl	0,05M HCl
Elution Yield	60-75%	70-75%	80%	(>80%) *)
Breakthrough **)	<0,01%	<0,001% **)	<0,007%	(<0,001%)

- Typical chelators: DOTA, NOTA, NODAGA



Copper-64:

- $T_{1/2} = 12,7$ h with 18 % β^+ emission
- Production in Tübingen, weekly, $^{64}\text{Ni}(p,n)^{64}\text{Cu}$:
 - Electroplating of ^{64}Ni onto platin sheet ($\text{Ni}^{2+} + 2 e^- \rightarrow \text{Ni}^0$)
 - Proton bombardment in solid cyclotron target (4 h, low energy)
 - Incubation over night, decay of shorter-lived isotopes (Co-61, 1.6 h)
 - $^{64}\text{Ni}/^{64}\text{Cu}$ mixture is recovered with hydrochloric acid (oxidation)
 - Chloro-complexes are separated by **anion** exchange chromatography
 - $^{64}\text{Cu}^{2+}$ in diluted hydrochloric acid



0.2 M HCl in 96 % methanol → Ni-64 not bound
↓
1.3 M HCl in 30 % isopropanol → Cu-64 eluted

- Typical chelators: DOTA, NOTA, NODAGA, CBTE2A, ...
- Liver uptake reported when unstable chelators are used



Zirconium-89 (Copenhagen, Amsterdam, Ulm, ...):

- Proton bombardment of Yttrium-89 (natural isotope)
- $^{89}\text{Y}(\text{p},\text{n})^{89}\text{Zr}$
- $T_{1/2} = 78.4 \text{ h}$ with 22.7 % β^+ (higher positron energy than Cu-64)
- Typical chelator: Deferoxamine, currently new developments
- Bone uptake when Zr is released from the chelator

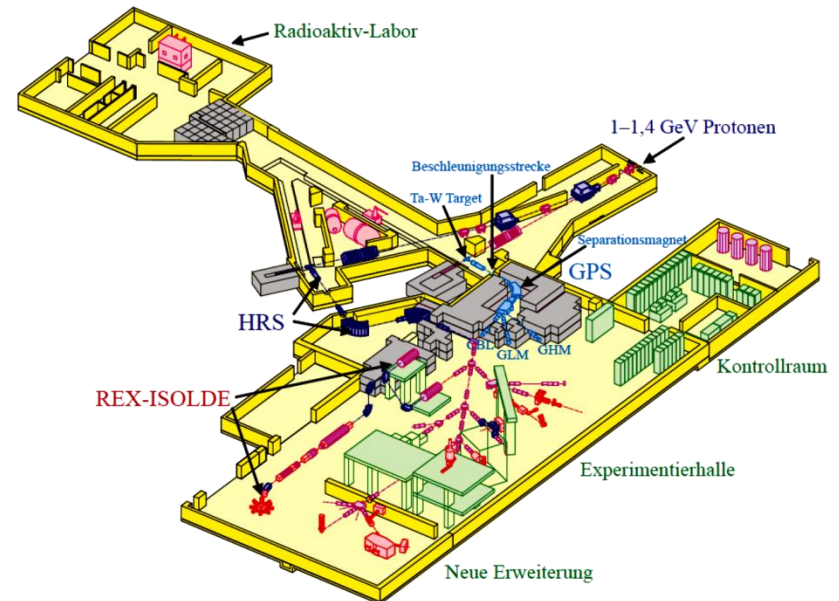
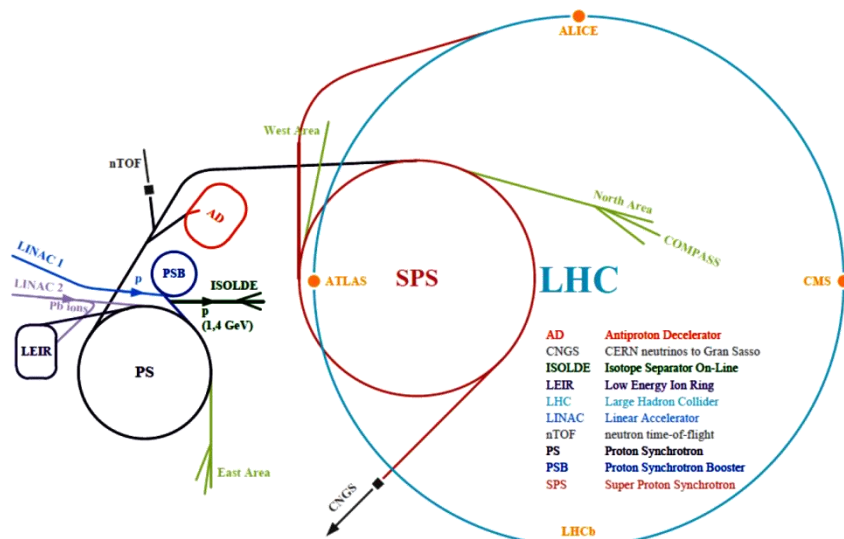


<https://www.radboudumc.nl/Research/Organisationofresearch/Departments/NuclearMedicine/Pages/prostatecancer.aspx>



Neodyme-140 (CERN, ISOLDE):

- $^{141}\text{Pr}(p,2n)^{140}\text{Nd} \xrightarrow[T_{1/2} = 3.37 \text{ d}]{\text{Auger emission}} ^{140}\text{Pr} \xrightarrow[T_{1/2} = 3.35 \text{ min}]{\beta^+} ^{140}\text{Ce}$
- ,*in vivo*' generator of Praseodyme-140 which emits the positrons for imaging
- Theranostics



Synthesis of ^{68}Ga -NODAGA-RGD:

1. Elute the generator with 0.1 M HCl, collect and measure fractions
2. Combine the fractions with the highest activity
3. Add 0.1 volume of 1.9 M sodium acetate, check for pH (~5)
4. Add 20 μg of the peptide
5. Incubate for 10 minutes (room temperature)
6. Push the reaction through a C18 cartridge
7. Wash the cartridge with 0.9 % saline
8. Elute the labeled peptide with 0.5 ml 95 % ethanol
9. Dry the peptide (70 ° C, argon stream)
10. Dissolve the product in 125 μl phosphate-buffered saline



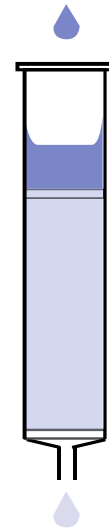
QC: Radio-TLC

- stationary phase: silica plates
- mobile phase: 0.5 M citrate pH 5

Knetsch et al., Eur J Nucl Med Mol Imaging (2011) 38:1303–1312

Chelator conjugation and radiolabeling of antibodies with Cu-64:

1. removal of metal ions potentially bound to the protein.
2. covalent attachment of the chelator (typically active ester or isothiocyanate): Antibody + buffer + Chelator-NHS. Suitable buffer?
3. Removal of small molecules (excess chelator): Ultrafiltration with ~30 kDa cut-off.
4. Buffering of the (acidic) radioisotope solution and incubation with antibody for 1 h at 37 ° C. Which buffer?
5. Quality control by Radio-TLC and Radio-HPSEC. Which column and buffer?
6. Removal of free radioisotope if necessary (size exclusion chromatography or ultrafiltration).



- Schatz & Ji, Nat. Rev. Immunol. 2011
- Collingridge *et al.*, Cancer Res. 2002
- Cai *et al.*, Cancer Res. 2006
- Malviya *et al.*, Mol. Imaging Biol. 2012
- Nayak *et al.*, PLoS ONE 2011
- Tamura *et al.*, J. Nucl. Med. 2013
- Harris *et al.*, Biochem. 1997
- Salsano *et al.*, Res. Rep. Nucl. Med. 2013
- Nobel Prize homepage
- CERN homepage
- Wikimedia

- Facts about IgG structure and antibody generation (B cell diversity).
- Conjugation chemistry with focus on amino group labeling
- Common chelators and their structure (DFO, DOTA, NOTA, DTPA, CBTE2A, ...)
- Important PET radioisotopes and their half lives
- General procedure for antibody radiolabeling