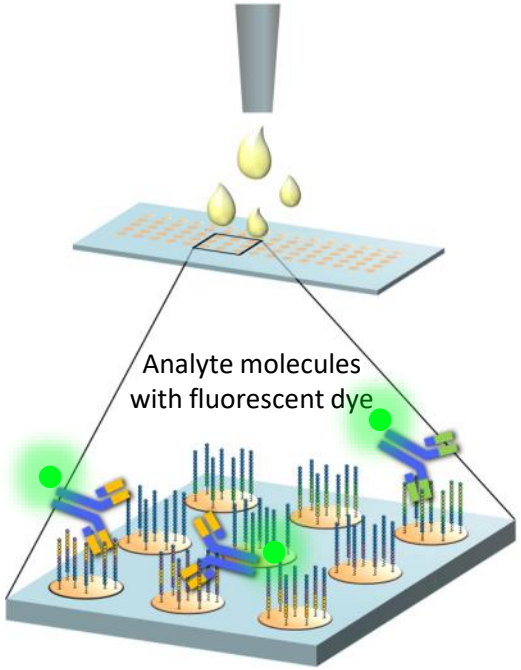
Goals for lecture – Molecules, interactions, surfaces

1. Revisited – Assay principle (specific binding)
2. Molecules
3. Interaction forces
4. Affinity, avidity, multivalency
5. Functionalizations and surface chemistry
6. Quantitative vs. qualitative

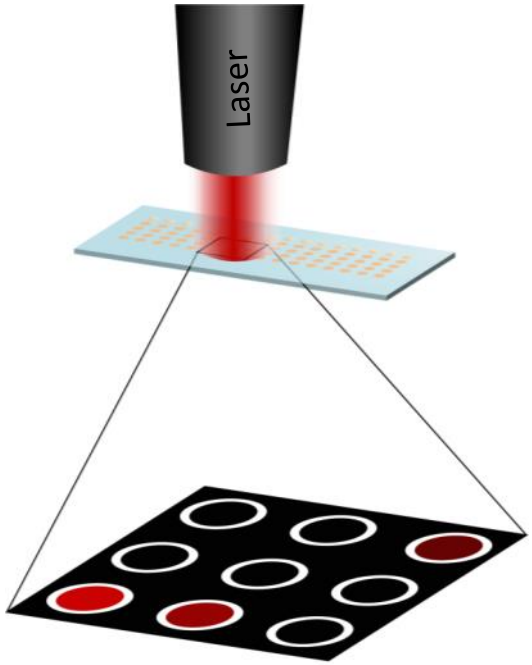
1. Assay principle

Microarray assay principle

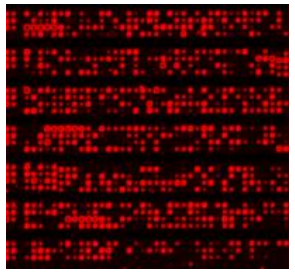
- Highly parallelized analysis of **interactions**/reactions



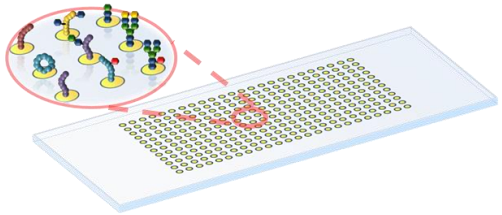
Incubation with diluted analyte molecules → interaction



Fluorescence scan and quantification



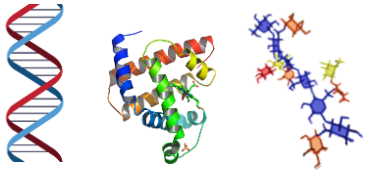
Microarrays – Big picture



Microarrays for screening

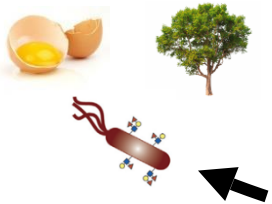
Spotting & coupling of ready compounds onto array

In-situ (= directly on the array) high-throughput synthesis of compounds



Microstructuring methods (*e.g.*, spotting, printing…)

Extraction & purification from "natural" sources



Chemically or biologically synthesized compounds

Building blocks + Enzymes

Liquid phase or Solid phase

Building blocks + Chemical reagents

2. Overview of immobilized molecules

Which ones do you know?

Overview of molecules

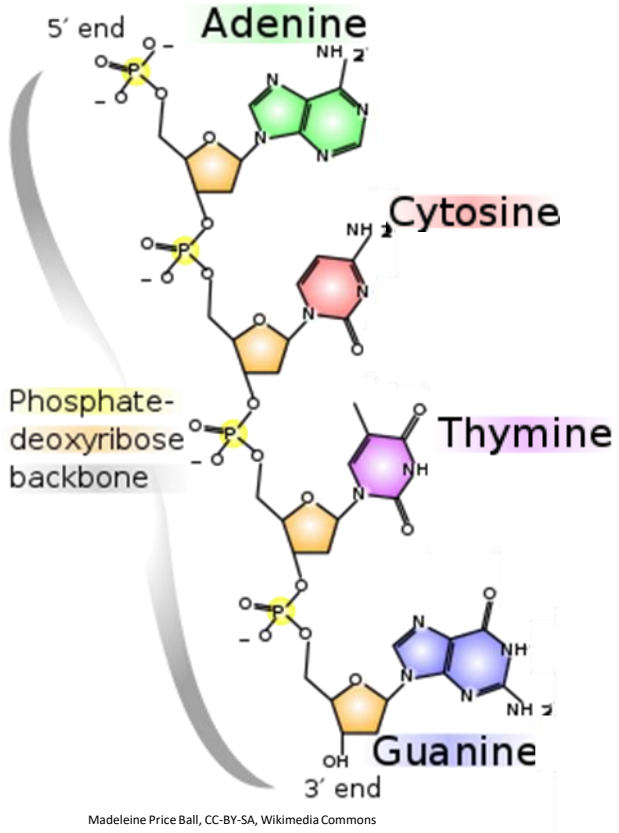
Molecules of interest for microarrays

- Oligonucleotides (DNA, RNA), aptamers
- Proteins & peptides
  - Anti-/Nanobodies
- Glycans
- Lipids
- Small molecules

How to immobilize molecules on a surface?

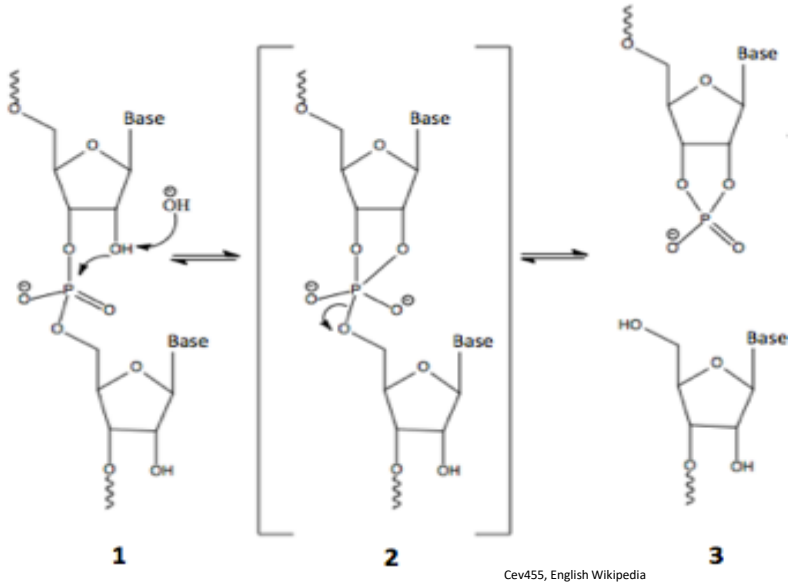
Oligonucleotides

- Oligonucleotides (DNA, RNA) Variants of n-mer? → 4^n



Madeleine Price Ball, CC-BY-SA, Wikimedia Commons

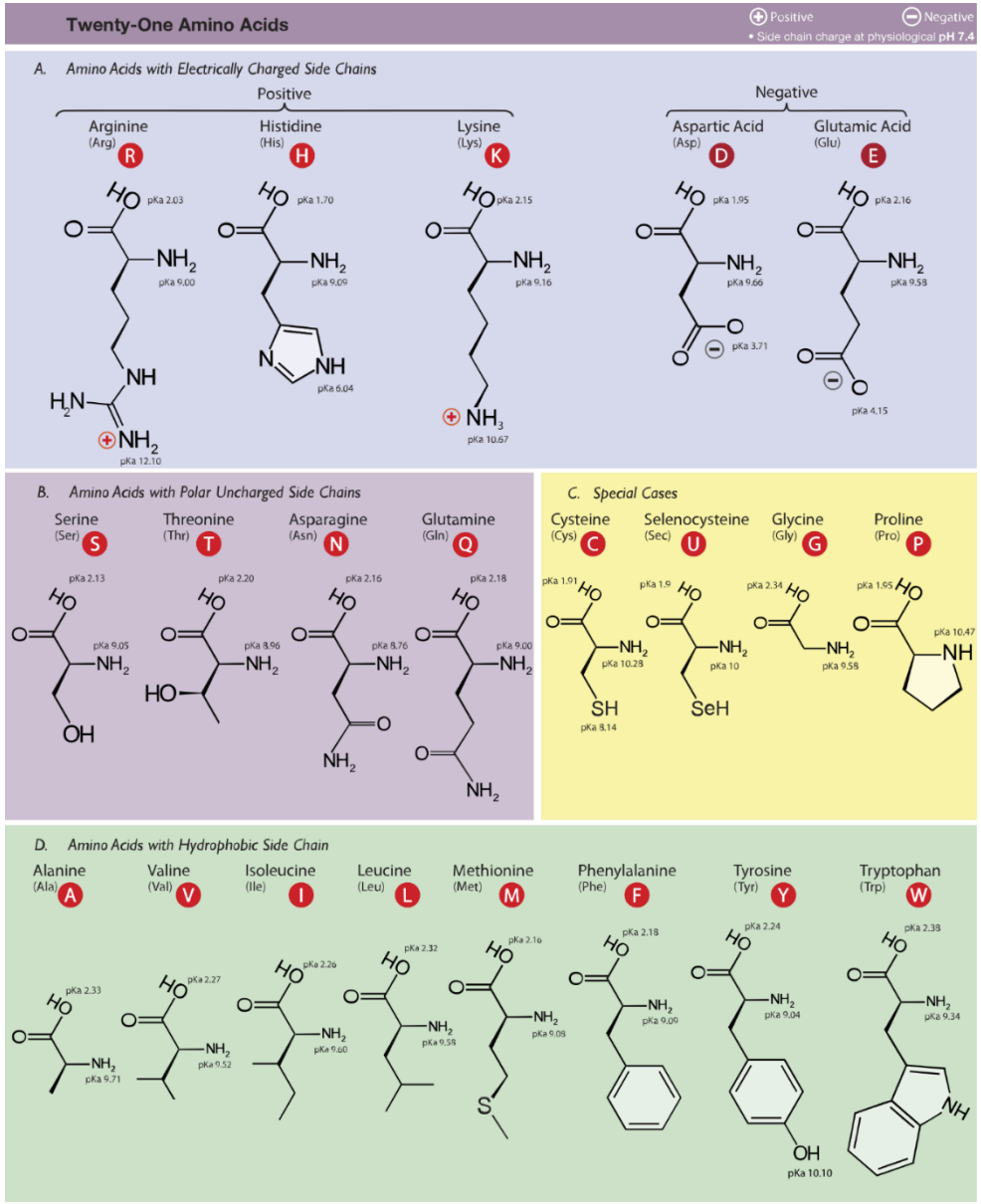
RNA unstable? Hydrolysis



Cev455, English Wikipedia
https://en.wikipedia.org/wiki/File:RNA_Hydrolysis.png

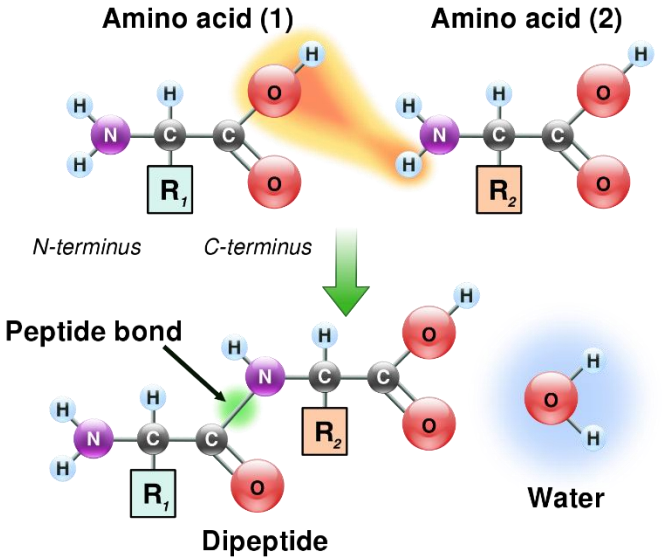
- pH 7: backbone negatively charged, bases neutral
- Special reactive groups often added to 3’ or 5’ end

Peptides & proteins



Author: Dancojocari, https://en.wikipedia.org/wiki/Amino_acid

Peptide bond formation

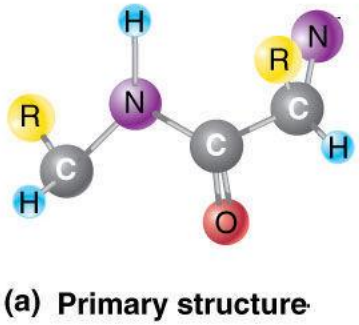


Variants of n-mer?

- Reactive groups:
  - NH_2 (amine), COOH (carboxyl), OH (hydroxyl), SH (thiol)

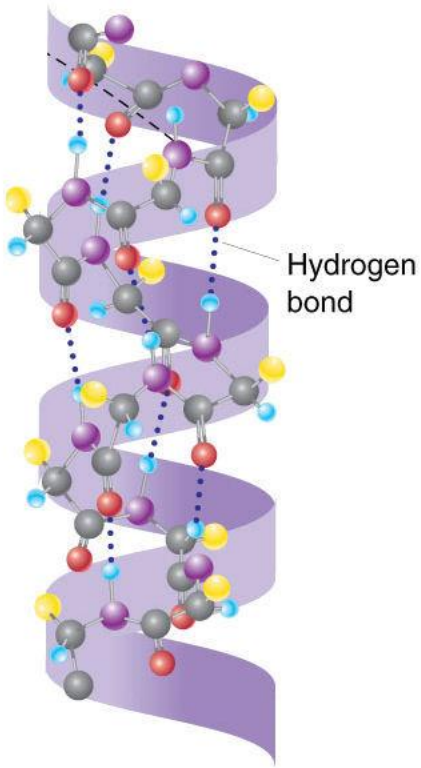
Peptides & proteins

- Proteins
  - Hormones
  - Enzymes
  - Receptors
  - Antibodies
  - …

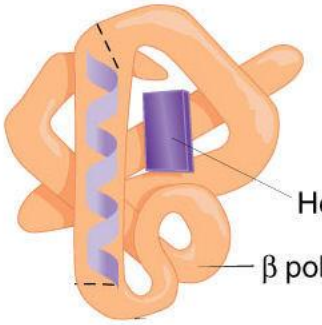


(a) Primary structure

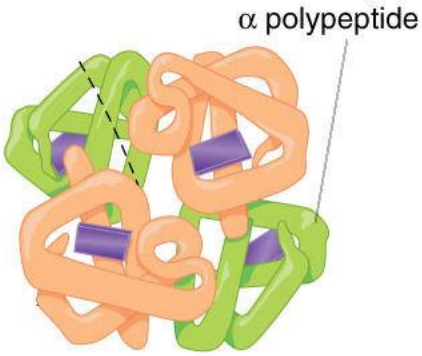
Protein structure



(b) Secondary structure



c) Tertiary structure

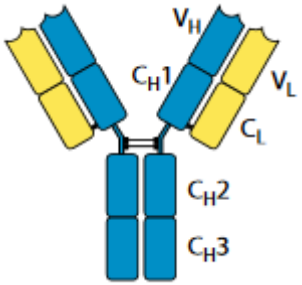


(d) Quaternary structure–

https://www.mun.ca/biology/scarr/iGen3_06-04_Figure-L.jpg

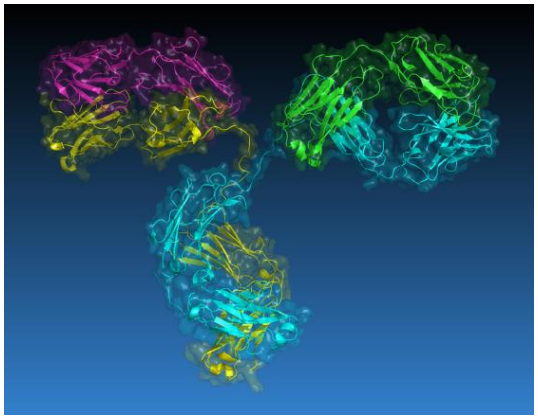
Antibodies

- Antibody (IgG, IgD, IgE)



Janeway, Immunologie, 2018

Heavy chain
Light chain
Constant (Fc)

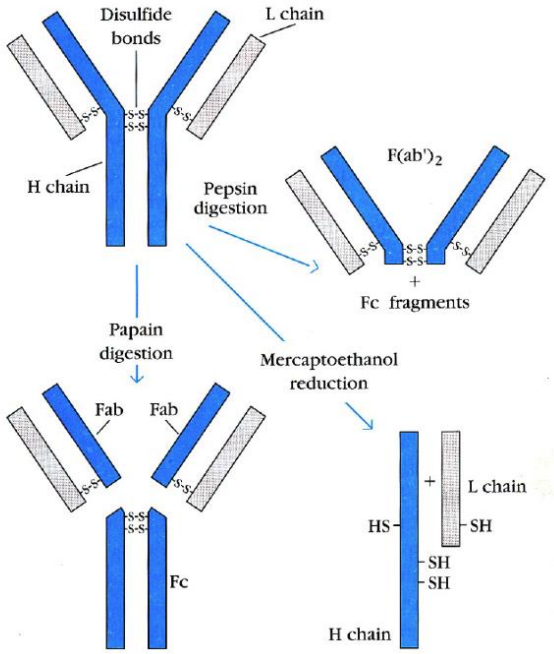


Structure of antibody in PyMol (Schrödinger, pymol.org/dsc/), based on RCSB PDB (www.rcsb.org) ID 1IGT

E.g. Adalimumab (Humira) for rheumatoid arthritis, developed via phage display

- Porter & Edelman, Nobel prize 1972

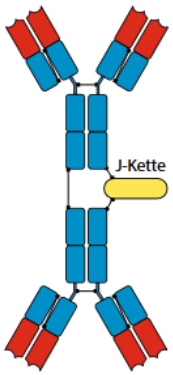
  → Structure of antibodies



Antibodies

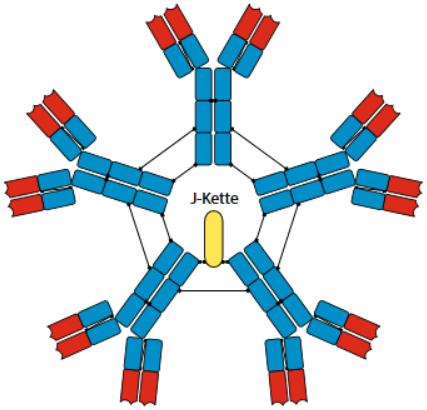
Other variants

IgA



Janeway, Immunologie, 2018

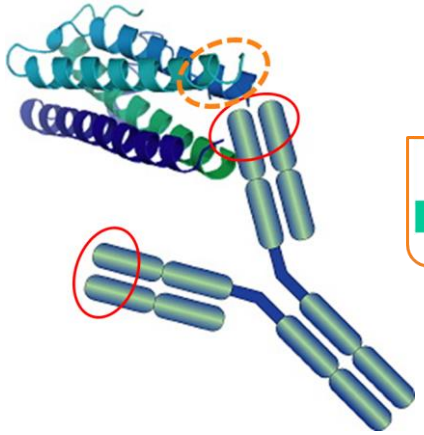
IgM



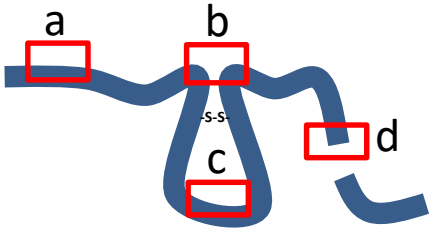
Janeway, Immunologie, 2018

Specific binding

- Epitope & paratope



Antigenic polypeptide



a: continuous epitope

b: conformational epitope

c: hidden antigen

d: neoantigen

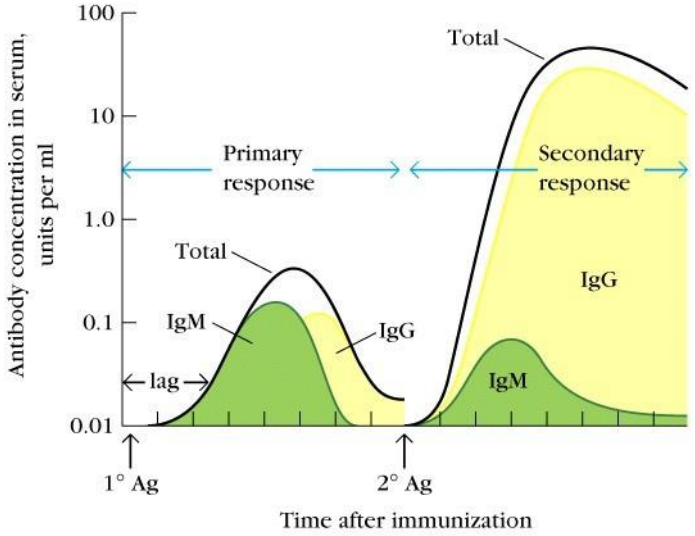
Antibodies

- Human antibody classes

	IgG1	IgG2	IgG3	IgG4	IgM	IgA1	IgA2	IgD	IgE
Heavy chain name	γ_1	γ_2	γ_3	γ_4	μ	α_1	α_2	δ	ε
Molecular weight [kDa]	146	146	165	146	970	160	160	184	188
Average adult serum concentration [mg/mL]	9	3	1	0,5	1,5	3,0	0,5	0,03	5×10^{-5}
Half life in serum [days]	21	20	7	21	10	6	6	3	2

Janeway, Immunologie, 2018

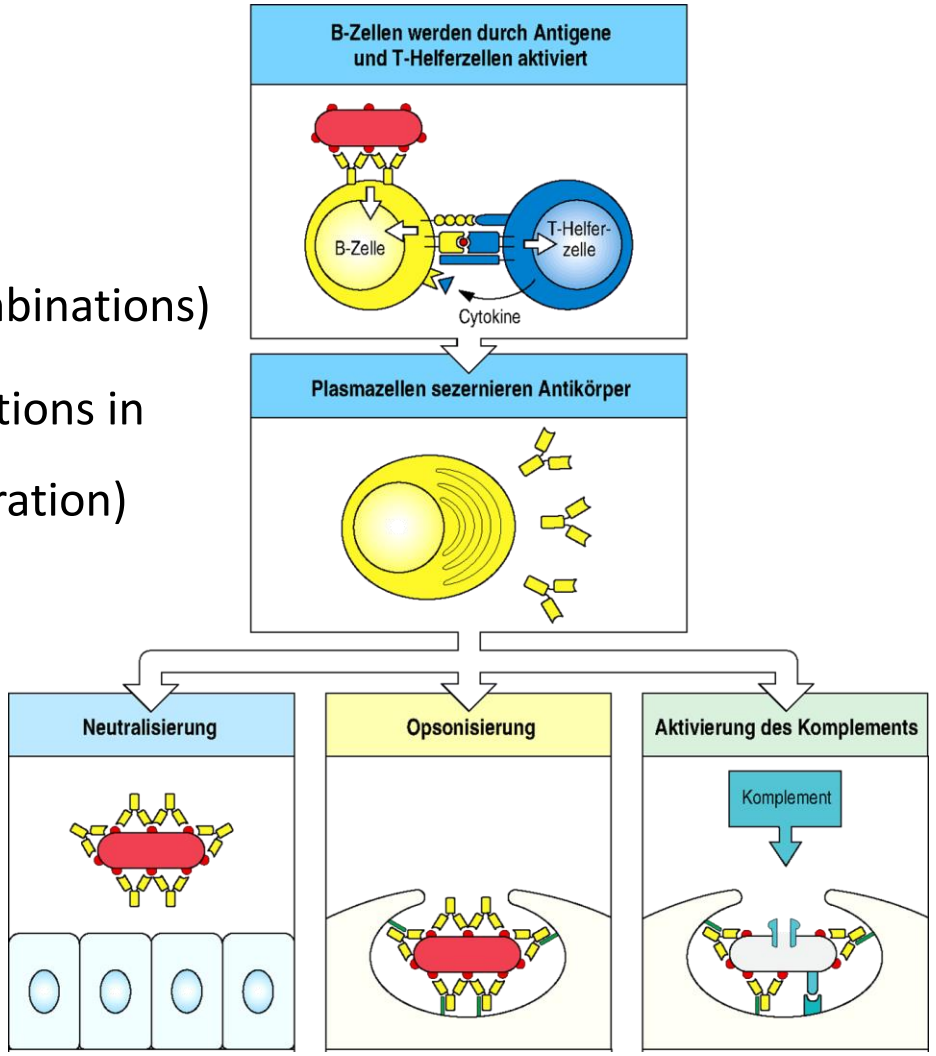
- Typical Ig immune response



https://www.newhealthadvisor.com/Primary-Immune-Response.html

Antibody diversity

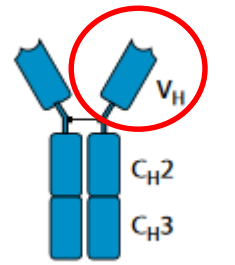
- Specificity *via* diversity
- Evolutionary process
  - V(D)J recombination (~3×10^{11} combinations)
  - Somatic hypermutation (1-2 mutations in antigen binding site, per cell generation)
  - Clonal selection (autoimmunity!)



Janeway, Immunologie, 2018

Anti-/Nanobodies

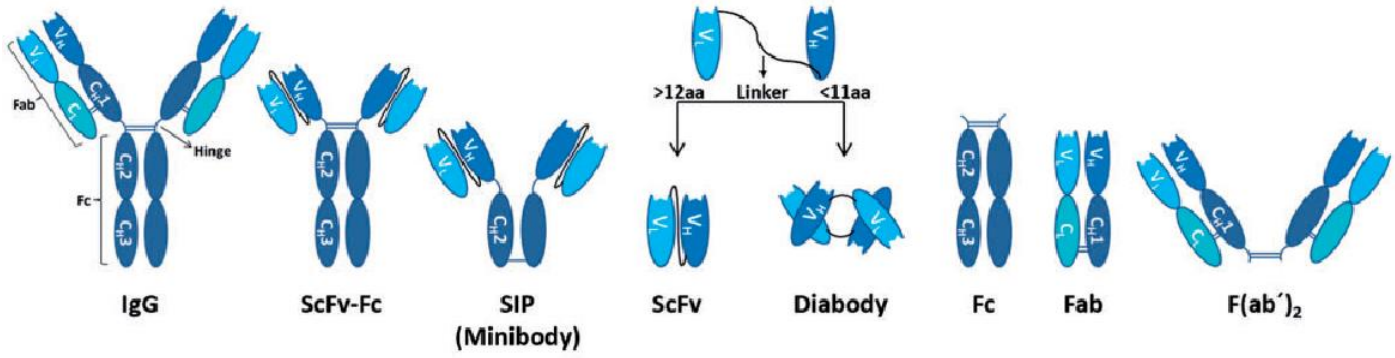
- Nanobody
  - *E.g.* camels or sharks
  - Only heavy chains
  - Small, stable, cheaper
  - Lower immunogenicity



Janeway, Immunologie, 2018

Philippe Lavoie, Wikipedia

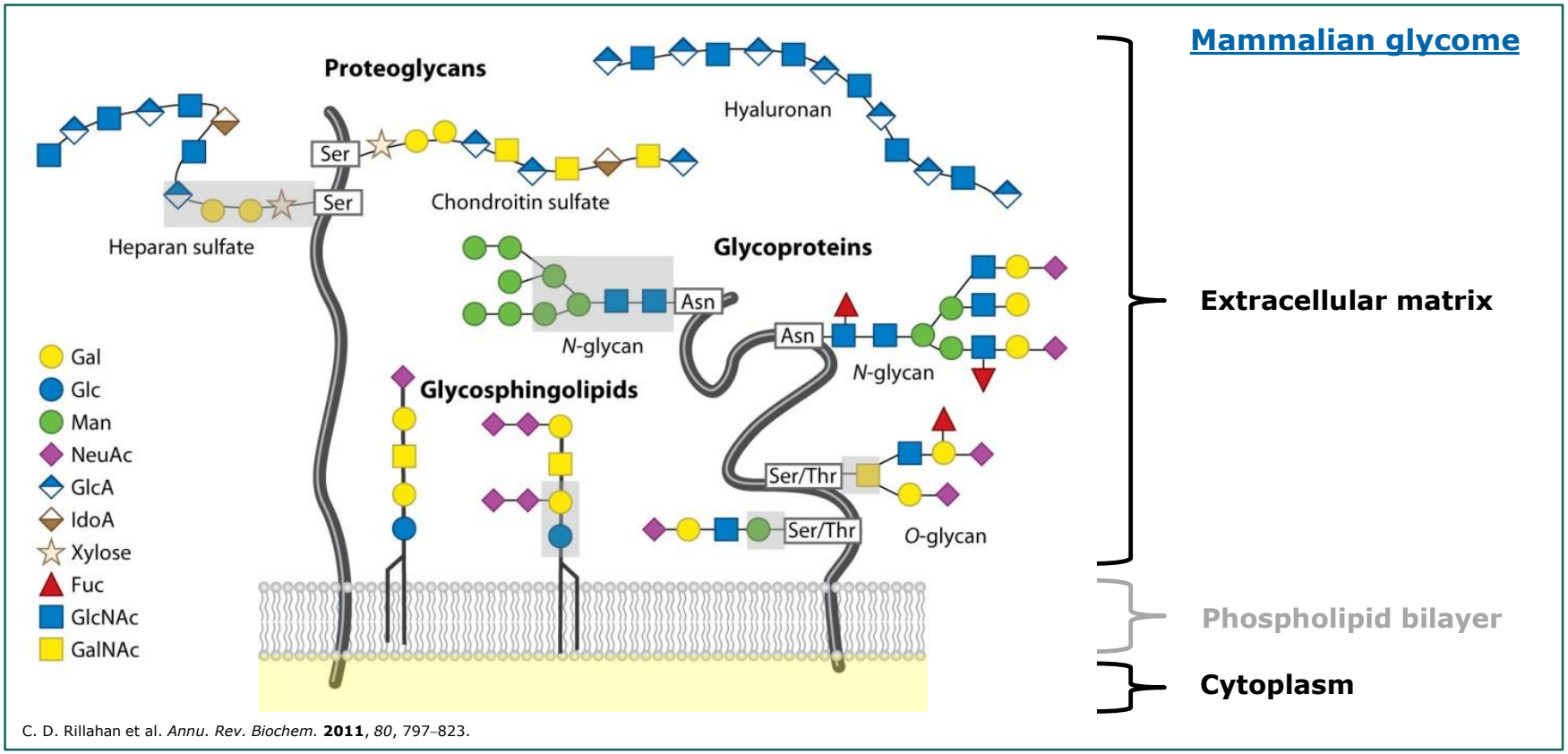
→ Other antibody (fragment) types



Fercher *et al.*, Experimental biology and medicine **2018**

Glycans

- Cell surfaces covered with various complex carbohydrates
- Glycans involved in many cell-cell interactions (e.g. cell motility or adhesion)

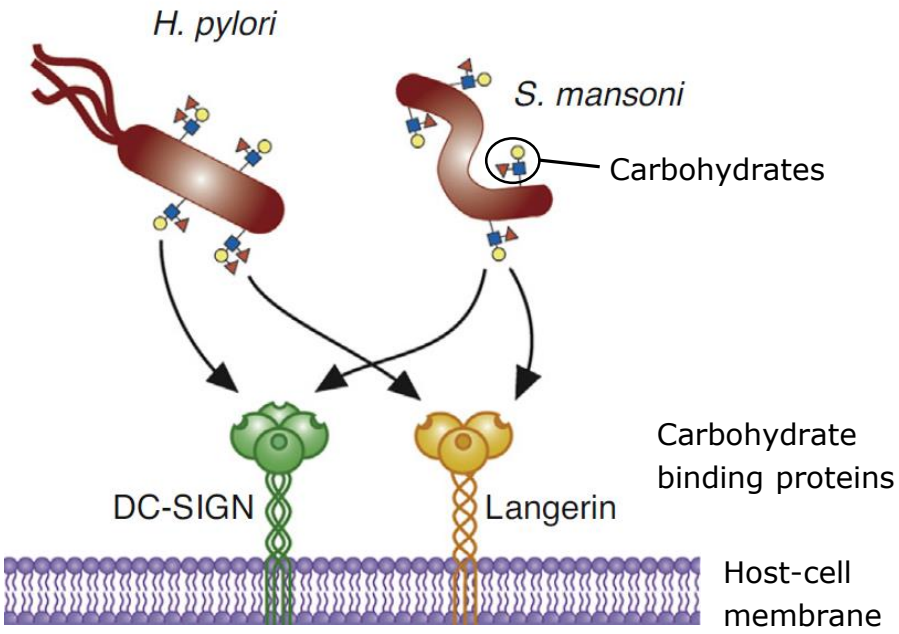


C. D. Rillahan et al. *Annu. Rev. Biochem.* **2011**, *80*, 797–823.

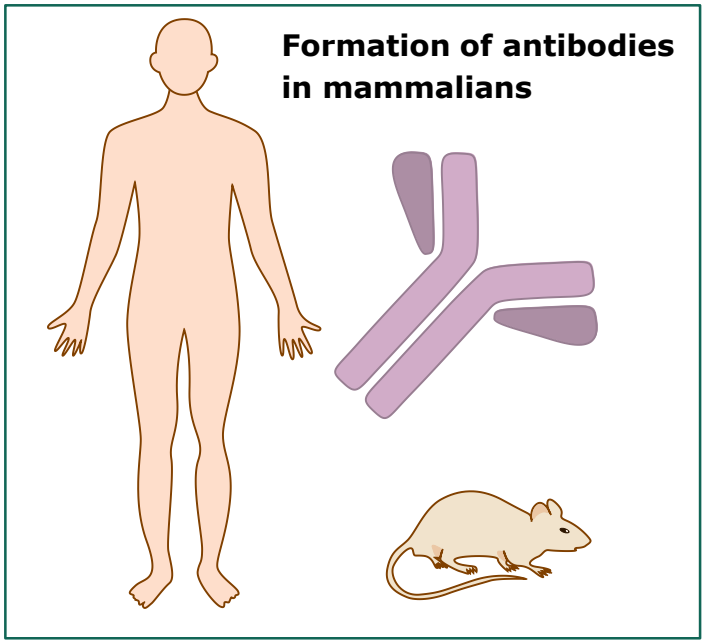
Glycans

- Pathogens often use surface-bound carbohydrates for invasion
- Pathogen glycans cause anti-glycan antibodies

  → Carbohydrate-based vaccines



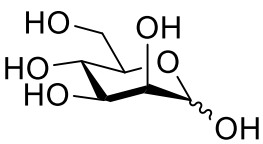
R. J. E. Li et al. *Curr. Opin. Biotechnol.* **2018**, *51*, 24–31.



Glycans – Carbohydrates - Saccharides

- "Carbohydrates" derived from hydrates of carbon (1844)

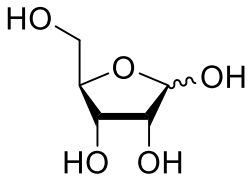
$$C_n(H_2O)_n$$



Mannose

$C_6H_{12}O_6$

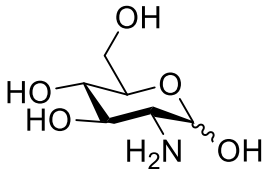
$C_6(H_2O)_6$



Ribose

$C_5H_{10}O_5$

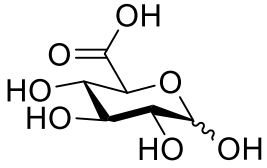
$C_5(H_2O)_5$



Glucosamine

$C_6H_{13}NO_5$

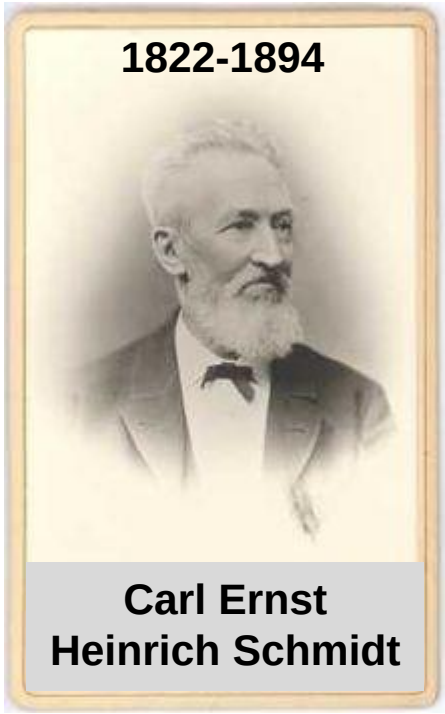
???



Glucuronic acid

$C_6H_{10}O_7$

???



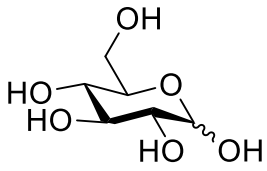
Old term but still used

Glycans - Monosaccharides

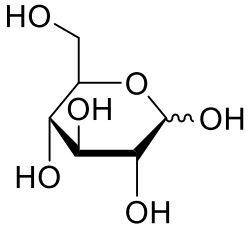
- Different possibilities how to draw them

All drawings display the stereochemistry!

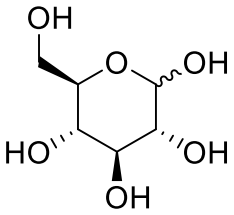
Glucose $C_6H_{12}O_6$



Chair conformation

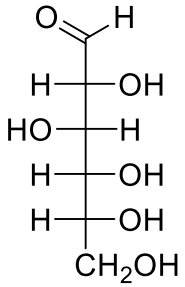


Haworth projection

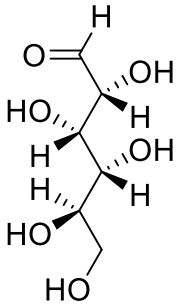


Stereochemical view from top

Ring form



Fischer projection



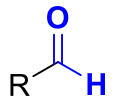
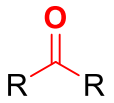
Natta projection

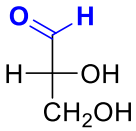
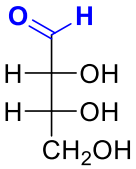
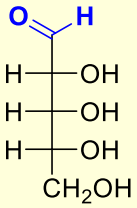
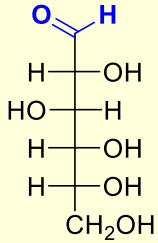
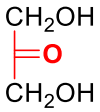
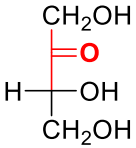
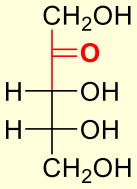
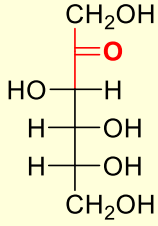
Open chain form

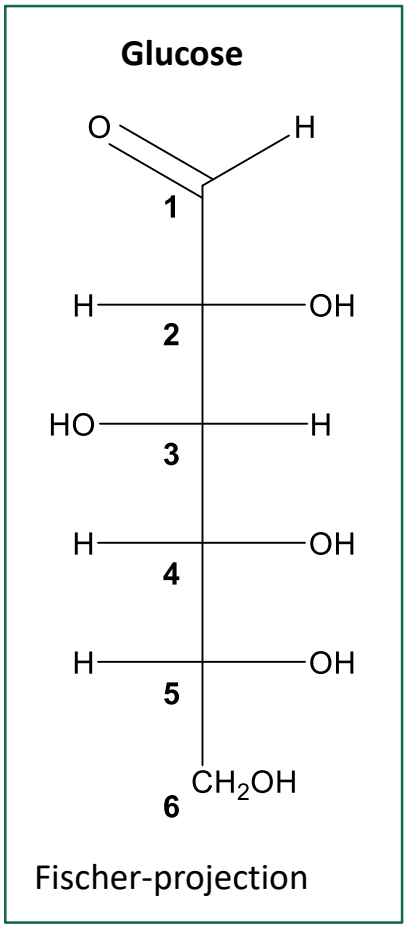
Reactive groups?

Glycans

- Two groups of monosaccharides: Aldoses and Ketoses

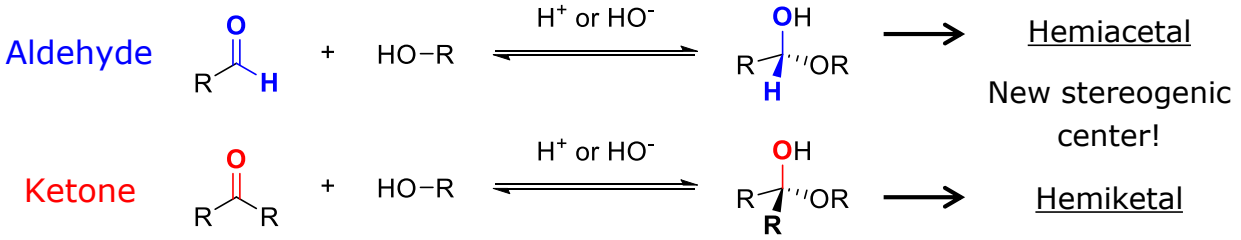
Aldehyde  Ketone 

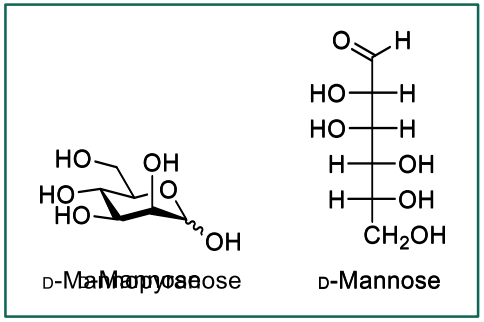
	Trioses	Tetroses	Pentoses	Hexoses
Aldoses	 Glyceraldehyde	 Erythrose	 Ribose	 Glucose
Ketoses	 Dihydroxyacetone	 Erythrulose	 Ribulose	 Fructose



Glycans

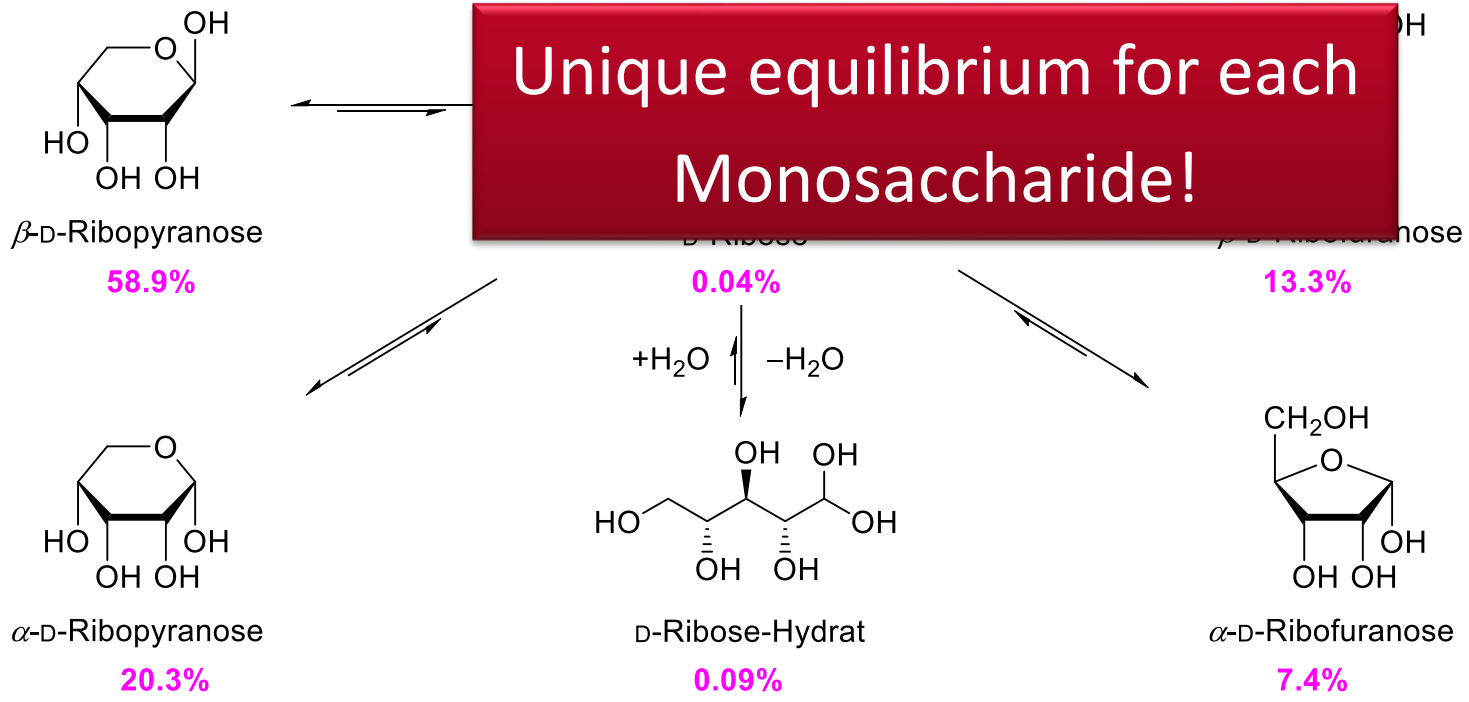
- What is the story behind all these different structures?

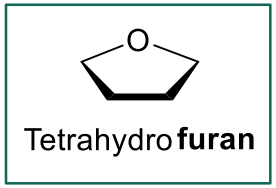


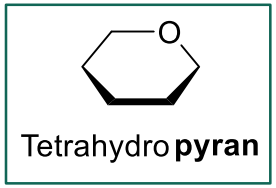


6 Forms can exist in solution!

Unique equilibrium for each Monosaccharide!

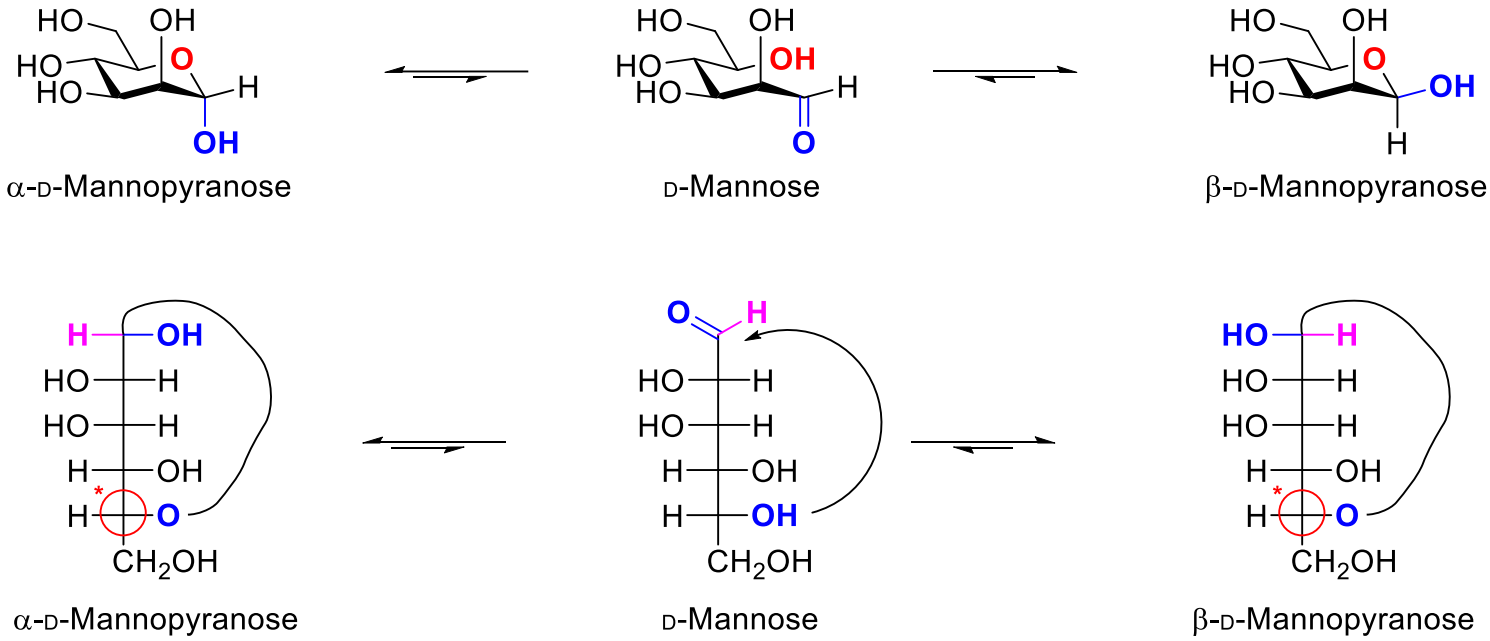






Glycans

- What is the α- and β-anomer?



Same side means α

Attention!

Up does not always mean β

Down does not always mean α

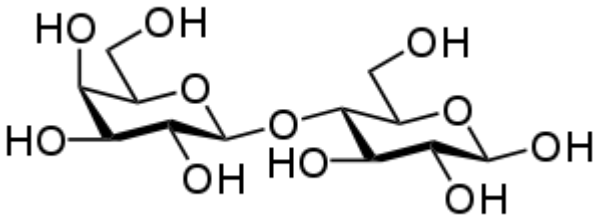
Opposite side means β

Glycans

- Monosaccharides

→ Oligo-/Polysaccharides or Glycans

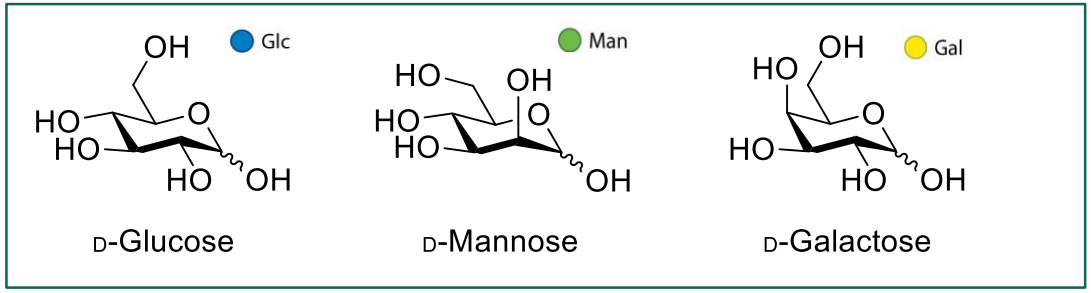
Lactose: β-D-galactose-(1→4)-D-glucose

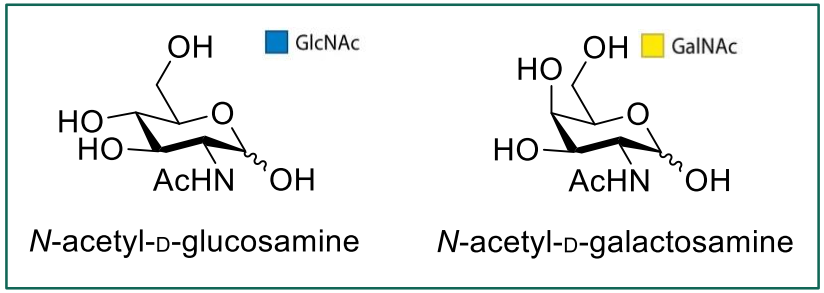


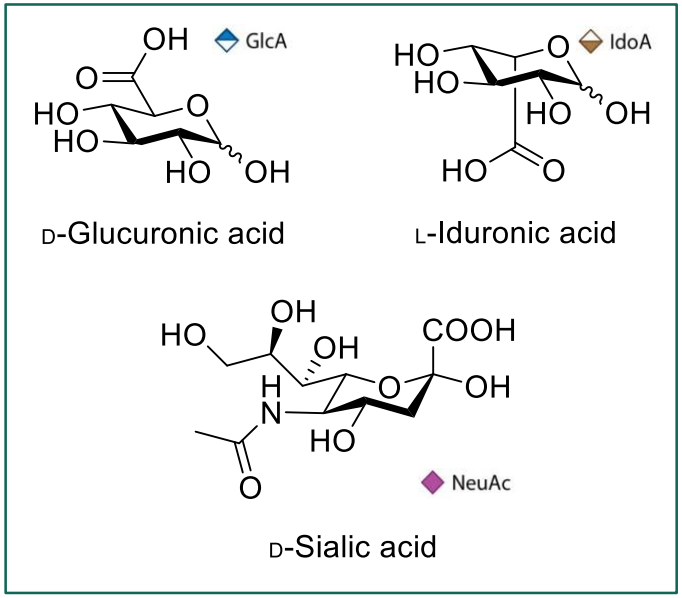
β-1→4 glycosidic bond

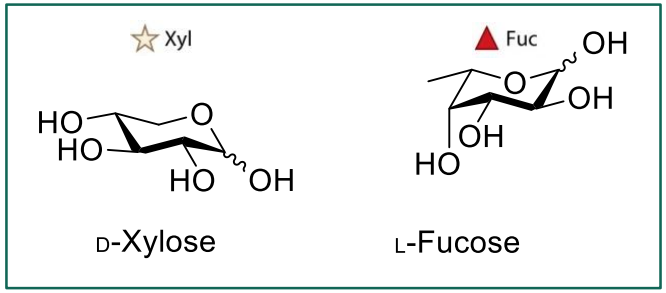
Glycans

- In mammals only 10 different sugar building blocks







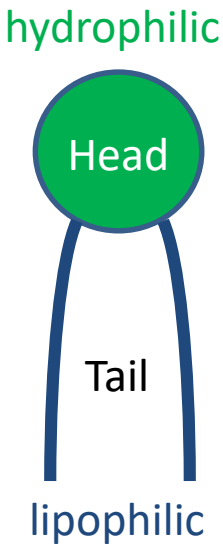


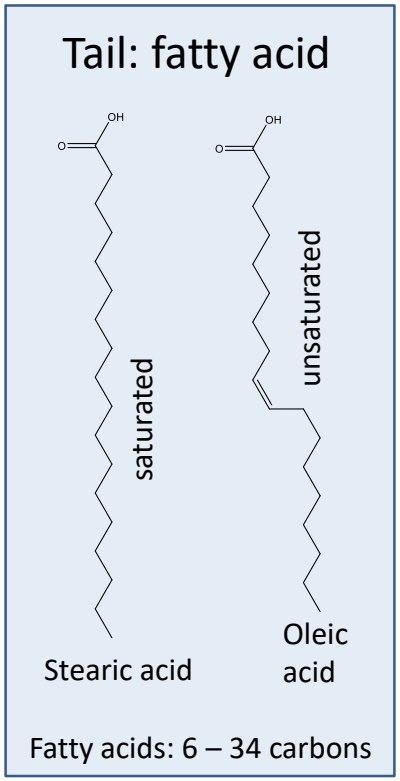
Why does a human glycan n-mer have > 76^n variants?

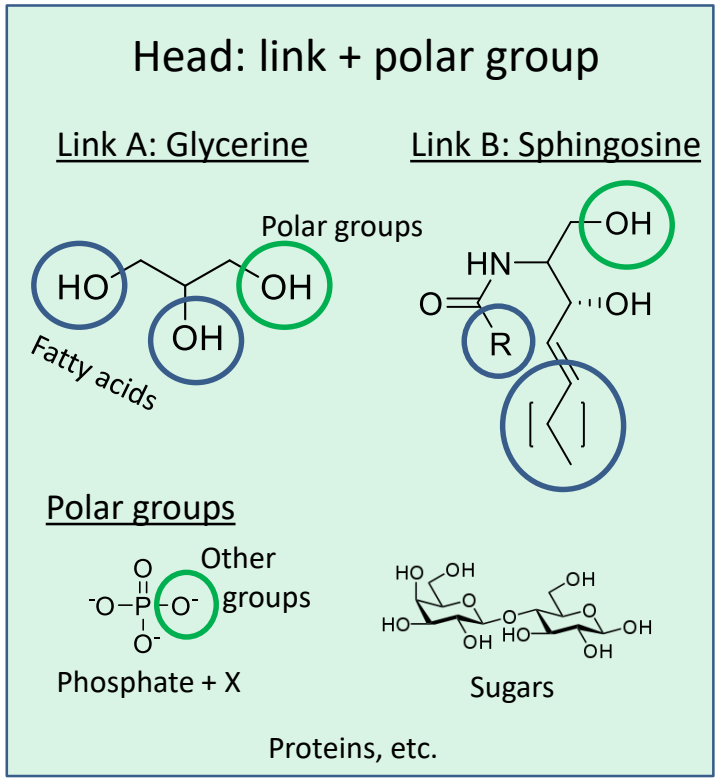
Lipids

- Biological lipids: amphiphilic compounds (hydrophilic head, lipophilic tail)
- Required for structure (membrane), energy, signaling
  - Phospholipids (phosphoglycerides, sphingolipids)
  - Glycolipids (phosphoglycolipids, sphingoglycolipids)

Immobilization difficult
→ Unspecific adhesion





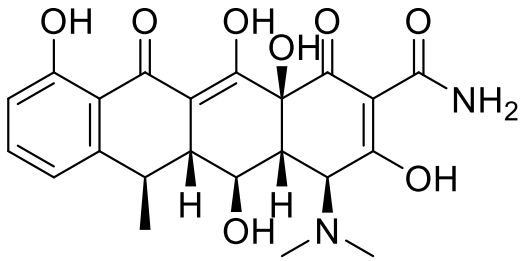


Small molecules

- Most drugs are small molecules (e.g. antibiotics, anti-malarial drugs)
- Low molecular weight (~< 900 Da)

  → oral (< 500 Da) vs. intravenous
- Can pass the lipid membrane
- Bind specific biological macromolecules
- Natural or synthetic compounds

Doxycyline
(antibiotic)



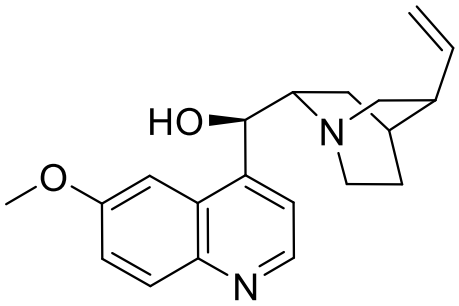
Molecular Weight: 444.44 Da

Immobilization depends on available groups

Giganitc number of small molecules (full chemical space)!

GeoTrinity, Wikipedia, Tonic Water

Quinine
(anti-malarial drug)



Molecular Weight: 324.42 Da

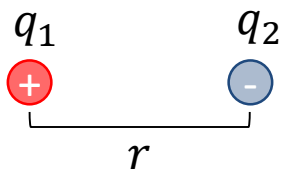
3. Interacting forces

Molecular interaction forces

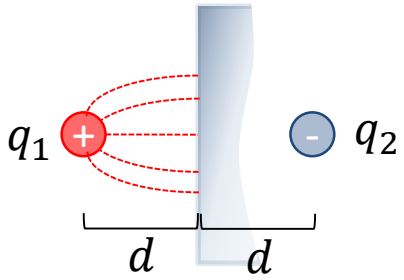
- Coulomb's law

$$F_c = k_e \frac{q_1 q_2}{r}$$

k_e: Coulomb constant



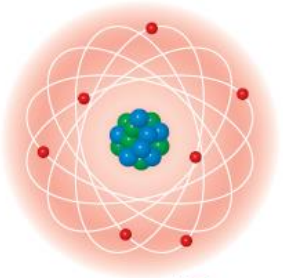
Metal plate



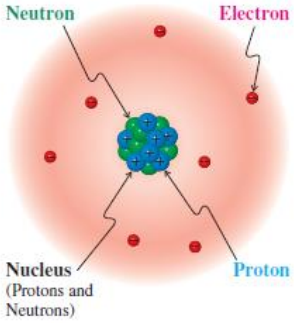
Induced mirror charge (virtual image of opposite charge)

→ Similar in molecules?

Classical model

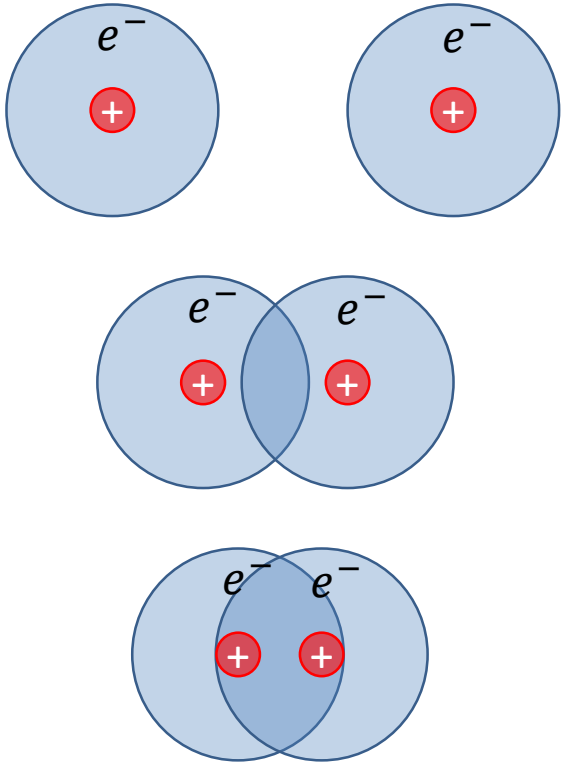


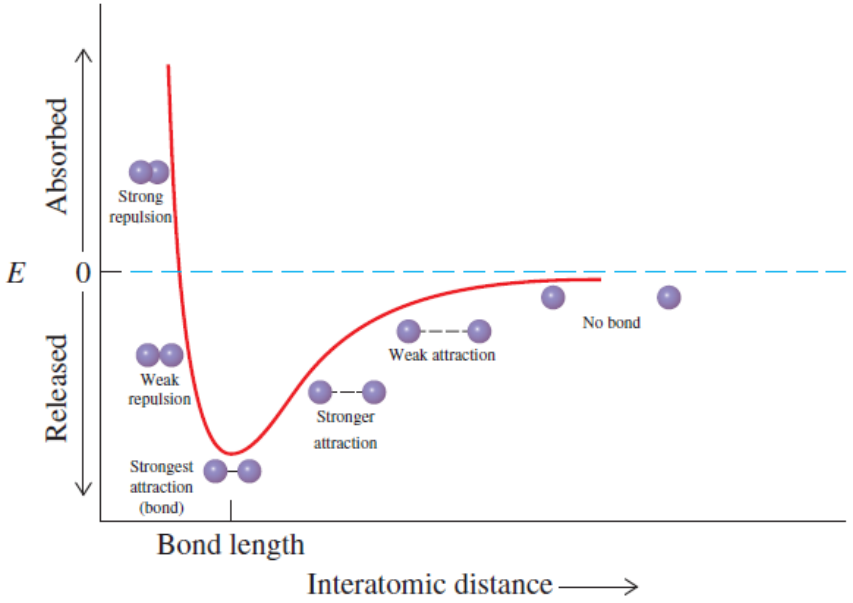
Quantum mechanical model



Molecular interaction forces

- Atomic attraction



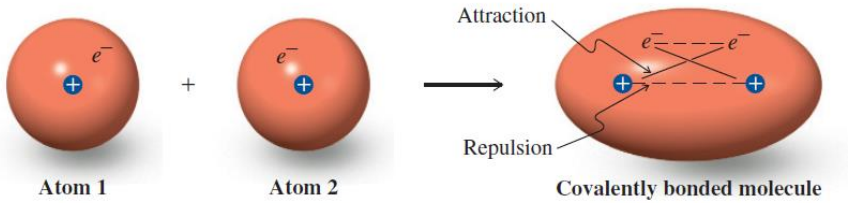


Vollhardt, Organic Chemistry, 2015

Molecular interaction forces

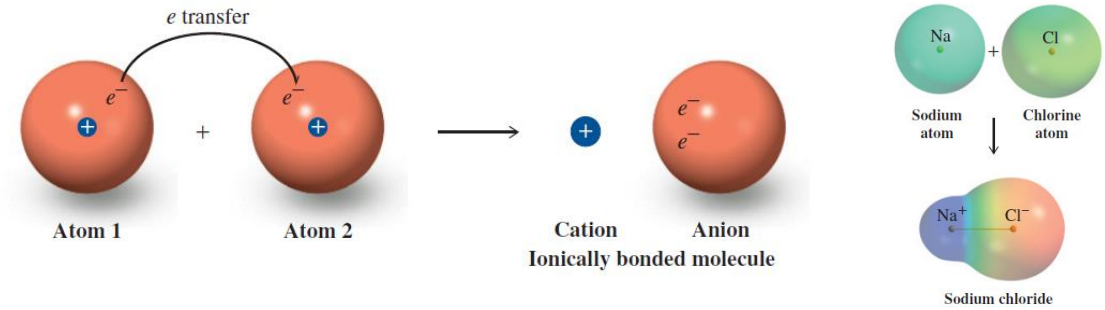
- Covalent

  130 – 1100 kJ/mol

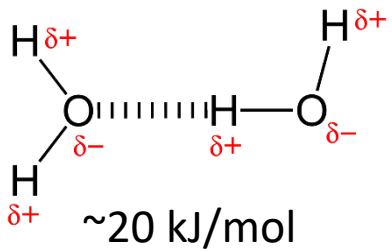


- Ionic

  > 5 kJ/mol

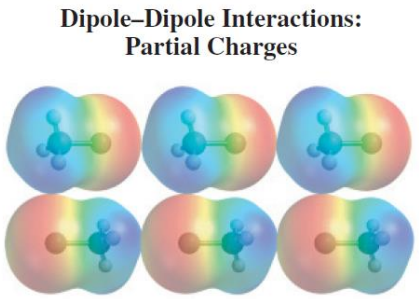


- Hydrogen bonds (→hydrophobic/hydrophilic forces), 4 – 50 kJ/mol
  - Special dipole-dipole case (H bonds to N, O, F atoms)



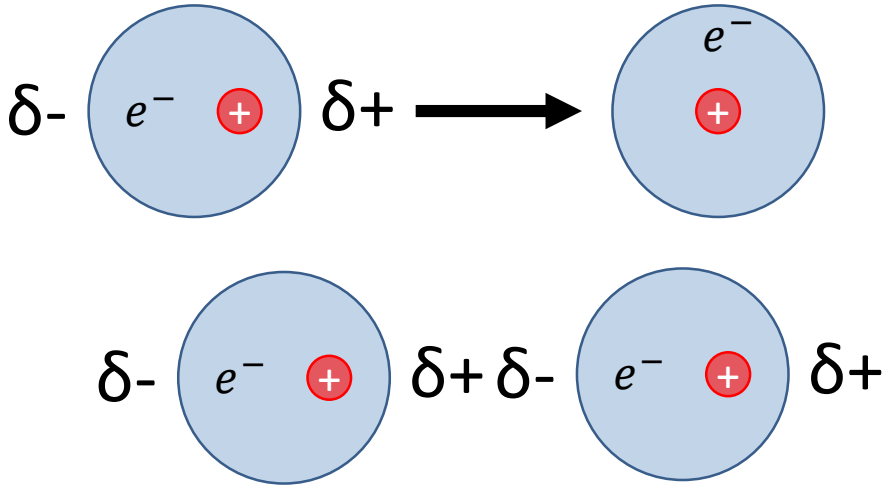
Molecular interaction forces

- Dipole-dipole, ~4 – 50 kJ/mol
  - Partial charges (electronegative)



Solid chloromethane
Vollhardt, Organic Chemistry, 2015

- Van-der-Waals (induced dipole) 0.4 – 4 kJ/mol (*e.g.* → Hexane liquid)
  - Random fluctuating polarizations (quantum dynamics)

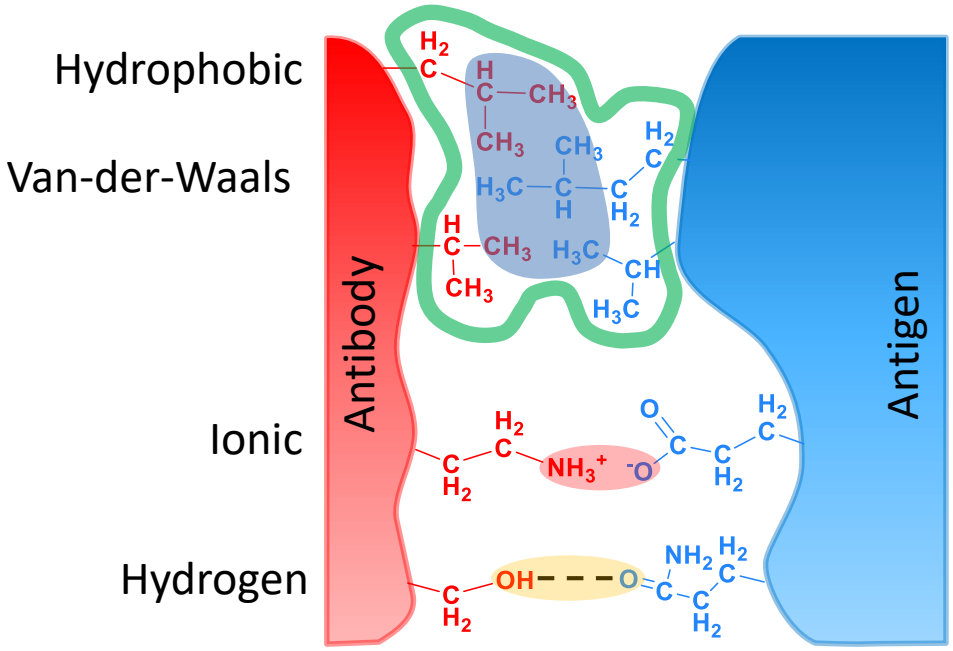


Author: Yintan, https://en.wikipedia.org/wiki/Gecko

- π-π stacking (aromatic ring interaction, part of dipole interaction)

Antibody-antigen interactions

- Antibody-antigen binding is non-covalent → reversible



4. Affinity, avidity, multivalency

Affinity

- Goal: Quantitative measurements of biological binding
- Non-covalent binding of <u>one</u> binding event of two molecules

$$A + B \underset{k_{off}}{\overset{k_{on}}{\rightleftharpoons}} AB$$

- In equilibrium: Constant concentrations

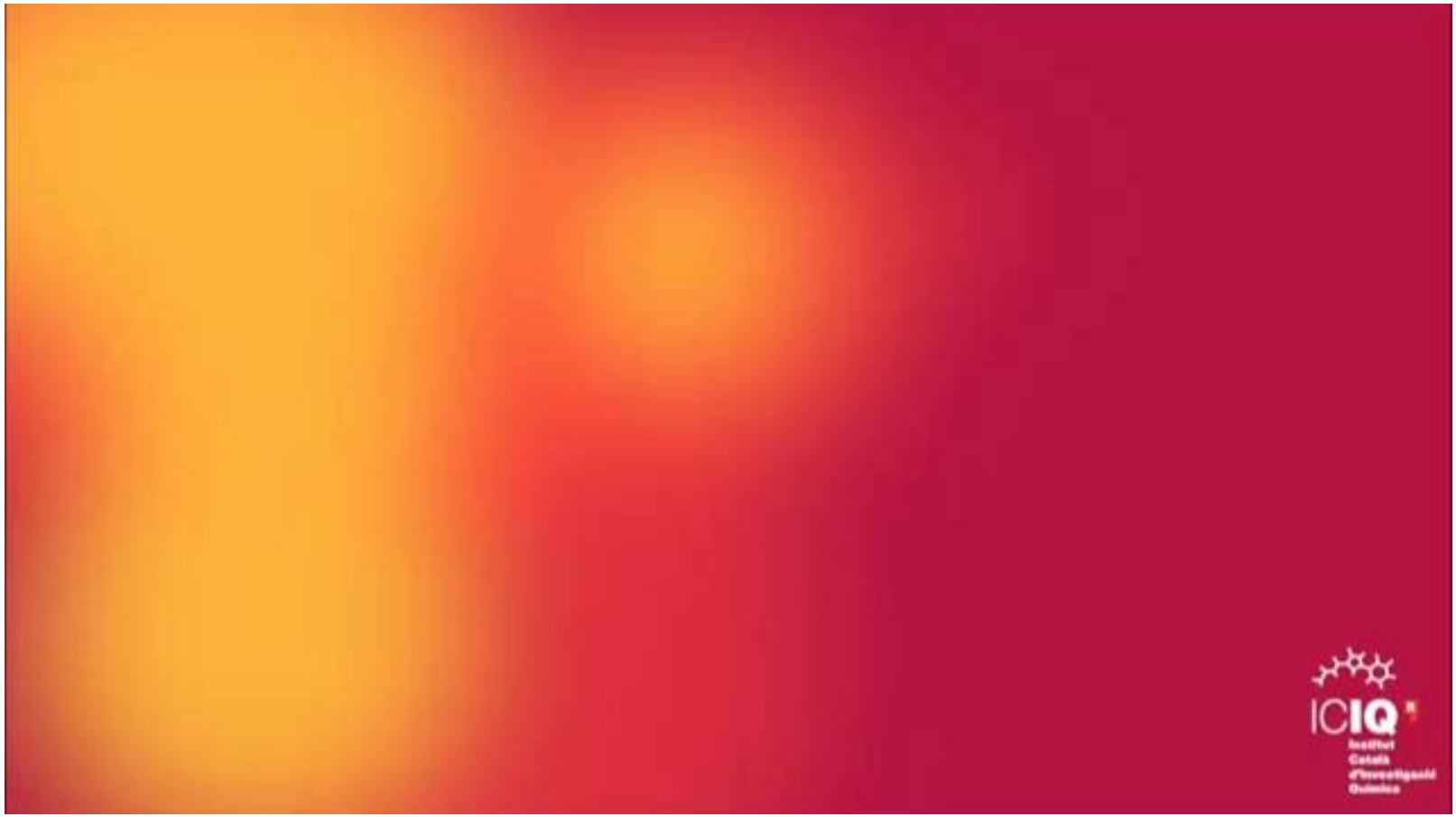
$$\frac{d[AB]}{dt} = [A] \cdot [B] \cdot k_{on} - [AB] \cdot k_{off} = 0$$ (Law of mass action)

$$[A] \cdot [B] \cdot k_{on} = [AB] \cdot k_{off}$$

- Equilibrium is not a static state (on/off continues)

Non-equilibrium reactions

- Oscillating Belousov-Zhabotinsky reaction



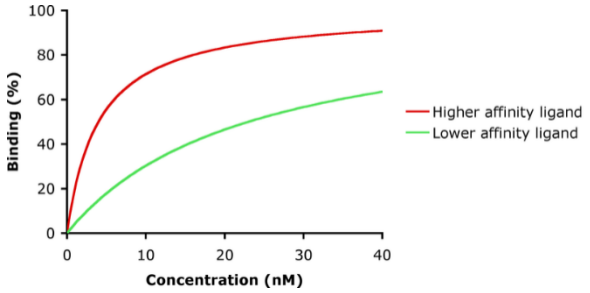
https://www.youtube.com/watch?v=PYxInARIhLY

→ Periodic color changes due to the formation and consumption of bromine

Affinity

- Affinity definition:

  → 50 % of B is bound to A

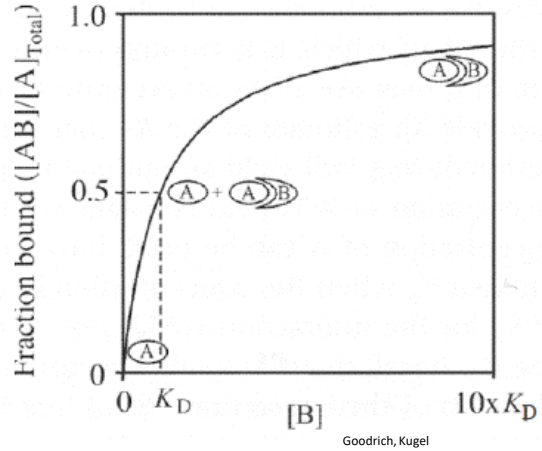


Keith Larence Brain, https://en.wikipedia.org/wiki/Ligand_(biochemistry)

- Equilibrium dissociation constant K_D

$$[A] \cdot [B] \cdot k_{on} = [AB] \cdot k_{off}$$

$$\rightarrow \quad K_D = \frac{k_{off}}{k_{on}} = \frac{[A] \cdot [B]}{[AB]}$$



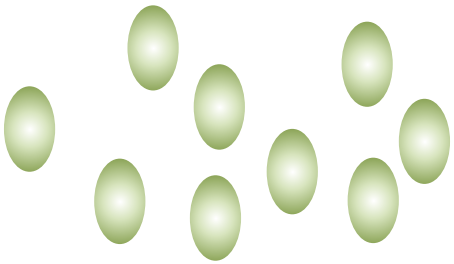
Goodrich, Kugel

K_D measured in: molar (mol/L = M)

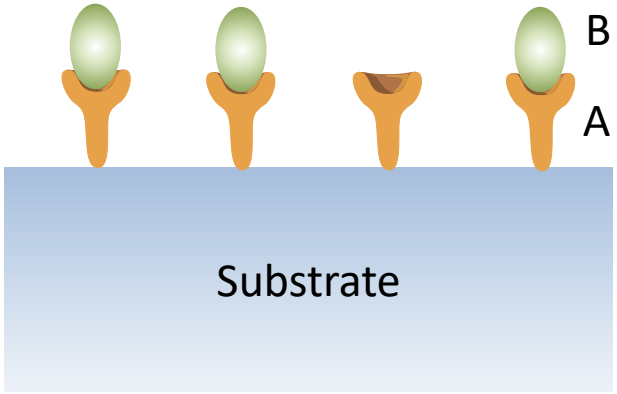
Antibodies: < 1 µM; 1 µM → quick dissociation
Very good antibodies: ~1 nM
Exceptionally good antibodies: ~10 – 100 pM
→ „longer“/stronger binding
Biotin-streptavidin: 10 fM

Measurement:
$[A]_{Total} << K_D$
Keep [A] constant, titrate B

Affinity



B (*e.g.* protein)
in solution (in excess)

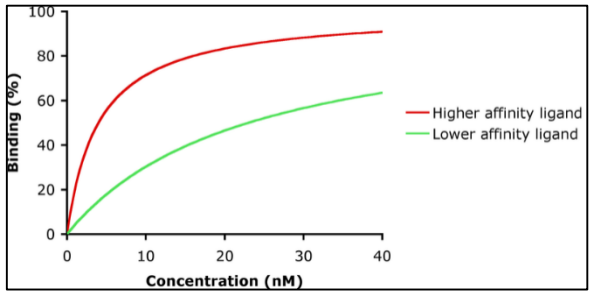


B (*e.g.* protein)

A (*e.g.* peptide)

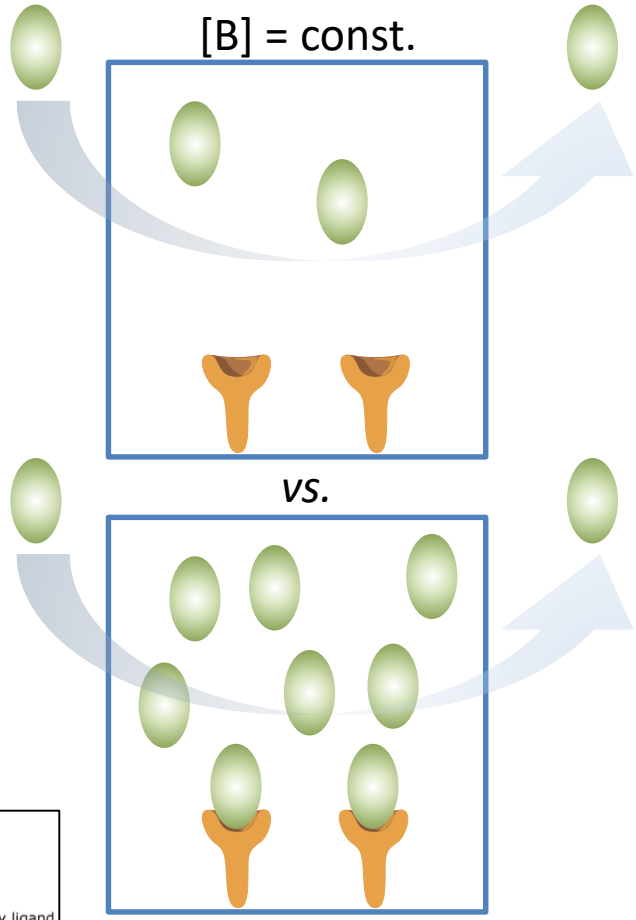
Concentration of A = [A] is small, because immobilized to surface

<u>Measurement:</u>
→ [A] constant, titrate B

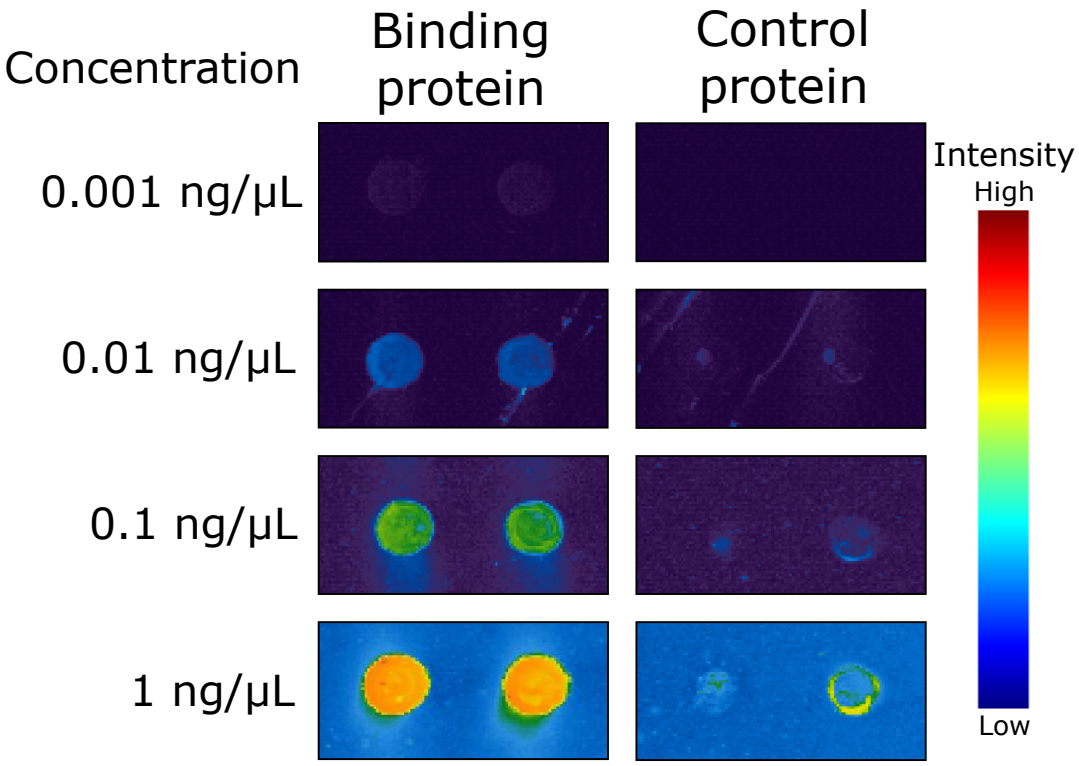


Keith Larence Brain, https://en.wikipedia.org/wiki/Ligand_(biochemistry)

Thought experiment
[A] = const.
[B] = const.



Affinity



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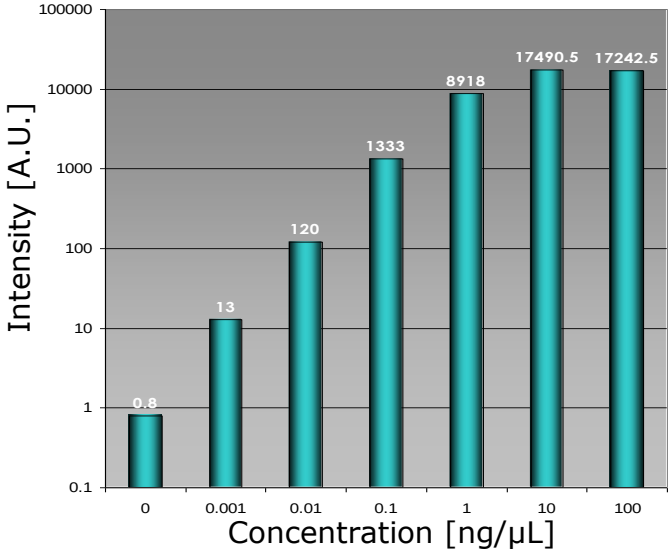
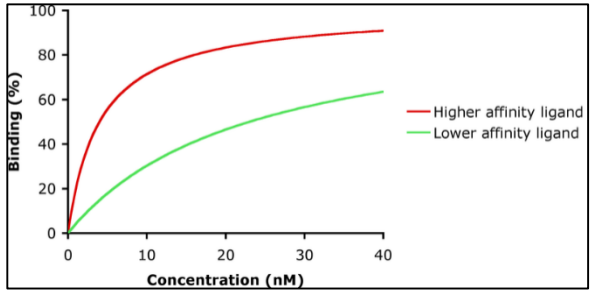




Keith Larence Brain, https://en.wikipedia.org/wiki/Ligand_(biochemistry)

Affinity measurement

- Direct labeling
  - Biotin
  - Radioisotopes
  - Fluorophores/quantum dots
  - Enzymes

→ Can cause steric hindrance or change structural/chemical configurations of analyte

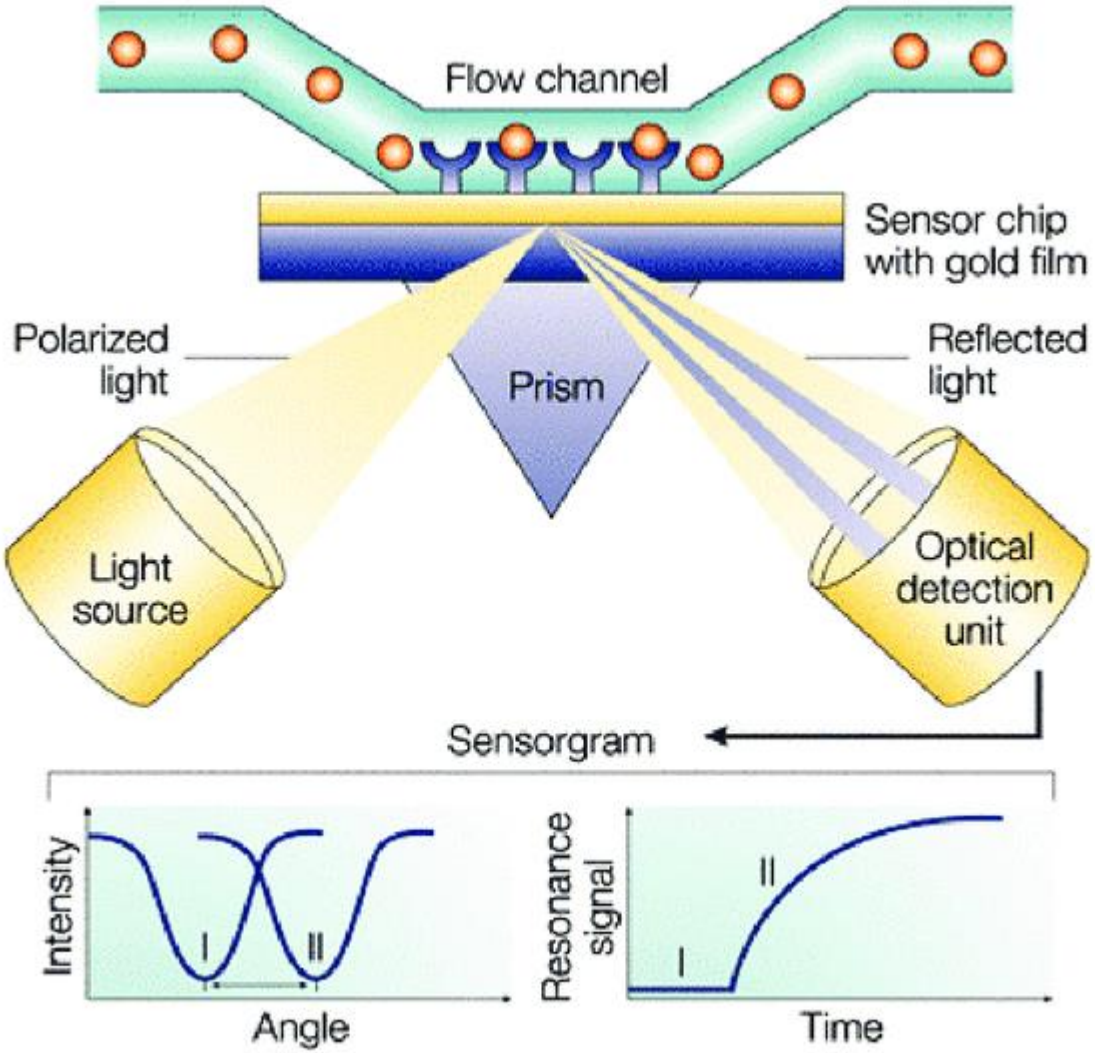
- Label-free detection
  - Surface plasmon resonance (SPR)
  - Isothermal titration calorimetry (ITC)
  - Quarz crystal microbalance (QCM)
  - Ellipsometry
  - Reflectrometric interference spectroscopy (RIFS)
  - Oblique-incidence reflectivity difference (OI-RD) scanner

→ Sensitive measurement of native biomolecules

→ Requires special devices

Affinity measurement – Biacore

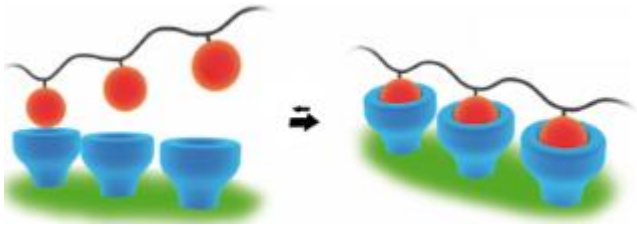
- Surface plasmon resonance (*e.g.* Biacore by GE Healthcare)



Multivalency

- Biomolecules can bind to each other at more than one site

- Multivalency:
  - Simultaneous binding of multiple ligands on one biological molecule to multiple receptors
  - Strong (in total), weak(er) single interactions, but also reversible
  - Multiple similar (IgM) or different (virus) binding sites can contribute



Haag, *Beilstein J. Org. Chem.* **2015**

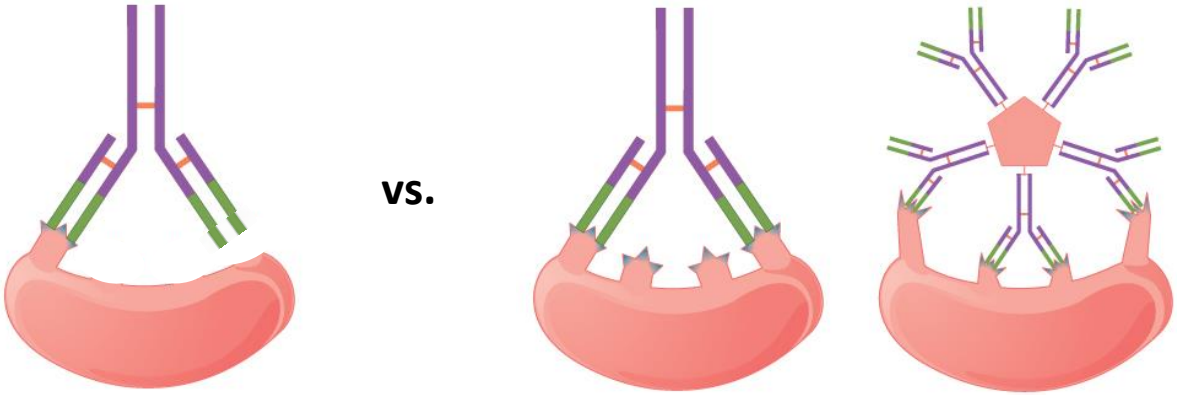
  - Virus-host
  - Ligand-receptor
  - Cell-cell

Avidity & multivalency

- Affinity does not represent:
  - Complex antigens with many (repetitive) binding sites and e.g. antibodies with 2 – 10 binding sites
  - Interaction of antigen and antibody in one site increases probability to bind at a second site (if present)

- **Avidity:** Binding strength of multiple interactions together

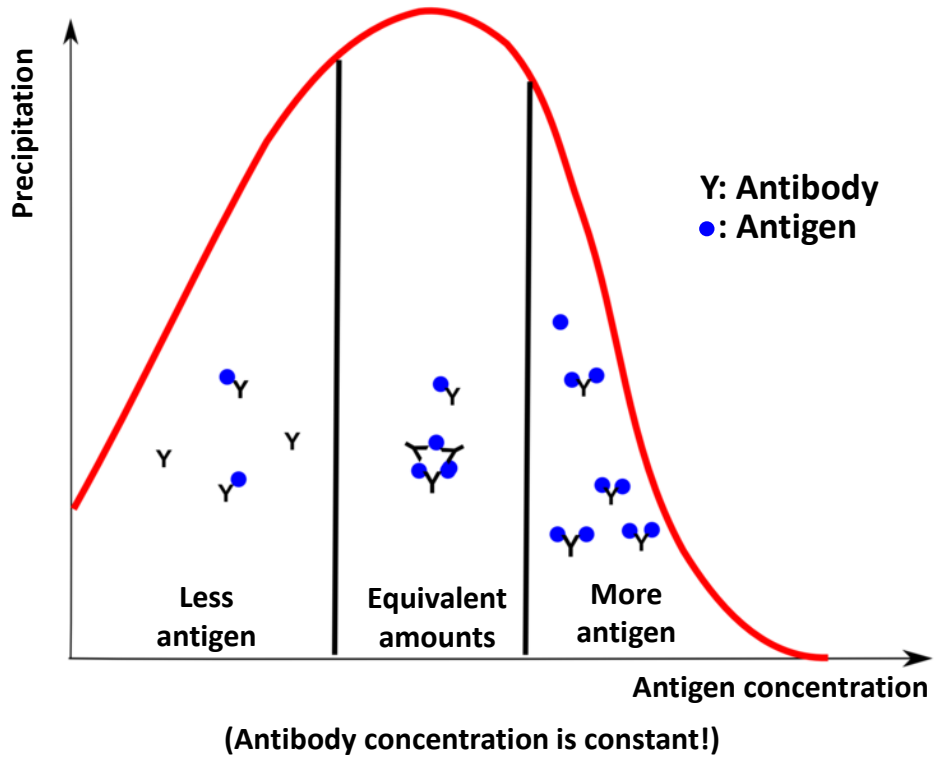
  → High avidity compensates for low (single) affinity



Biology 2e, OpenStax

Antibody-antigen interactions

- Precipitation (Heidelberger) curve

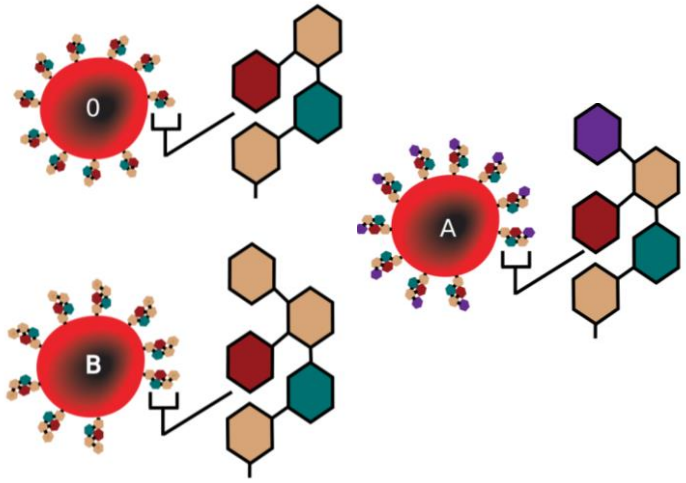


https://www.abiweb.de/biologie-immunologie/vielfalt-des-immunsystems/antikoerpervielfalt/antigen-antikoerper-interaktion.html

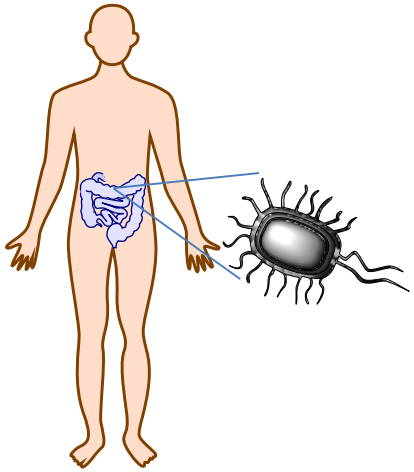
→ Saturation can cause lower binding signal in assay

Cross reactivity

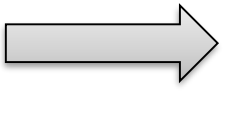
- Despite specificity of antigen-antibody interaction:
  - Antibody may cross react with other antigens (unrelated epitope with similar binding properties)
- Example 1: ABO blood group antigens (glycans!)
  - A and B blood type defined by different glycan antigens

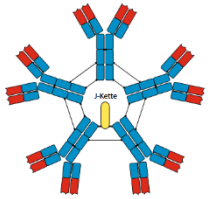


https://de.wikipedia.org/wiki/Datei:ABO_blood_group_diagram-de.svg



Blood type A





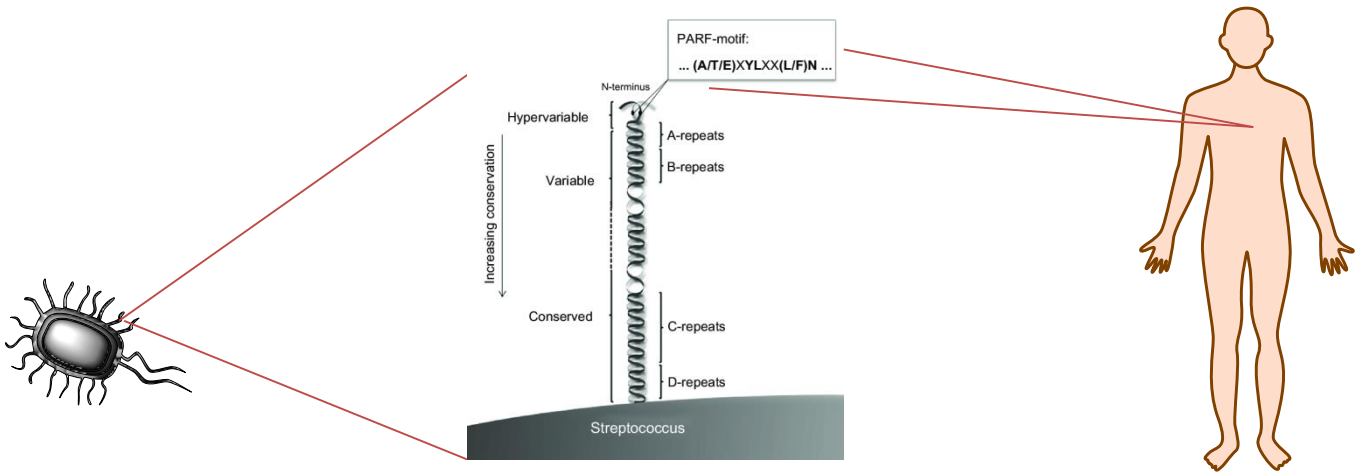
Cross reactive
anti-(blood group B)-IgM

→ Microbes cause development of complementary blood group antibodies

Cross reactivity

- Viral and bacterial antigens similar to human components
  → Induced autoimmune reaction (rheumatic fever)

- Example 2: Streptococcus pyogenes cell wall antigens (M protein)
  → Antibodies may cross react with heart and skeletal muscles
  (<u>theory is still debated!</u>)



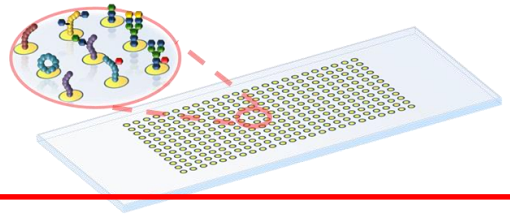
– Break –

Elevator Pitch

Describe your Bachelor thesis project in 30 seconds to a non-expert scientist

5. Surface functionalizations, surface chemistry, reactive groups

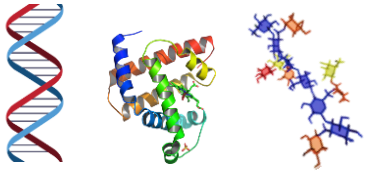
Microarrays – Big picture



Microarrays for screening

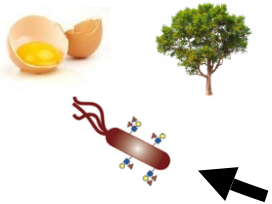
Spotting & coupling of ready compounds onto array

In-situ (= directly on the array) high-throughput synthesis of compounds



Microstructuring methods (*e.g.*, spotting, printing…)

Extraction & purification from "natural" sources



Chemically or biologically synthesized compounds

Building blocks + Enzymes

Liquid phase or Solid phase

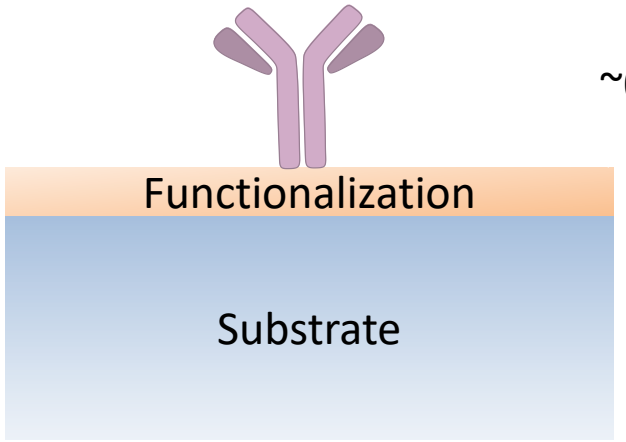
Building blocks + Chemical reagents

Surfaces

- No “one fits all“ for the ideal immobilization
  → Unique solutions for each molecule/application

Components

- Substrate (support material)
- Functionalization (linker)
- Molecule (catcher/binder)



Substrate

Polymers

Polystyrene (PS)
Polyacrylamide
Polyethylenterephthalate (PET)
Polyurethane
Polyvinylalcohol (PVA)
Nylon
Polypyrrole
Sephadex LH 20
Perfluoropolymer
Polypropylene (PP)

http://vp-sci.com/products/reagent-cleaning-reservoirs-and-specialty-labware/microplates/vp-416.html

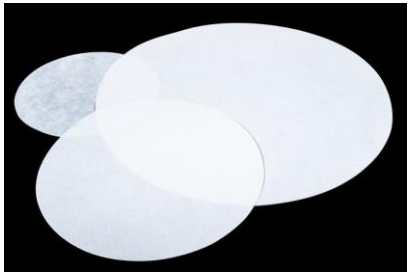
Inorganic

Gold
Glass
Titanium
Aluminium
Metal oxides

https://www.mirka.com/glass_sanding/

Organic/Biological

Cotton
Cellulose
Latex
Nitrocellulose
Carboxymethyldextran
Chitosan



https://www.bestvaluevacs.com/cellulose-filter-paper-50-micron-5-pack.html

→ Mixtures or composites

Substrate – glass

- Glass most versatile for chemical functionalization
  - Temperature stability
  - Inert
  - Chemical resistance to most organic solvents, salt, pH
  - Planar and smooth surface (roughness < 10 nm locally)
  - Low background fluorescence
  - Cheap
  - Environmental compatibility

https://www.mirka.com/glass_sanding/

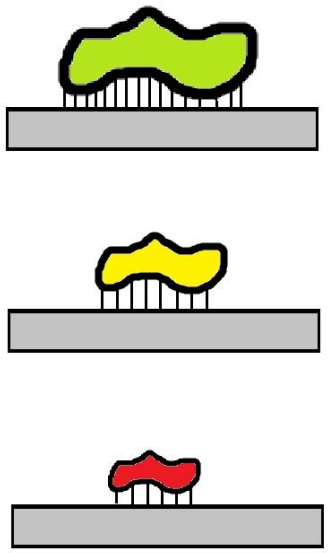
Immobilization strategies – non-covalent

Adsorption, physisorption

- Non-covalent binding (less stable), *e.g.* electrostatic or hydrophobic
- Fast, simple, (mainly) nondenaturing
- No molecular orientation
- Examples:
  - ELISA (polystyrene)
  - Western blot (nitrocellulose)

<u>Vroman effect:</u>
- First smaller (quicker), then, larger proteins adsorb to surfaces

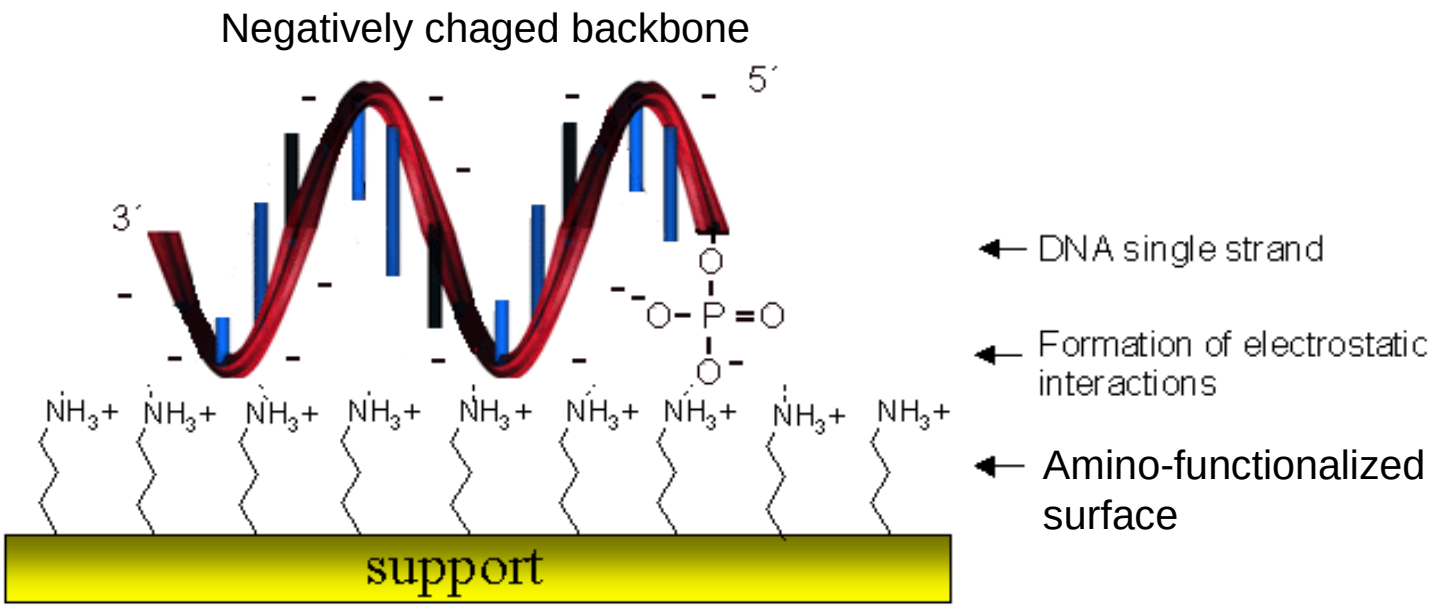
- In equilibirum mainly larger molecules are adsorbed



SurfaceChem446, https://de.wikipedia.org/wiki/Physisorption

Immobilization strategies – non-covalent

- Ionic/polar binding (nucleic acids and some proteins)



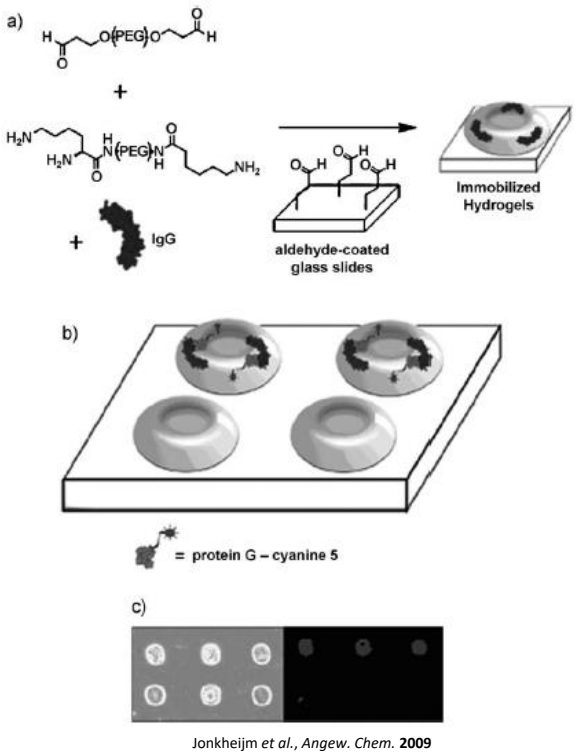
Immobilization strategies – non-covalent

Inclusion

- Trapping molecules in gels/hydrogens (agar or polyacrylamide)
- Nondenaturing
- Gel pores must be smaller than trapped molecule
  - Not suitable for smaller molecules

Example

- Aldehyde-, lysine-polyethyleneglycol, IgG mixture spotted on surface
- Incubation with Cy5-labeled protein G

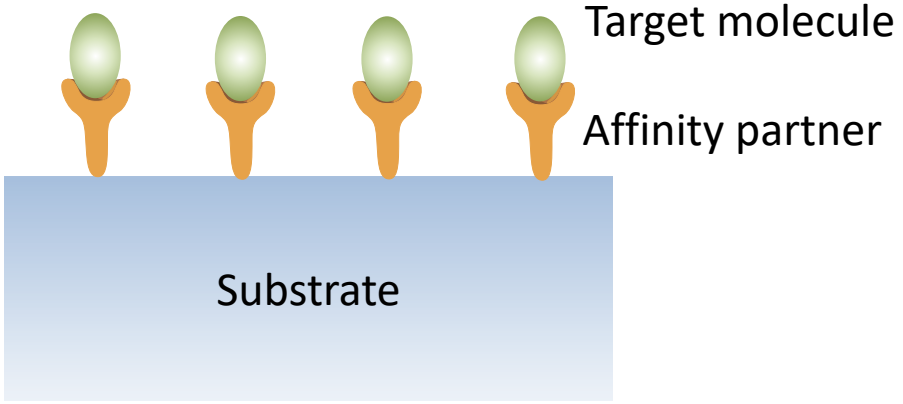


Jonkheijm *et al., Angew. Chem.* **2009**

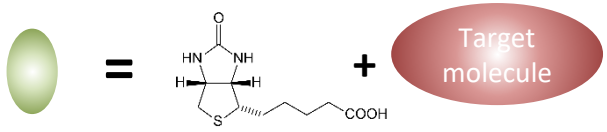
Immobilization strategies – non-covalent

Affinity immobilization

- (Strept-)avidin/biotin
- Protein A/G
- Nucleotides
- Fatty acids, flourinated alkanes
- PolyHis-Tag & nickel

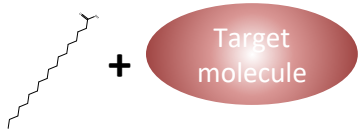


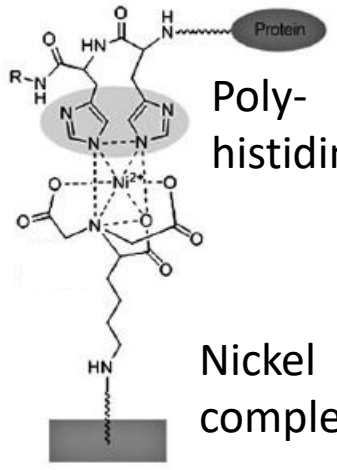
Biotin



IgG

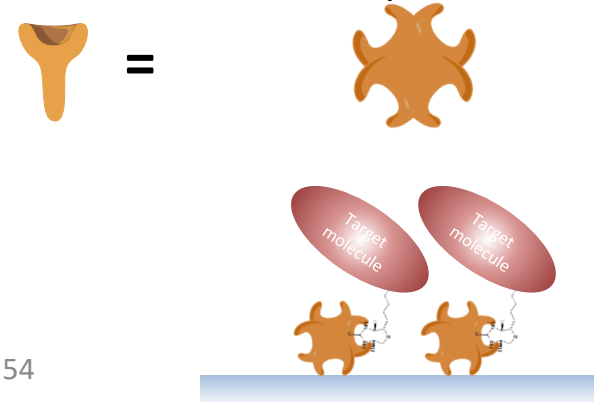
Fatty acid



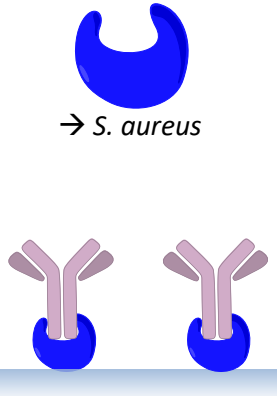


Jonkheijm *et al.*, *Angew. Chem.* **2009**

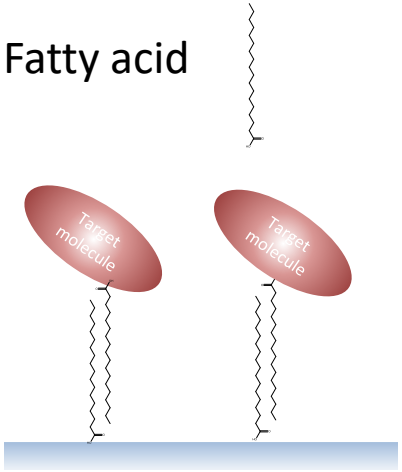
Streptavidin



Protein A



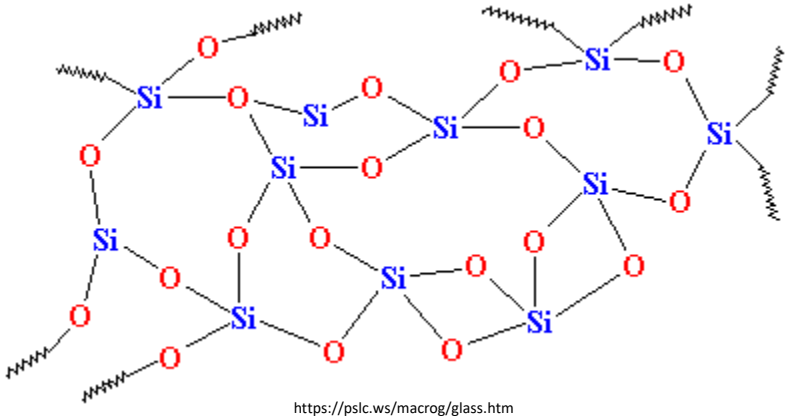
Fatty acid



Immobilization strategies – covalent

- Forming a covalent chemical bond to a surface
  - Polymer
  - Glass

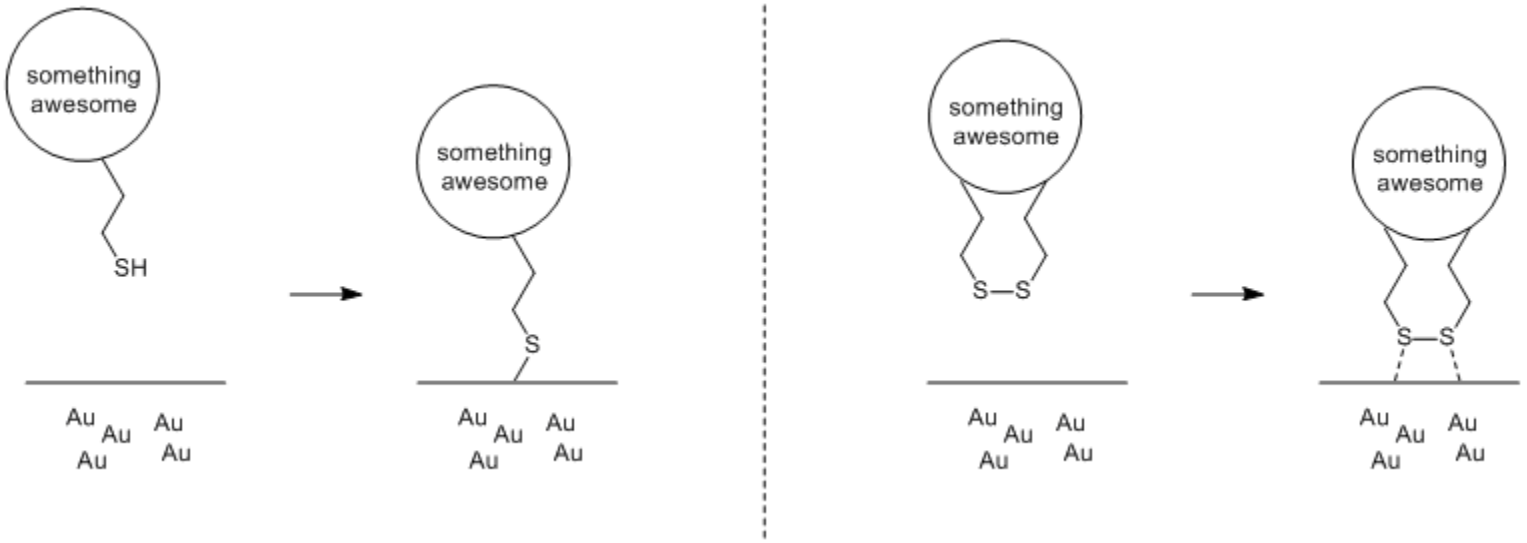
- Reactive groups required
  - Sulfur-gold binding (easy, quick, but less stable)
  - Introduction of reactive groups
    - → Polymers: introduce co-polymers
    - → Glass: requires harsh chemicals



https://pslc.ws/macrog/glass.htm

Immobilization strategies – covalent

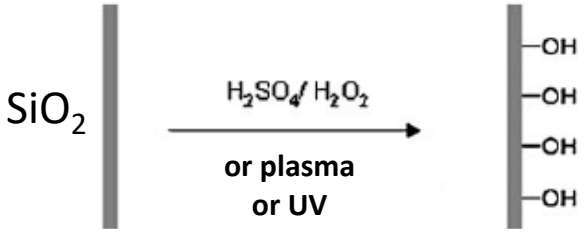
- Sulfur-gold binding



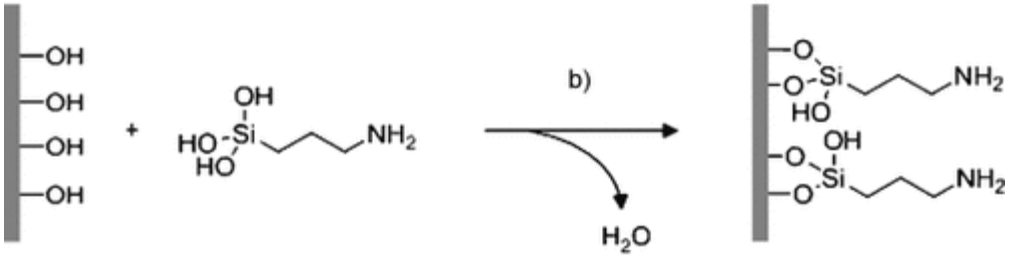
https://syntheticremarks.com/what-is-the-true-nature-of-gold-sulfur-bonds/

Immobilization strategies – covalent

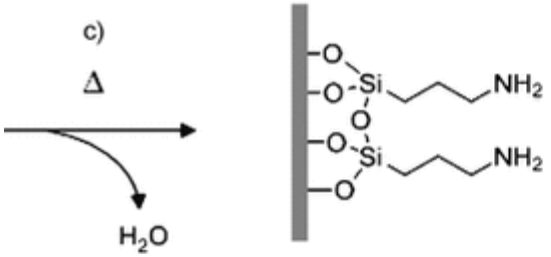
- Silanization:



Cleaning and generation of free OH groups on glass (SiO_2) with Piranha solution (sulfuric acid & hydrogen peroxide) or plasma/UV cleaning



(Amino)-silane coupling reaction & heating up to 130 °C for 2h

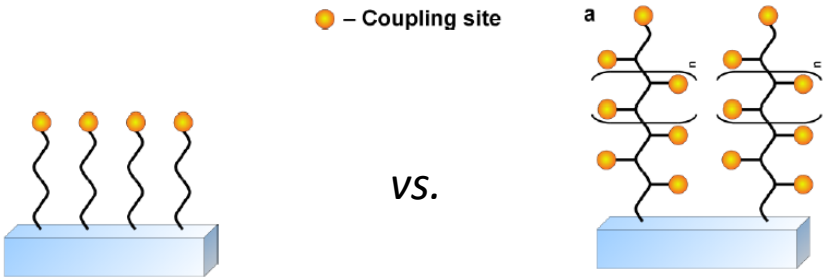


→ Many different silanes possible!?

Jonkheijm *et al.*, *Angew. Chem.* **2009**

3d surfaces (polymer on glass)

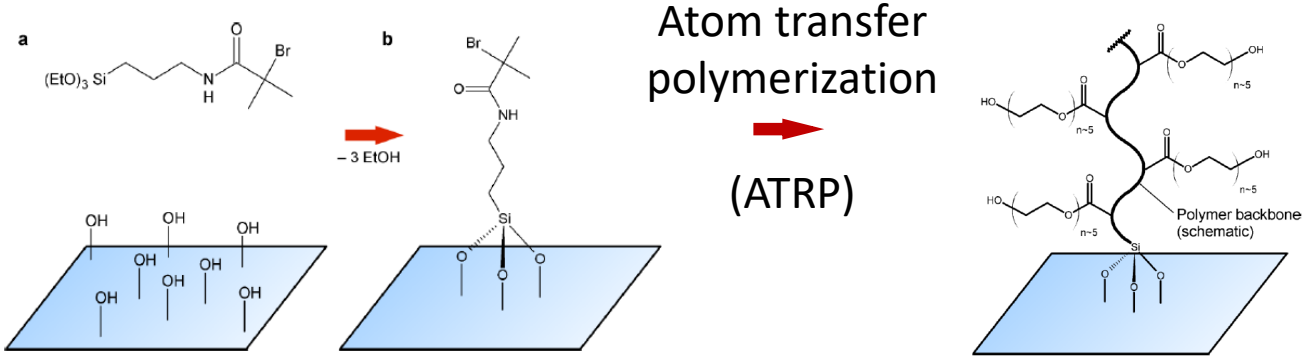
- Glass surfaces with Functional polymer surfaces



2d functionalized surface

3d functionalized surface

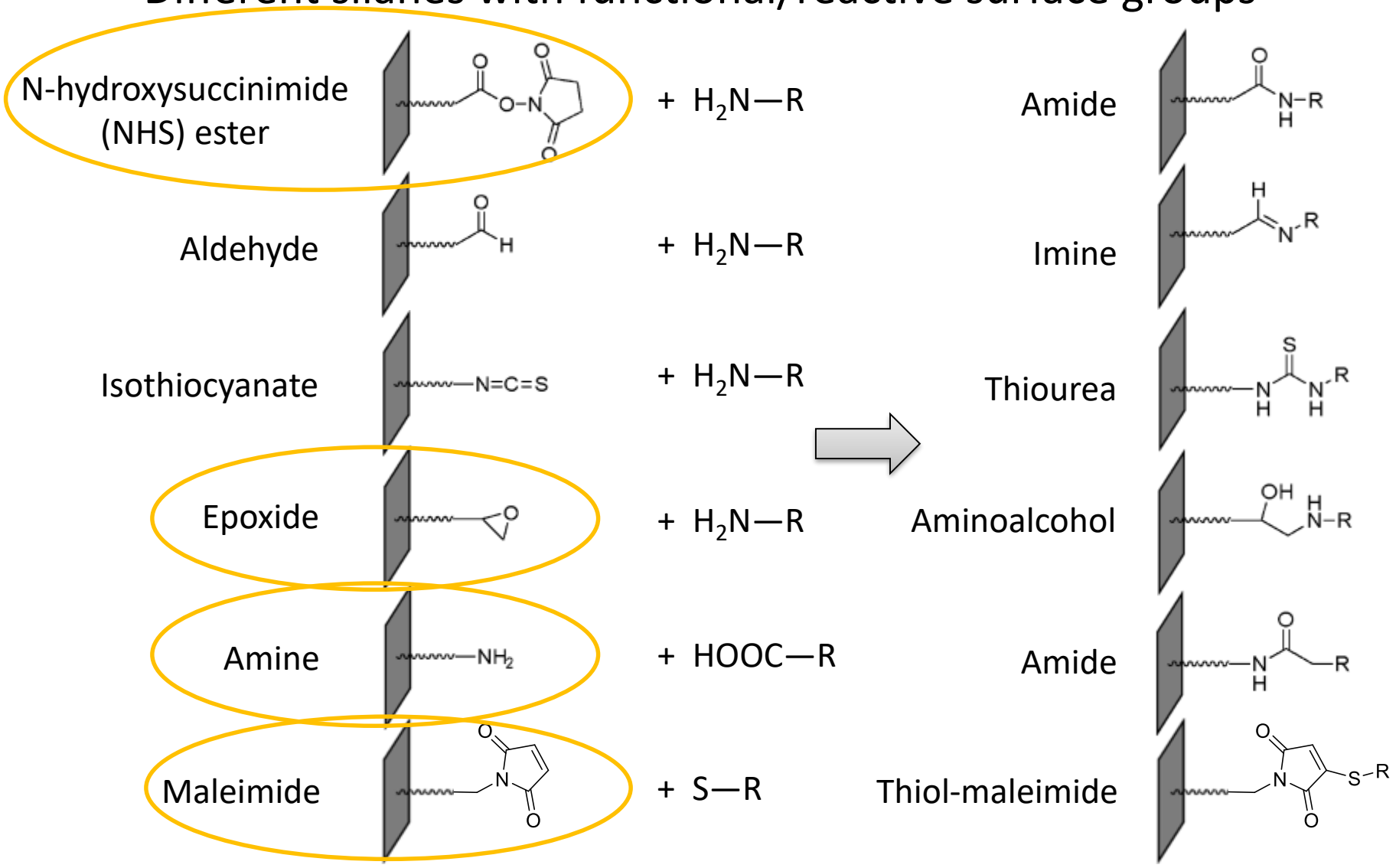
- Functional polymer surfaces



Many companies sell functional microarray surfaces (Schott, Surmodics, PolyAn, Pepperprint, etc.)

Functional surface groups 1

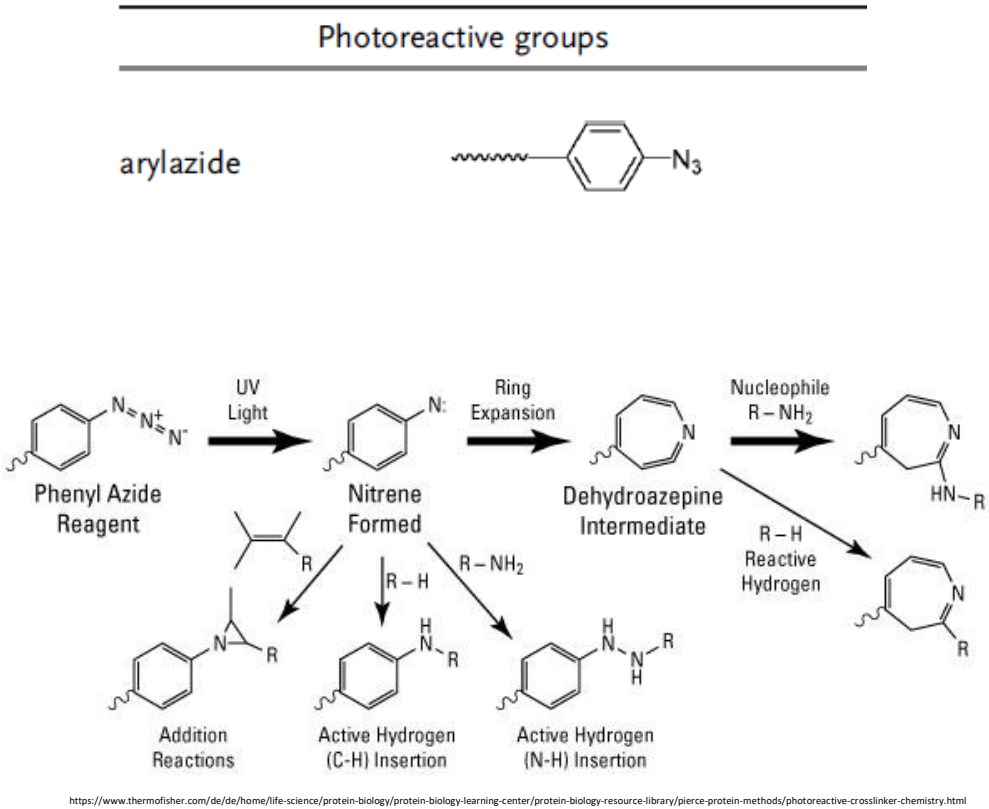
- Different silanes with functional/reactive surface groups



Jonkheijm *et al.*, *Angew. Chem.* **2009**

Functional surface groups 2

- Photoreactive group(s)



https://www.thermofisher.com/de/de/home/life-science/protein-biology/protein-biology-learning-center/protein-biology-resource-library/pierce-protein-methods/photoreactive-crosslinker-chemistry.html

Disadvantages:

- May react unspecifically
- May destroy molecules

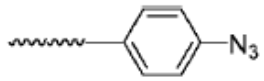
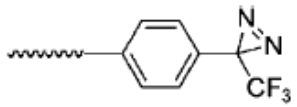
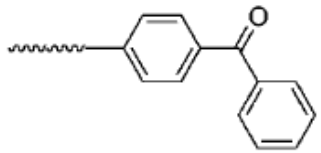
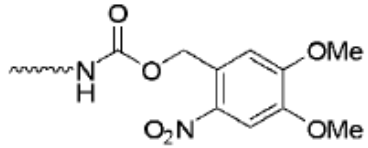
Advantages:

- Rapid attachment (no chemical modification required)
- High resolution (light → semiconductor industry)

Jonkheijm *et al.*, *Angew. Chem.* **2009**

Functional surface groups 2

- Photoreactive group(s)

Photoreactive groups	
arylazide	
diazirine	
benzophenone	
azidophenylalanine	
disulfide	
nitrobenzyl[a]	

[a] Photocleavable protecting group.

Jonkheijm *et al.*, *Angew. Chem.* **2009**

Disadvantages:

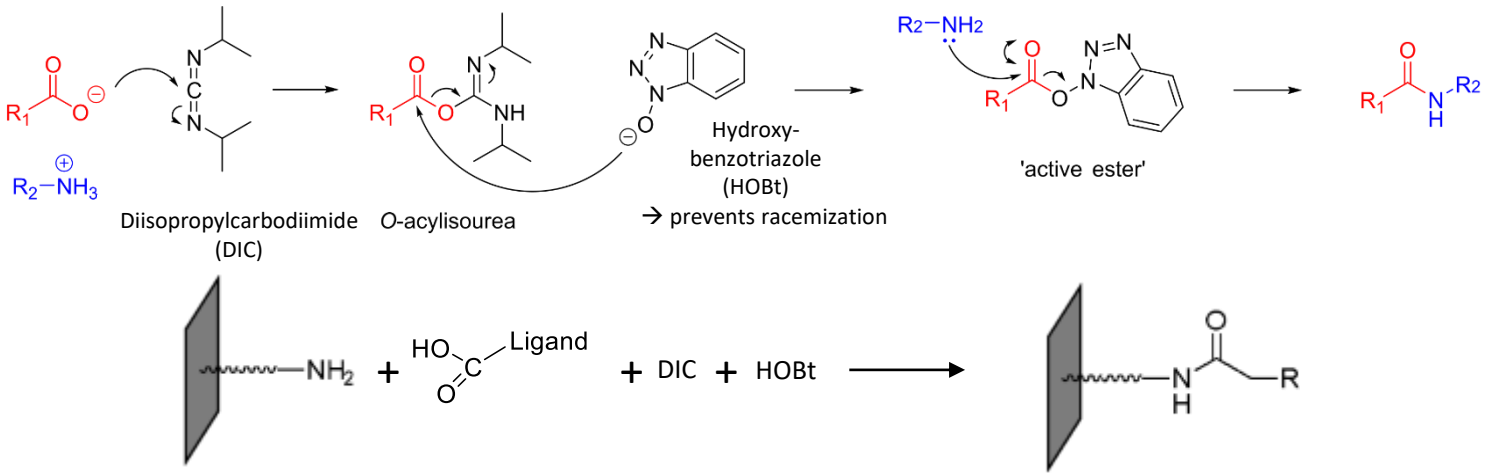
- May react unspecifically
- May destroy molecules

Advantages:

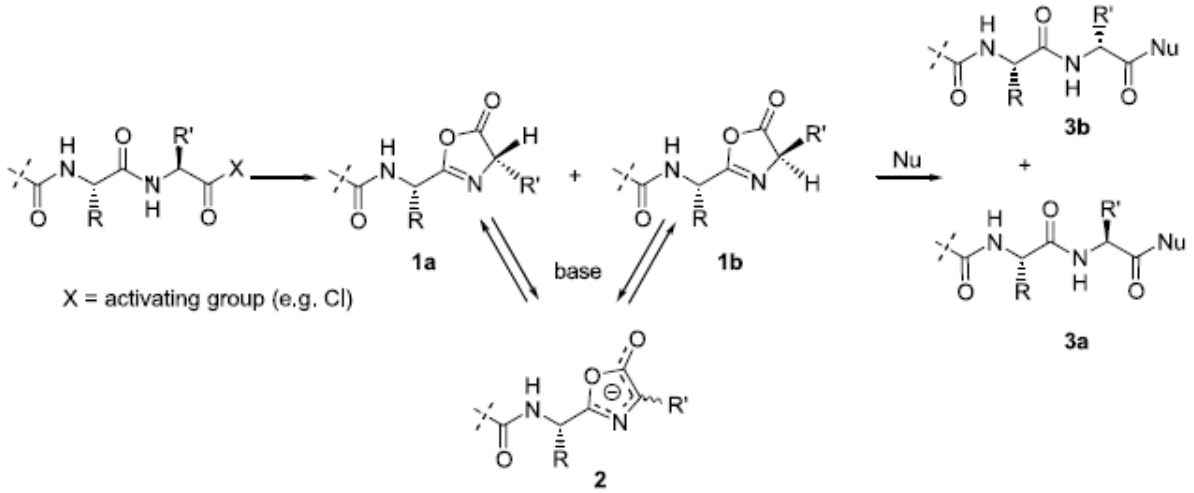
- Rapid attachment (no chemical modification required)
- High resolution (light → semiconductor industry)

Covalent coupling

- Covalent coupling *via* amide bond (amine)



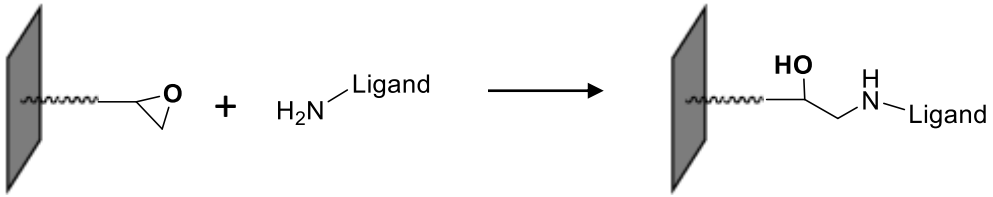
- Racemization (epimerization) with carbodiimides only



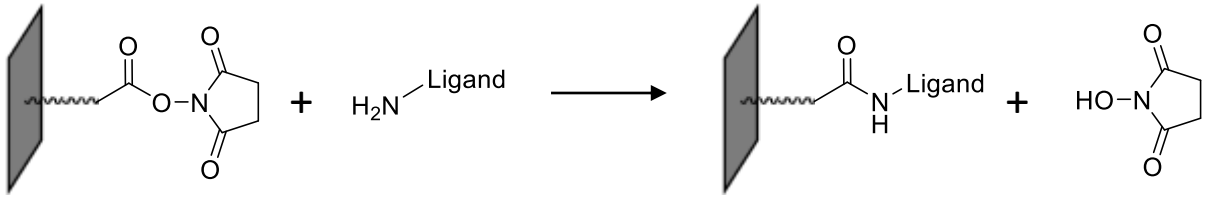
Montalbetti & Falque, *Tetrahedron*, **2005**

Covalent coupling

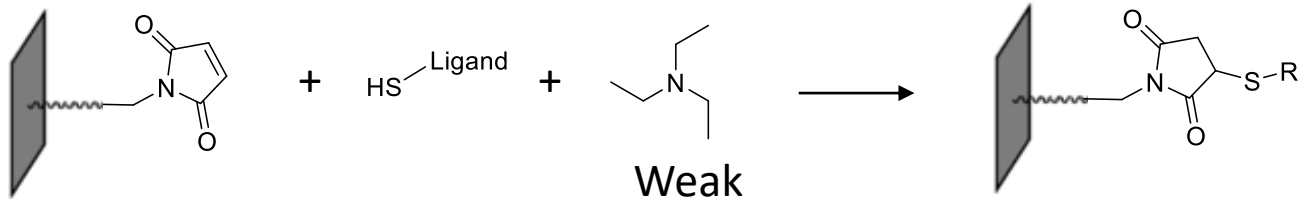
- Covalent coupling *via* epoxide group



- Covalent coupling *via* N-hydroxysuccinimide (NHS) group



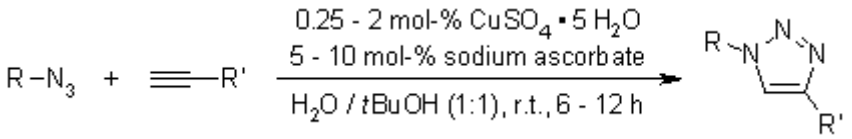
- Covalent coupling *via* thiol-maleimide



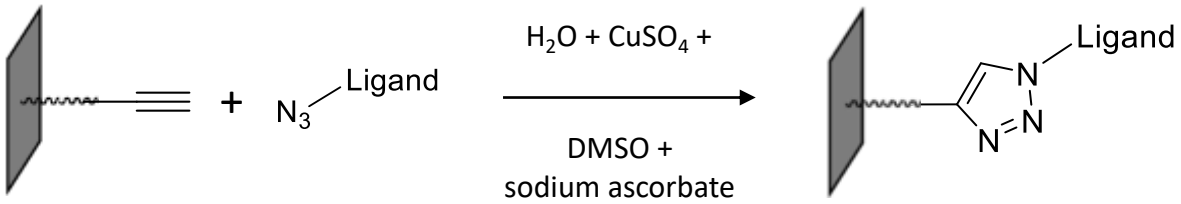
Covalent coupling

- Click chemistry
  - Introduced by K. B. Sharpless in 2001
  - Reactions that are high yielding
  - Byproducts can be removed without chromatography
  - Stereospecific
  - Simple
  - Easily removable/benign solvents (some are even biocompatible!)

Copper-catalyzed azide-alkyne cycloaddition (CuAAC)



https://www.organic-chemistry.org/namedreactions/click-chemistry.shtm



6. Qualitative *vs* quantitative

What do you think is a quantitative measurement?

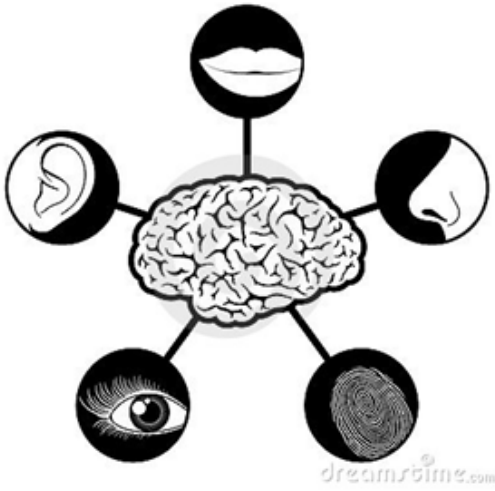
Qualitative vs. quantitative

Quantitative Data

are made with instruments such as rulers, balances, graduated cylinders, beakers, and thermometers.
These results are measurable.
(numbers)

Qualitative Data

use your senses to observe the results.



https://kenandeen.wordpress.com/2015/01/18/quantitative-vs-qualitative-data/

Quantitative experimental data should be highly reproducible!

What you should have learned – Molecules, interactions, surfaces

1. Molecules
   - Which ones and how can they be immobilized (reactive groups)
   - Typical surface functionalizations and surface chemistry
2. Interaction forces
3. Affinity, avidity, multivalency
   - Difference between affinity and avidity
   - Antibodies & nanobodies, types
4. Surfaces and key coupling groups
5. Quantitative vs. qualitative