Affinity Chromatography (AC)

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- Principles of AC
- Main stages in Chromatography
- How to prepare Affinity gel - Ligand Immobilization - Spacer arms – Coupling methods – Coupling tips
- Types of AC
- Elution Conditions
- Binding equilibrium, competitive elution, kinetics
- Industrial Examples: Protein A/G for Therapeutic proteins
- Future Considerations

What is affinity chromatography?

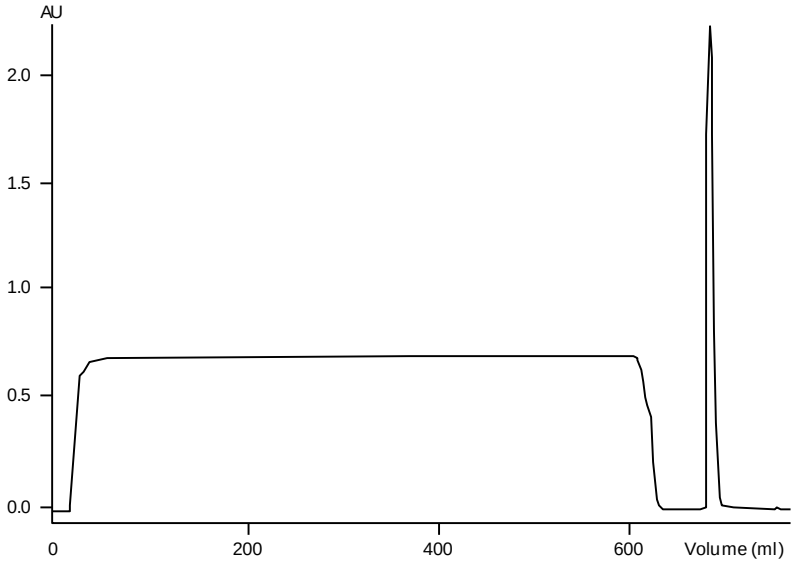
- Affinity chromatography is a technique of liquid chromatography which separates molecules through biospecific interactions.
- The molecule to be purified is specifically and reversibly adsorbed to a specific ligand
- The ligand is immobilized to an insoluble support (“matrix”): resin, “chip”, Elisa plate, membrane, western, etc
- Introduction of a “spacer arm” between the ligand and the matrix to improve binding
- Elution of the bound target molecule: a) non specific or b) specific elution method

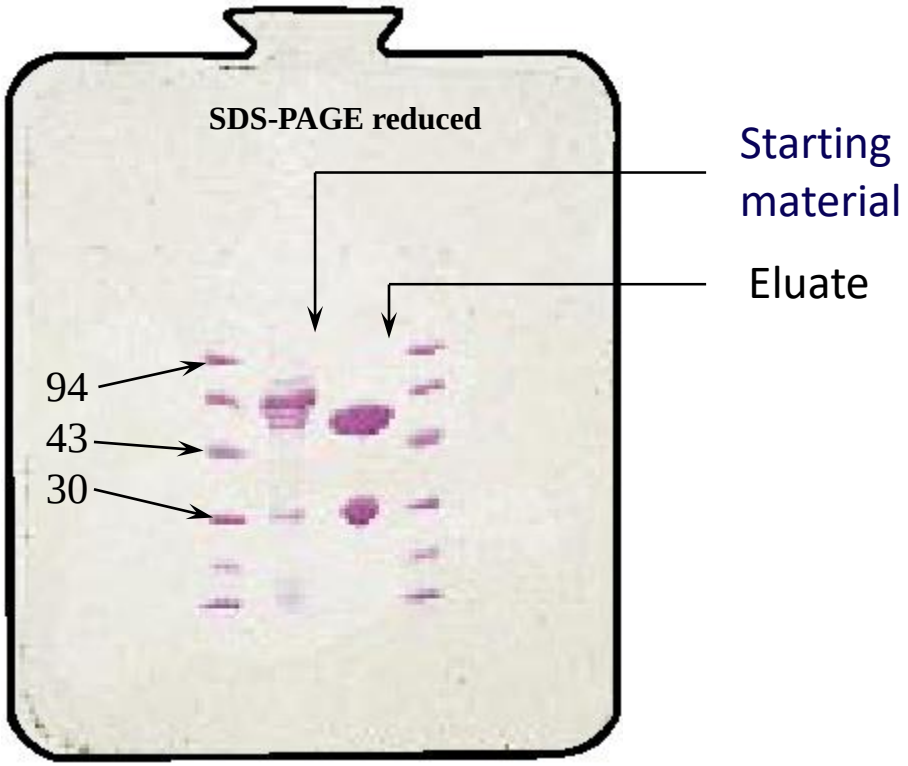
What is it used for?

- Monoclonal and polyclonal antibodies
- Fusion proteins
- Enzymes
- DNA-binding proteins . . . . . .
- ANY protein where we have a binding partner!!

Purifying monoclonal antibodies

Sample:	600 ml mouse monoclonal IgG $_{2a}$
Gel:	rProtein A Sepharose Fast Flow
Elution:	20 mM Sodium Citrate, pH 4.0
Result:	**83 mg Mab, recovery 95%**

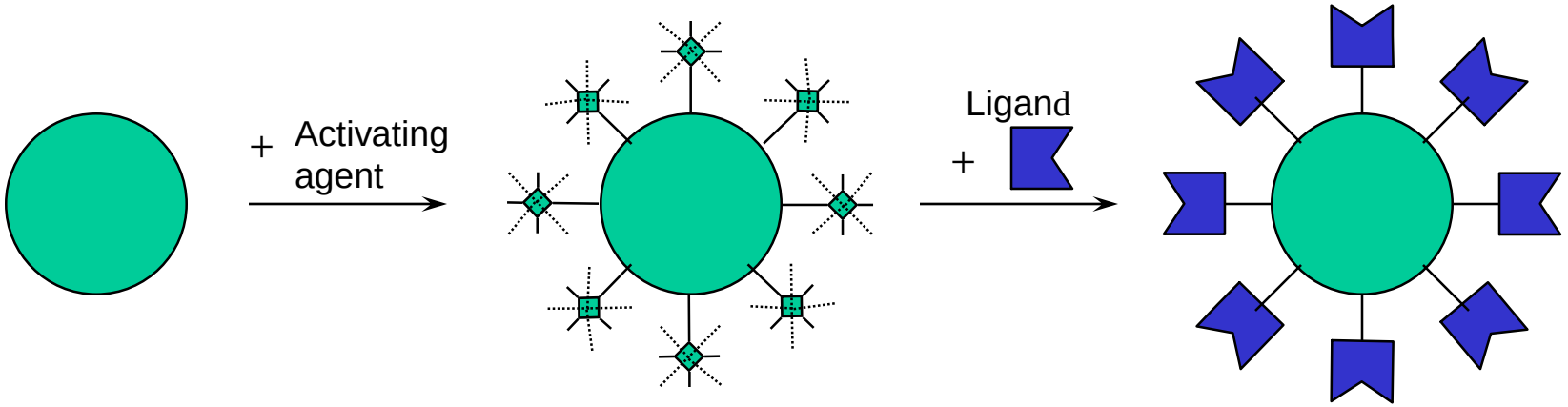




Designing and preparing an affinity gel

- Choosing the matrix
- Designing the ligand - Spacer arms
- Coupling methods

Ligand Immobilization



Matrix

Activated matrix

Immobilised ligand

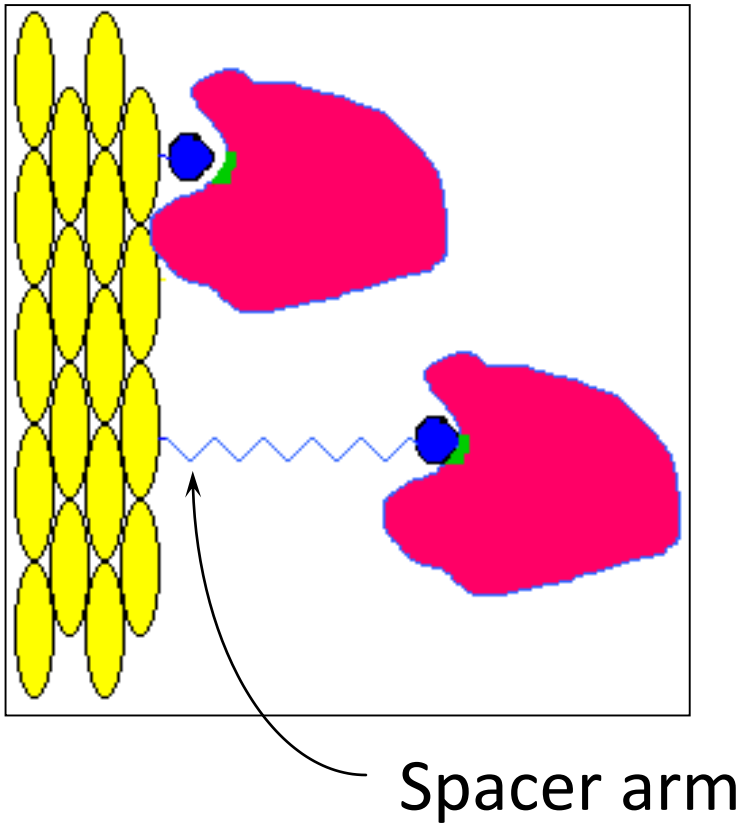
Designing the ligand

- Essential ligand properties: interacts selectively and reversibly with the target

- Carries groups which can couple it to the matrix without losing its binding activity

- Available in a pure form

Steric considerations & spacer arms



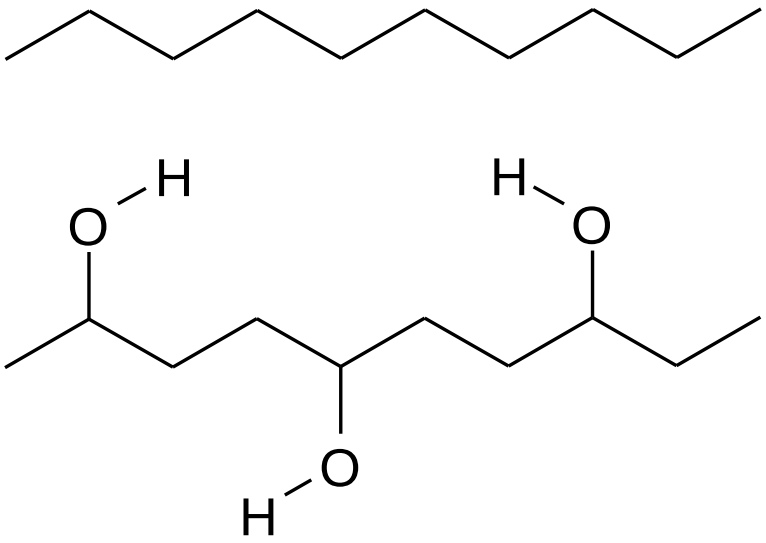
✓**Small ligand (<1,000)**

Risk of steric interference with binding between matrix and target molecule

✓Often need spacer arm

✓**but watch out for adsorption to the spacer!**

Design of spacer arms



Alkyl chain

Real risk of unspecific interactions between spacer and target molecule

Hydrophilic chain

Risk of unspecific interactions greatly reduced

Best choice

No coupling reaction will use 100% of the available binding sites. Using a gel with pre-attached spacer arms will leave some uncoupled spacers, increasing the possibility of non-specific interactions. The unused spacer arms should not be too much of a problem if they are hydrophilic. If available, this problem may be eliminated by using a ligand with a pre-attached spacer arm.

Choosing a coupling group

- Ligands are coupled to the matrix via reactive groups

Amino	$-NH_2$
Hydroxyl	-OH
Aldehyde	-CHO
Thiol	-SH
Carboxyl	-COOH

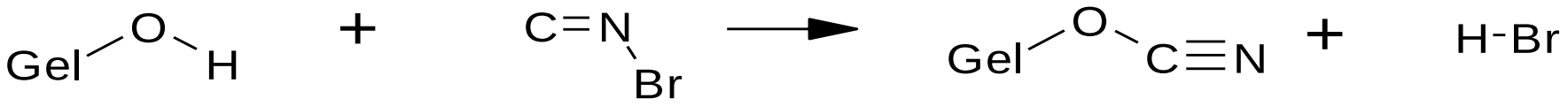
- The group coupled must not be too near the site for binding the target

Useful Coupling Methods

Coupling chemistry	Kinds of group coupled	Ligand
N-hydroxysuccinimide	$-NH_2$	Protein ,Peptide. Sugar.Polynucleotide.Cofactor
CNBr	$-NH_2$	Protein , Sugar.Polynucleotide.Cofactor
Carbodiimide	$-NH_2$ via hydrophilic spacer arm	Small ligands. Non polar ligand in organic solvents. Derivatized material
Epoxide	$-SH-NH_2-OH$ via hydrophilic spacer arm	Sugars. Small ligands
Thiol exchange	$-SH$ Mild Conditions	Protein ,Peptide. Polynucleotide Low MW thiol containig ligand

Useful Coupling Methods

Cyanogen bromide activation

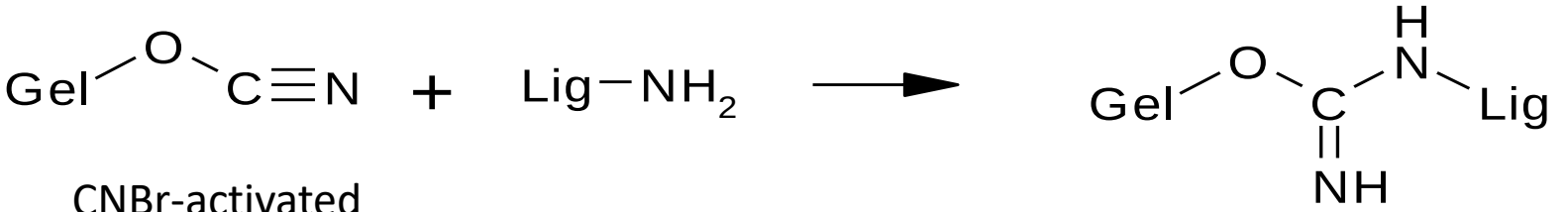


Sepharose

Cyanogen bromide

Cyanate ester formed

Cyanogen bromide coupling



CNBr-activated Sepharose

Protein ligand

N-substituted isourea

Reaction at pH 8. Isourea may be cleaved by nucleophilic attack at high pH.

Multiple bonds formed with protein ligands. No spacer arm present.

WARNING!: Cyanogen bromide is dangerous. Consult safety data before start working

A general protocol for ligand coupling

- Prepare the ligand solution in coupling buffer
- Prepare the activated matrix
- Mix the ligand solution and the activated gel in coupling buffer until coupling is complete
- Check total ligand before and after coupling: ligand/ml resin
- Block any remaining active groups
- Wash the coupled gel alternately at high and low pH to remove adsorbed ligand
- Transfer to storage buffer

Tips: Preparing the activated matrix - couple buffer

- If the **activated matrix** is freeze-dried, allow it to swell before washing
- Always use the recommended swelling and washing solutions!
- The swelling, washing and equilibration are best performed on a sintered glass filter. Facilitates quickly removal of excess liquid from the media
- **Couple buffer**: make sure there are no components, e.g. amines, that can couple in place of the ligand, e.g. Tris buffer has a primary amine
- To suppress ionic adsorption, add NaCl, 0.5 M.
- pH is important. Always use the recommended pH.

Tips: Coupling reaction, washing and storage

- Ligands which are poorly soluble in water, may usually be added in an organic solvent.
- The ligand should be available at a high level of purity. Any contaminants present in the ligand sample will likely be coupled to the matrix.
- Mix the activated gel and the ligand solution by swirling or end-over-end mixing - **Avoid magnetic stirrers** as these can crush the gel and produce fines!!
- Take samples of ligand before and after reaction: to calculate ligand per ml resin
- Time and temperature are in the standard protocol. Most coupling reactions are performed at room temperature
- Block any remaining active groups with a small neutral ligand (Tris buffer for amino reactions)
- Wash the coupled gel at alternating high and low pH to remove adsorbed substances.
- Keep in storage buffer (Na Azide , Thimerosol 0.02% or ethanol if possible)

G Protein Receptor Kinase

Technique		Purification factor	Comment
	Pig brain homogenate		
Ppt	Ammonium sulfate precipitation	7	A. Tobin et al. (1996) J. Biol. Chem. 271, 3907-3916
HIC	Butyl Sepharose Fast Flow	20	
AIEX	RESOURCE Q		
CIEX	RESOURCE S	2408	
AC	**HiTrap Heparin**	**18647**	• **10 mg homogenous protein**

Membrane Protein

Technique		Purification factor	Comment
	Placental extract in 1.5% Triton X-100		
AC	Blue Sepharose	3	• Concentration and capture
AIEX	DEAE Sephacel	4	
CIEX	SP Sepharose	6	
AC	Muc2 Sepharose	242	• Main purification step
CIEX	Mono S	1442	• Final polishing and purity check, 20 mg obtained

T. White et al. (1995)
J. Biol. Chem. 270, 24156-24165

DNA-binding Protein

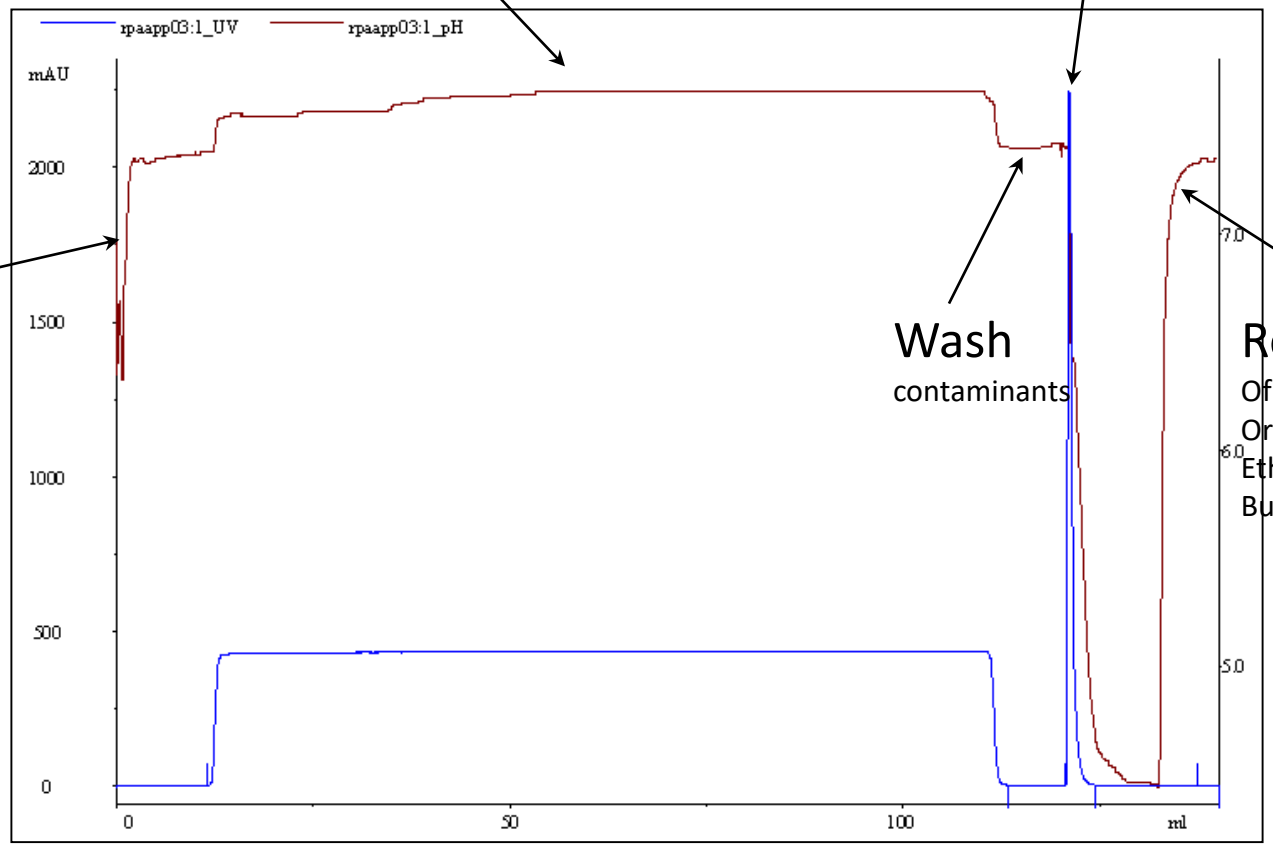
Technique		Purification factor	Comment
	HeLa cell nuclear extract		J. Berthelsen et al. (1996) J. Biol. Chem. 271, 3822-3830
CIEX	SP Sepharose High Performance	5	
AC	**Heparin Sepharose Fast Flow**	**8**	**• General AC step for DNA-binding proteins**
AC	**DNA-1 Sepharose**	**9**	**• Removal step, non-specific DNA binding activity removed**
AC	**DNA-2 Sepharose**	**2447**	**• Main purification step**
CIEX	Mono S	4943	• Final polishing, 20 mg obtained

Advantages and Disadvantages of affinity chromatography

- Easy to achieve otherwise difficult separations
- Often high purity in one step *(but not always)*
- Fast separations *(depends of the kinetics of binding and elution)*
- Can be use to remove specific contaminants

- Economics (scale-up)
- Buffer / elution limitations
- Sometimes needs harsh or denaturizing elution conditions
- **Do not resolve all the problems**

The main stages in affinity chromatography



Columns and equipment

Tips

- Column volume: according to amount of target and gel capacity
- Avoid using excess of resin: to avoid low affinity binding of impurities
- Column length: not usually critical, short and wide
- Equipment: no special demands
- Equilibrate column before applying sample and follow standard protocol

Sample preparation

Tips

- Adjust pH, buffer salts and additives to promote binding
- Filter or centrifuge to remove particles
- **Make sure that components known to interfere with binding are absent**
- Consider alternative capture steps before affinity purification (cost of resin, crude impurities or interfering substances, concentrate sup, etc)

Sample application

Tips

- Binding buffer: usually neutral pH, and high salt concentration when is possible (0.3-0.5M NaCl) to avoid hydrophobic interactions of non specific proteins to the resin. Use additives only if necessary.
- For batch binding, incubation of around 1.5 hours at 4°C is enough. For extremely slow binding kinetics, incubate overnight at 4°C.
- Loading directly on column
  - Strong affinity and fast binding: High flow rate
  - Weak affinity and/or slow binding: Low flow rate

Elution and re equilibration

Tips

- Follow standard protocol
- **Optimize alternative wash strategies to increase final purity**
- If eluting at extreme pH: collect the target protein in a small amount of concentrated buffer at a neutralizing pH
- Re-equilibrate column immediately with binding or storage buffer
- Regeneration: use buffers that do not harm the ligand

Column Regeneration

Tips

- Wash with basic and acidic buffers.
  - 10 col. vol. 0.1 M Tris.HCl, 0.5 M NaCl, pH 8.5
  - 10 col. vol. 0.1 M NaAc, 0.5 M NaCl, pH 4.5
  - Use chaotropic agents or detergents if needed
- Re-equilibrate with 10 column volumes starting buffer. Use more if needed
- Ni columns can be washed with 0.5N NaOH, neutralized, destriped with 100mM neutral EDTA, wash, charge with 100mM Ni SO_4, wash and store with 20% Ethanol

Column Storage

Tips

- To maximize the useful lifetime of an affinity resin, choose a storage buffer that does not harm the ligand
- Typical buffers are 20% ethanol solution or binding buffer containing a bacteriostatic agent (e.g. 0.02% Thimerosal or 0.02% sodium azide)
- Many of the commercial resins can be used hundreds of times: strong and stable ligands

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Type of Affinities

Mono-specific ligands

- Specific for a single substance

  Antigen antibody

  Hormone receptor

- Usually home-made gels
- Elution scheme must be worked out for each case:
- Often general elution
- Little help from the literature

Group-specific ligands

- Specific for a group of structurally or functionally similar substances:

  Lectins glycoproteins

  Protein G IgG antibodies

  Dye-stuffs enzymes

- Often ready-made gels
- Known elution schemes
- Standard tested elution protocols
- Often competitive elution

First choice

Group-specific ligands - 1

Ligand	Specificity
Protein A	Fc region of IgG
Protein G	Fc region of IgG
Concanavalin A	Glucopyranosyl & Mannopyranosyl groups
Peanut Lectin	Terminal galactose group
Cibacron Blue	Broad range of enzymes, serum albumin
Procion Red	$NADP^+$ dependent enzymes
Lysine	Plasminogen, ribosomal RNA

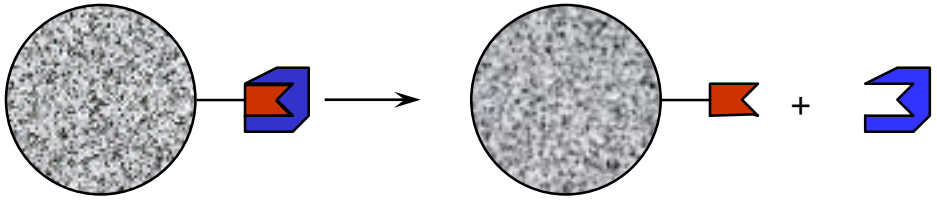
Group-specific ligands - 2

Ligand	Specificity
Arginine	Serine proteases
Benzamidine	Serine proteases
Calmodulin	Proteins regulated by calmodulin
Heparin	Coagulation factors, lipoproteins, lipases, hormones, steroid receptors, protein synthesis factors, Nucleic acid-binding enzymes
Metal chelate resins (Co, Ni, Zn, Cu, Fe)	Proteins and peptides which contain 6-12 Histidines

Designing the elution scheme

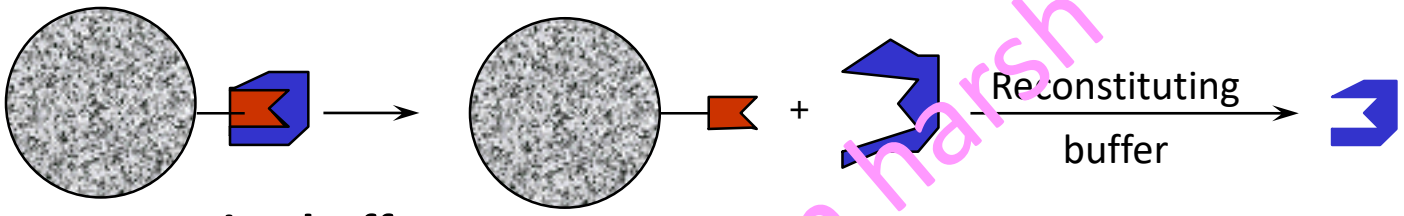
- General elution conditions
  - Low pH, salt, GuHCl, Urea etc.
  - Often harsh conditions
- Specific eluents
  - Competing free ligand
  - Competing binding substances
  - Sometimes expensive compounds (like peptides)

General elution conditions



Changing buffer conditions

- Usually decrease pH or increase ionic strength
- decrease polarity adding up to 10 % dioxane or up to 50 % ethylene glycol



Denaturing buffer

Usually extremes of pH or chaotropic agents

- There are no guaranteed general elution methods in affinity chromatography
- Low pH, e.g. glycine HCl, pH 2.8, is the closest approach

General elution conditions

- **pH**
- 100 mM glycine•HCl, pH 2.5-3.0
- 100 mM citric acid, pH 3.0
- 50-100 mM triethylamine or triethanolamine, pH 11.5
- 150 mM ammonium hydroxide, pH 10.5

- **Ionic strength and/or chaotropic effects**
- 3.5-4.0 M magnesium chloride, pH 7.0 in 10 mM Tris
- 5 M lithium chloride in 10 mM phosphate buffer, pH 7.2
- 3.0 M Potassium chloride
- 2.5 M sodium iodide, pH 7.5
- 0.2-3.0 sodium thiocyanate

- **Denaturing**
- 2-6 M guanidine•HCl
- 2-8 M urea
- 1% deoxycholate
- 1 % SDS

- **Organic**
- 10% dioxane
- 50% ethylene glycol, pH 8-11.5 (also chaotropic)

- **Competitor**
- >0.1 M counter ligand or analog

➢ *Elution conditions are intended to break the ionic, hydrophobic and hydrogen bonds that hold the ligand and the target together*

➢ *Successful eluting conditions will be dependent upon the specific ligand-target interaction that is occurring.*

➢ *Ideally, an elution condition effectively releases the target without causing permanent damage, but all eluting conditions result in some loss of functionality*

➢ *Empirical evidence is needed to determine which elution condition is the best.*

Elution at Low pH

IgG antibodies on rProtein A Sepharose

- Column: HiTrap rProtein A
- Binding: 20 mM sodium phosphate, pH 7.0
- Elution: 0.1 M glycine HCl, pH 3

Check for stabilizing elution conditions: slightly higher pH, stabilizing additives as Arginine, kosmotropic salts, etc

Note: Collect IgG into a small volume of strong buffer (1M pH 7.5) , to preserve its antibody activity.

Elution at High salt concentration

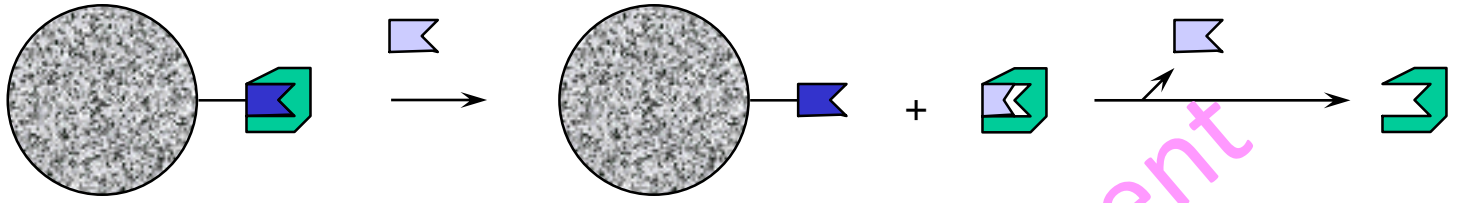
DNA-binding proteins on Heparin Sepharose

- Column: HiTrap Heparin
- Binding: 20 mM Tris-HCl, pH 8, 0.5 M NaCl
- Elution: Binding buffer + 1 to 2 M NaCl

Heparin Sepharose behavior is similar to CEIX

Consider use of CEIX

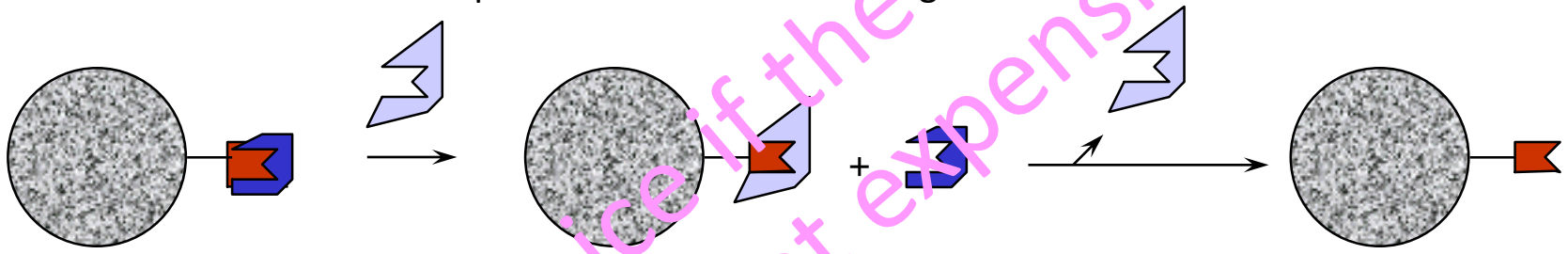
Specific eluents



Competing ligand in solution

Elution of enzymes from Blue Sepharose by free NADH

Glutathione elution of GST fusion protein from Glutathione-Agarose columns



Competing binding substance in solution

Elution of glycoproteins from Con A Sepharose by a-D-methylmannoside

Elution of 6His-Proteins from chelating columns (Ni-NTA) with Imidazole

Elution of Antigens from Antibody columns with specific peptides

- Competitive soluble ligands or binding substances can elute the bound target specifically
- Are usually far more gentle than general methods
- But sometimes could be very expensive (like specific peptides for antigen elution)

Lectins

Lectins are proteins which bind well-defined sugar residues of polysaccharides, glycoproteins, etc

Elution with competing free binding substance: sugar

Lectin	Specificity
Concanavalin A	α-mannose, α-glucose
Glycine Max (soybean)	N-Acetyl Galactosamine
Ulex Europaeus I	α-L-fucose
Wheat germ lectin	N-Acetyl Glucosamine & NeuNAc
Peanut lectin	α-mannose
Ricinus Communis, RCA 120	ß-Galactose
Arachis hypogaea	ß-Gal(1->3)GalNAc
Bandeira Simplicifolia, BS-I	α-Gal & α Gal NAc
Maackia amurensis	Sialic acid
Lymulus polyphemus	NeuNAc

Dye-stuffs

Many dye-stuffs mimic binding sites of natural biologically active molecules

Elution with High Salt

Dye-stuff	Sites recognised
Cibacron Blue 3G-A	NADH-binding and similar
Procion Red	NADPH-binding and similar

- The structural analogies are not exact.
- Related structures are also recognized.
- Blue Sepharose also binds serum albumin.

Binding equilibrium

At equilibrium

$$K_D = \frac{[L][T]}{[LT]}$$

K_D is the equilibrium dissociation constant,
[L] is the concentration of free ligand
[T] is the concentration of free target
[LT] is the concentration of the ligand/target complex

Binding equilibrium

It can be shown* that

$$\frac{\text{Bound target}}{\text{Total target}} \approx \frac{L_0}{K_D + L_0}$$

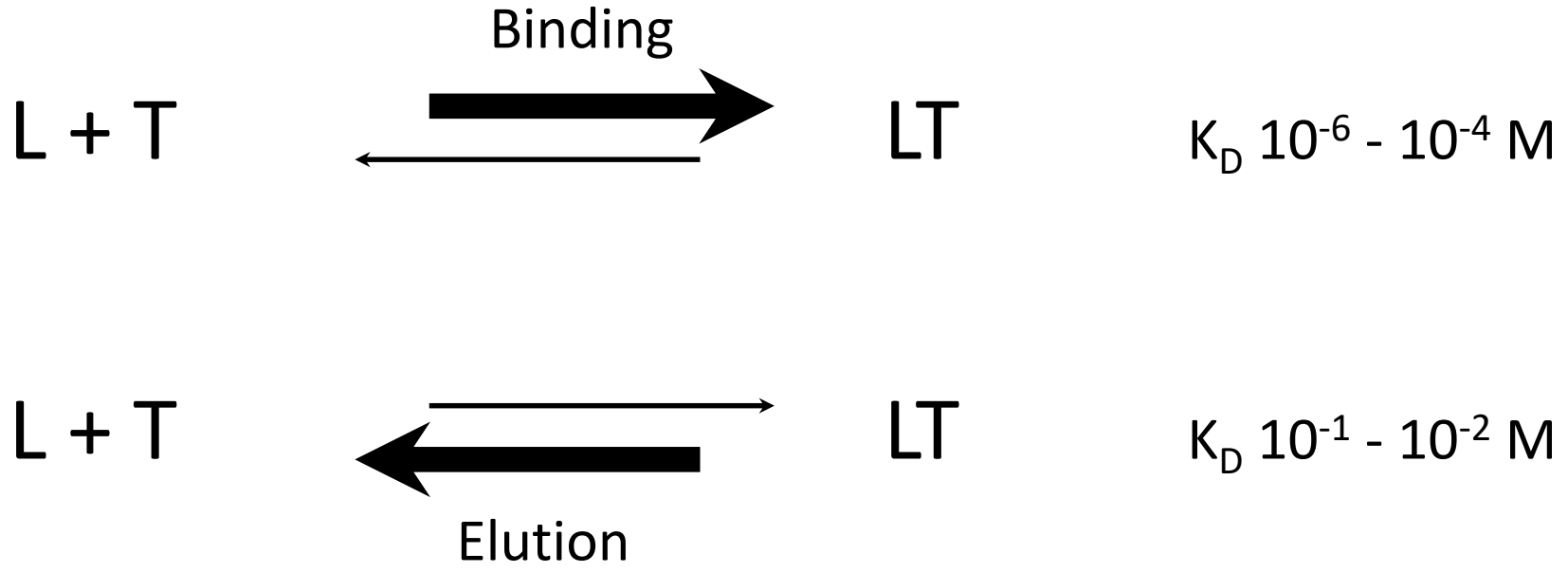
K_D is the equilibrium dissociation constant,
L_0 is the concentration of ligand, usually 10^{-4} - 10^{-2} M

For good binding, K_D should be at least two orders of magnitude less than L_0, i.e. 10^{-6} - 10^{-4} M

*Graves, DJ, Wu, YT Meth. Enzymol 34 (1974) 140-163

Binding equilibria

We can change K_D
by changing pH, temperature, salt concentration *etc.*



Target elutes as a sharp peak

Elution by changing K_D - Unexpected results

- K_D too high during binding (low affinity)

Target is retarded as a broad peak and elutes under binding conditions as a broad, low peak

Find better binding conditions or use ligand with higher affinity

- K_D not low enough during elution

Target elutes in a long, low 'peak'

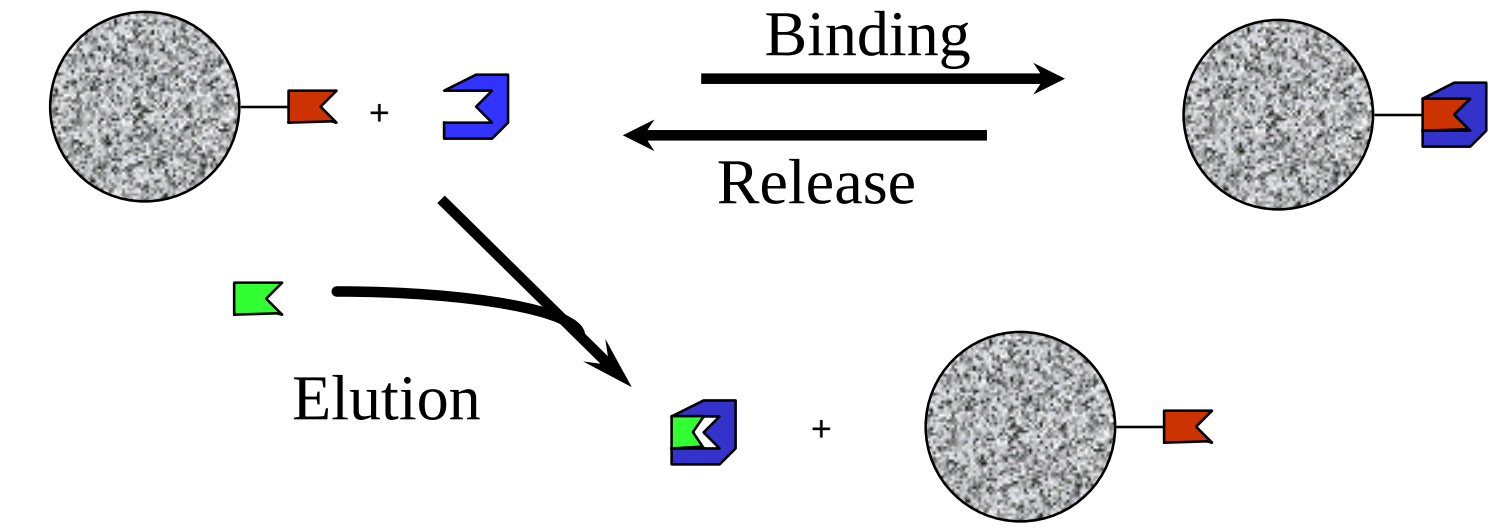
Try different elution conditions or use ligand with lower affinity

- K_D too low during binding (extremely high affinity)

Difficult to elute target. Difficult or impossible to increase K_D enough to elute the target without destroying its activity

Use ligand with lower affinity

Competitive elution



$$C + T \rightleftharpoons CT$$

Binding equilibrium for competing ligand

At equilibrium

$$K_{DComp} = \frac{[C][T]}{[CT]}$$

K_{DComp} is the equilibrium dissociation constant
[C] is the concentration of free competing ligand
[T] is the concentration of free target
[CT] is the concentration of the competing ligand/target complex

Kinetics of adsorption and desorption
Unexpected results

- Slow binding

Some of the target elutes under binding conditions as a broad, low peak

Slow down binding. Stopped flow binding to bind the target in portions

- Slow desorption

Target elutes as a long, low 'peak'

Stopped flow elution to elute the target in pulses. Change elution scheme

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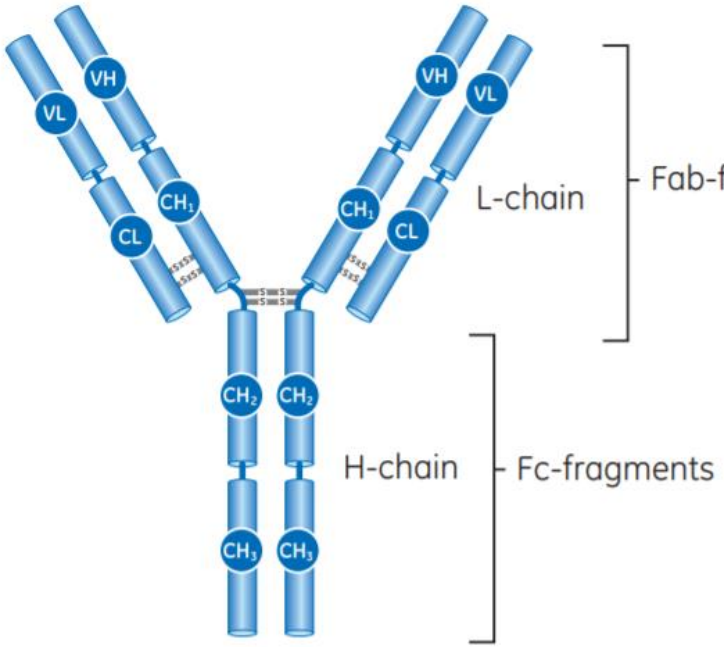
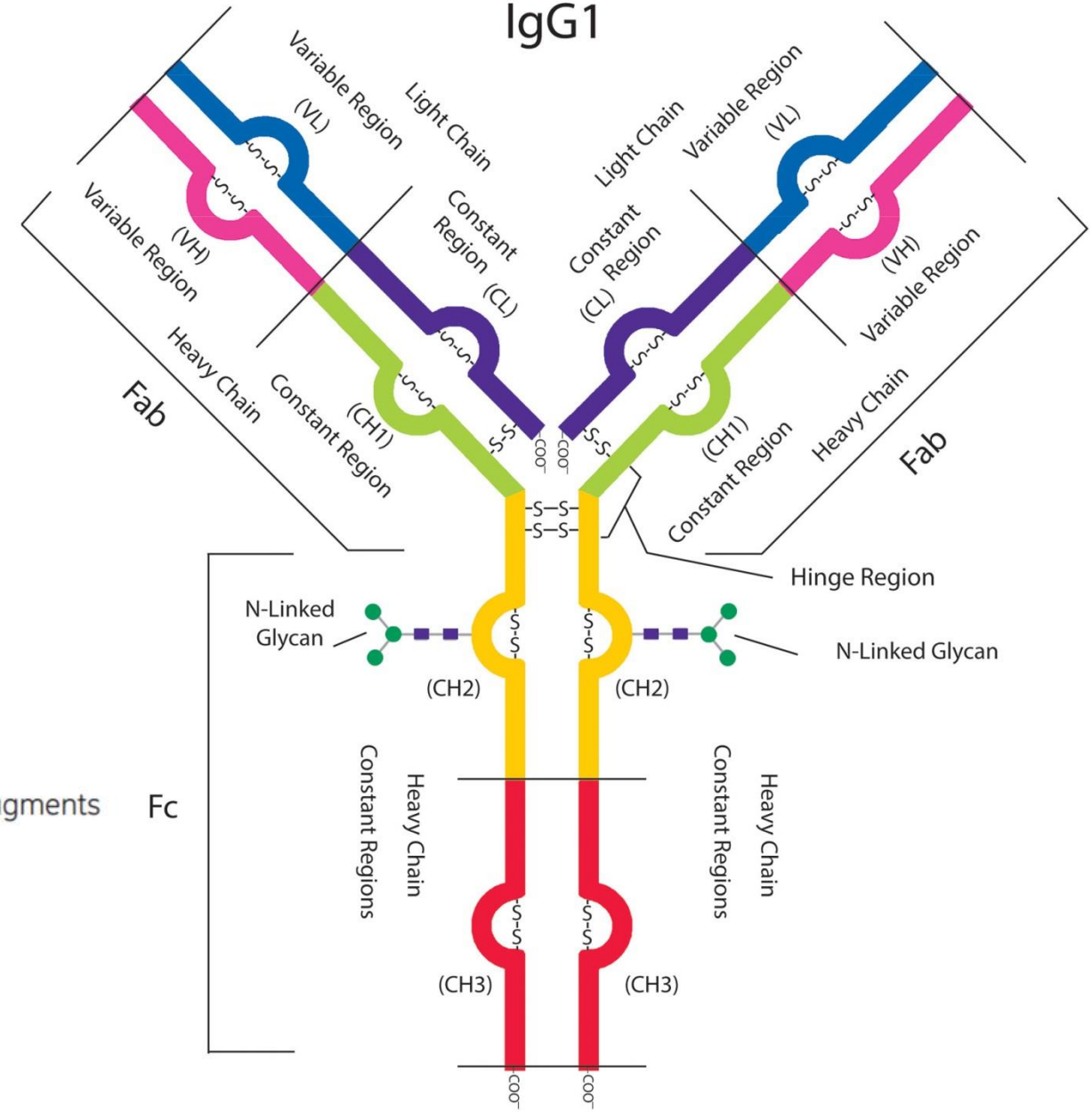
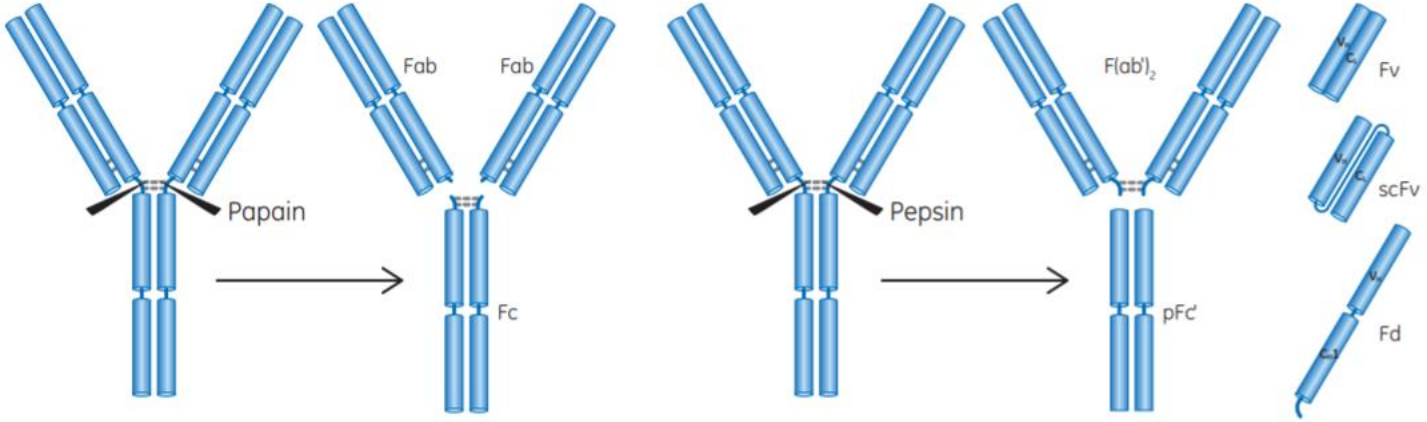
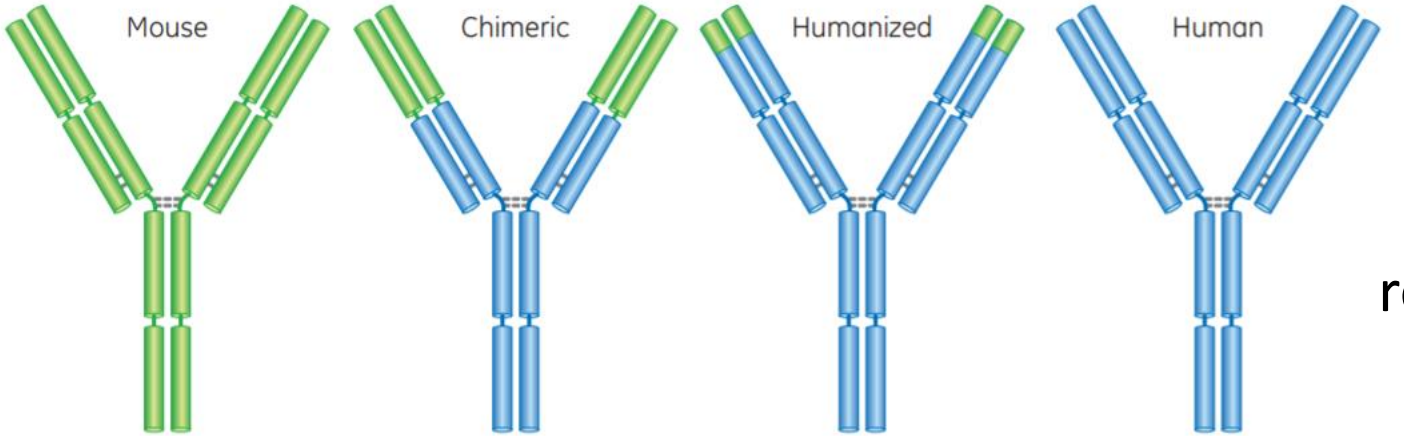

VH
VL
CH1
CL
CH2
CH3
L-chain
Fab-fragments
H-chain
Fc-fragments


IgG1
Variable Region
(VL)
Light Chain
Constant Region
(CL)
Variable Region
(VH)
Heavy Chain
Constant Region
(CH1)
Fab
Hinge Region
N-Linked Glycan
(CH2)
Fc
Constant Regions
Heavy Chain
(CH3)
COO-

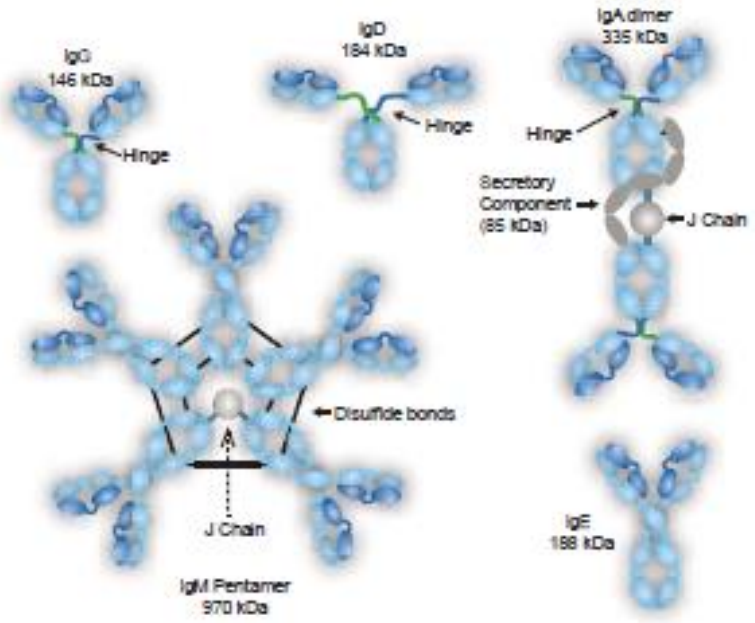
Antibody fragments are created by enzymatic cleavage or recombinant

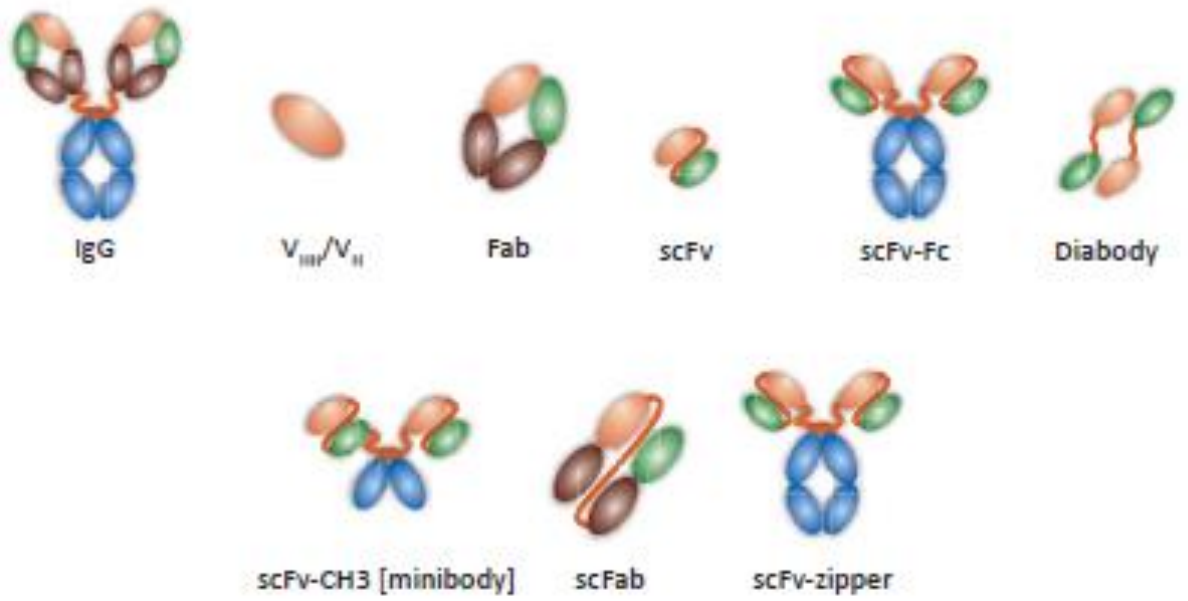




Various modifications of both native and recombinant antibodies are possible

Five major classes of Immunoglobulin and Single domain antibodies (sdAb)





GenScript®

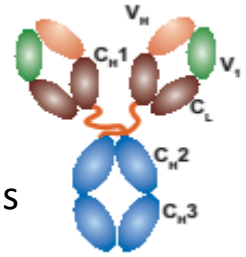
Antibody classes

Characteristic	IgG	IgM	IgA	IgE	IgD
Heavy chain	γ	μ	α	ε	δ
Light chain	κ or λ	κ or λ	κ or λ	κ or λ	κ or λ
Y structure					

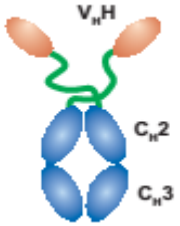
Advantages of target specific sdAb

www.GenScript.com

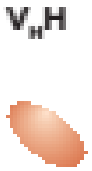
Conventional IgGs consist of two heavy chains and two light chains. Variable regions o both chains contribute to antigen binding.



Camelids produce antibodies composed of only two heavy chains, called heavy chain antibody (HCAb).



Single domain antibodies (sdAb) consist of only the variable region of HCAb, since it is the sole region responsible for binding. These are called VHH fragments



✓sdAb) retain all the function of conventional IgGs despite lacking the Vl binding region

✓Use only the V_H H fragment for binding

✓Benefits: 1/10 the size of convention IgGs, ~13 kDa vs ~150 kDa

✓Readily amenable to downstream engineering such as humanization

✓Access to hidden epitopes

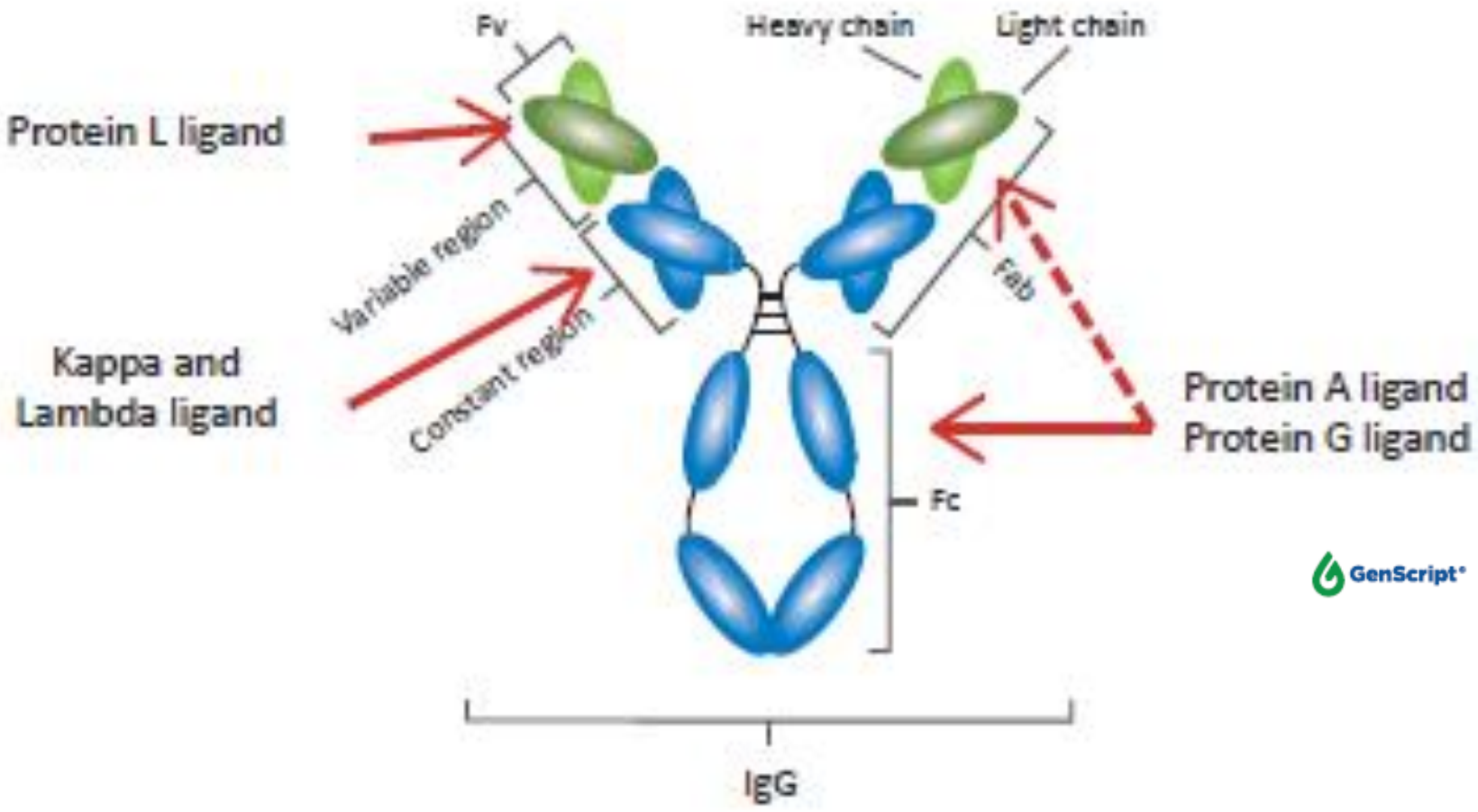
✓Better penetration into solid tissue

✓Ability to cross the blood brain barrier

✓High pH and temperature stability:

- Permits alternate routes of administration (i.e. oral)
- Use in other extreme environments (biosensors)
- **Use as a ligand for affinity resins**

Affinity ligands for antibody purification



Protein G and protein A bind to different IgG through the Fc region

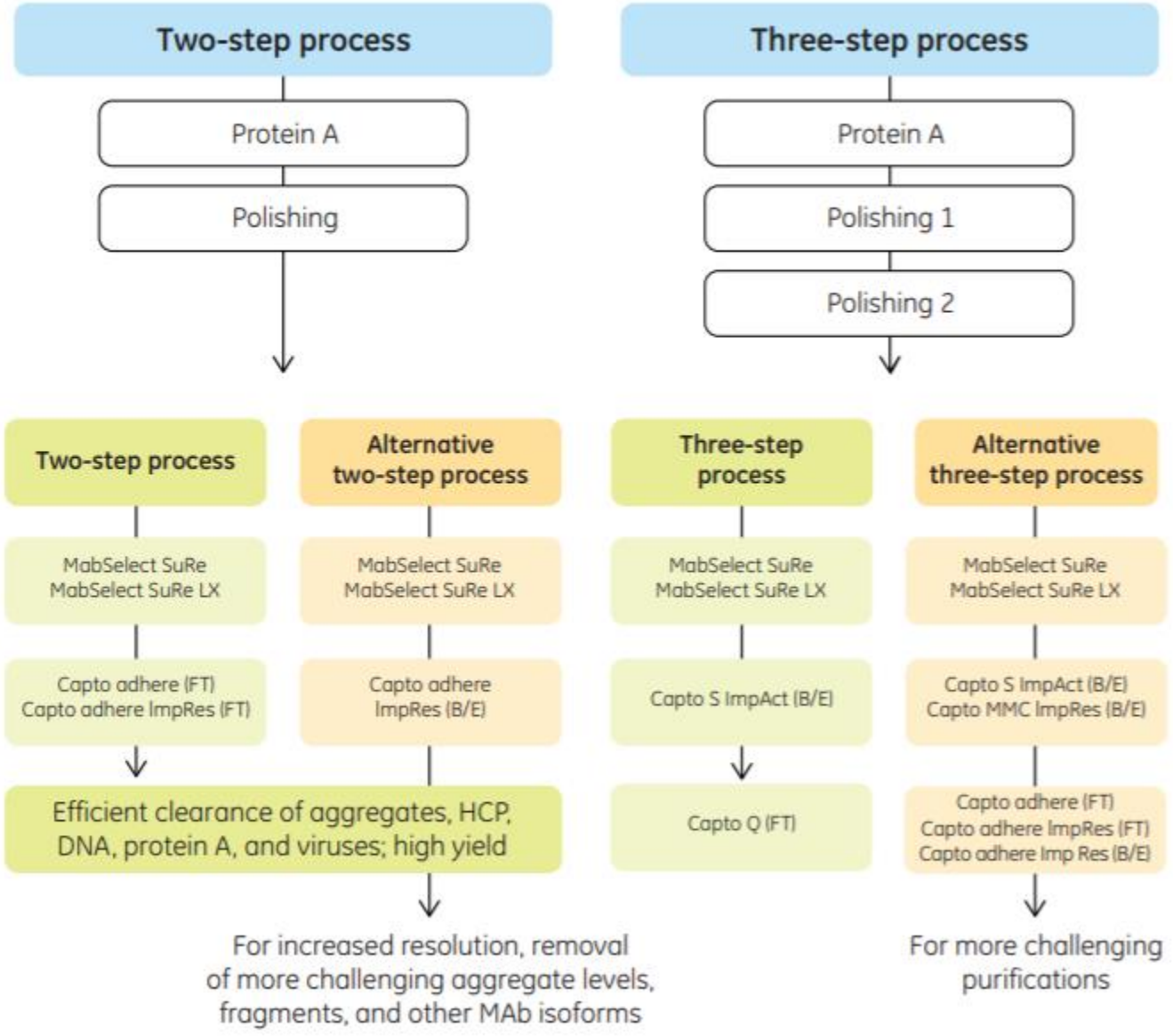
Protein G and protein A are bacterial proteins from Group G Streptococci and Staphylococcus aureus, respectively

Species	Subclass	Protein G binding	Protein A binding
Human	IgA	—	variable
	IgD	—	—
	IgE	—	—
	IgG_1	++++	++++
	IgG_2	++++	++++
	IgG_3	++++	—
	IgG_4	++++	++++
	IgM*	—	variable
Avian egg yolk	IgY†	—	—
Cow		++++	++
Dog		+	++
Goat		++	—
Guinea pig	IgG_1	++	++++
Hamster		++	+
Horse		++++	++
Koala		+	—
Llama		+	—
Monkey (rhesus)		++++	++++
Mouse	IgG_1	++++	+
	IgG_{2a}	++++	++++
	IgG_2	+++	+++
	IgG_3	+++	++
	IgM*	—	variable
Pig		+++	+++
Rabbit		+++	++++
Rat	IgG_1	+	—
	IgG_{2a}	++++	—
	IgG_{2b}	++	—
	IgG_3	++	+
Sheep		++	+/—

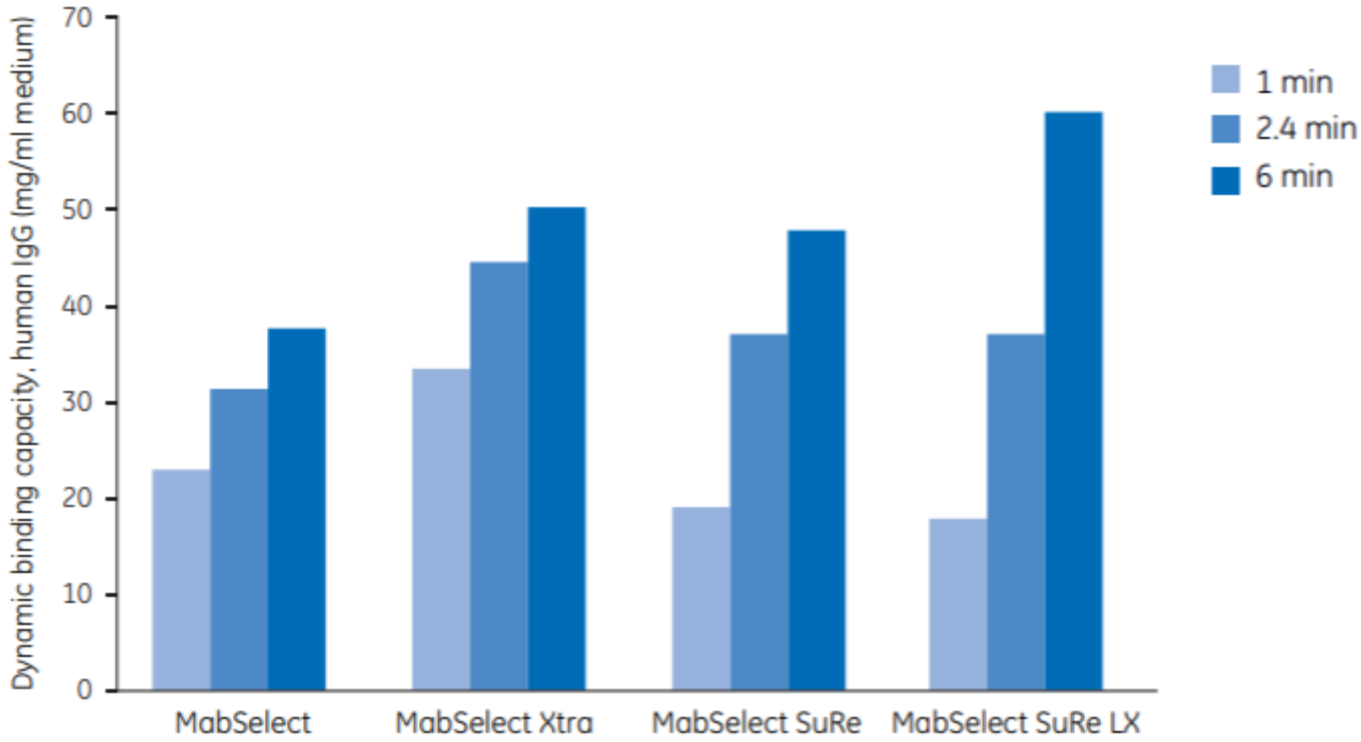
Overview of different combinations of chromatography unit operations in a MAb purification process

Removal of specific contaminants after initial purification

- Bovine immunoglobulins
- Albumin and transferrin
- α2 -macroglobulin and
- haptoglobulin
- DNA and endotoxins
- Dimers and aggregates
- Host cell proteins (HCP)
- Virus



Dynamic binding capacity of MAb at various residence times



Protein L binds to the variable region of the kappa light chain

- ✓It can be used to purify intact immunoglobulins conventional Fabs chain variable fragments (scFvs)
- ✓ Was first isolated from the surface of bacterial species *Peptostreptococcus magnus*

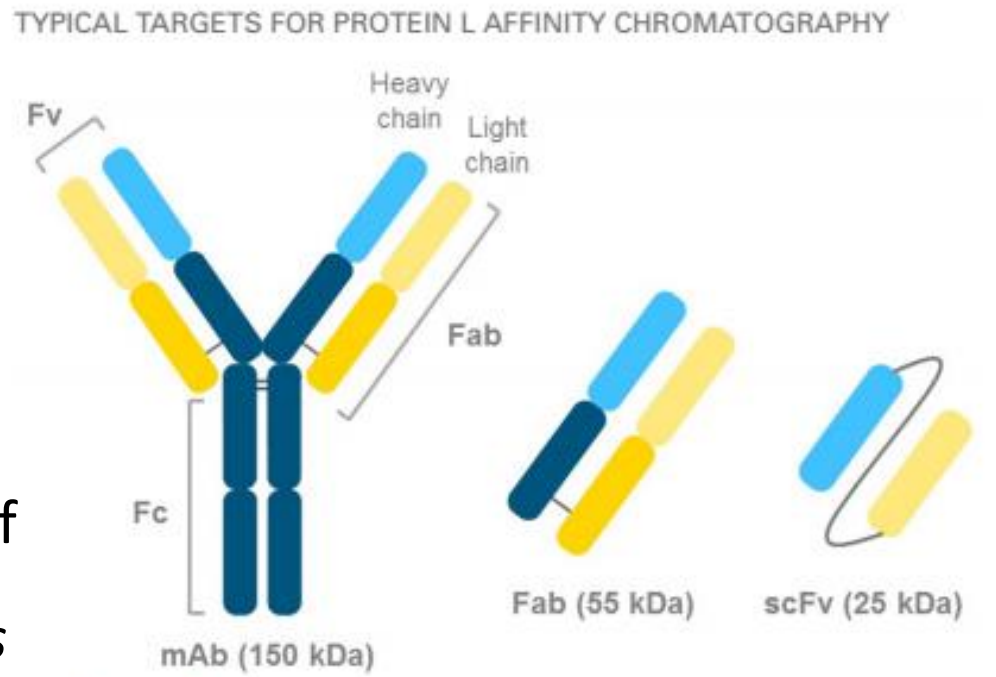
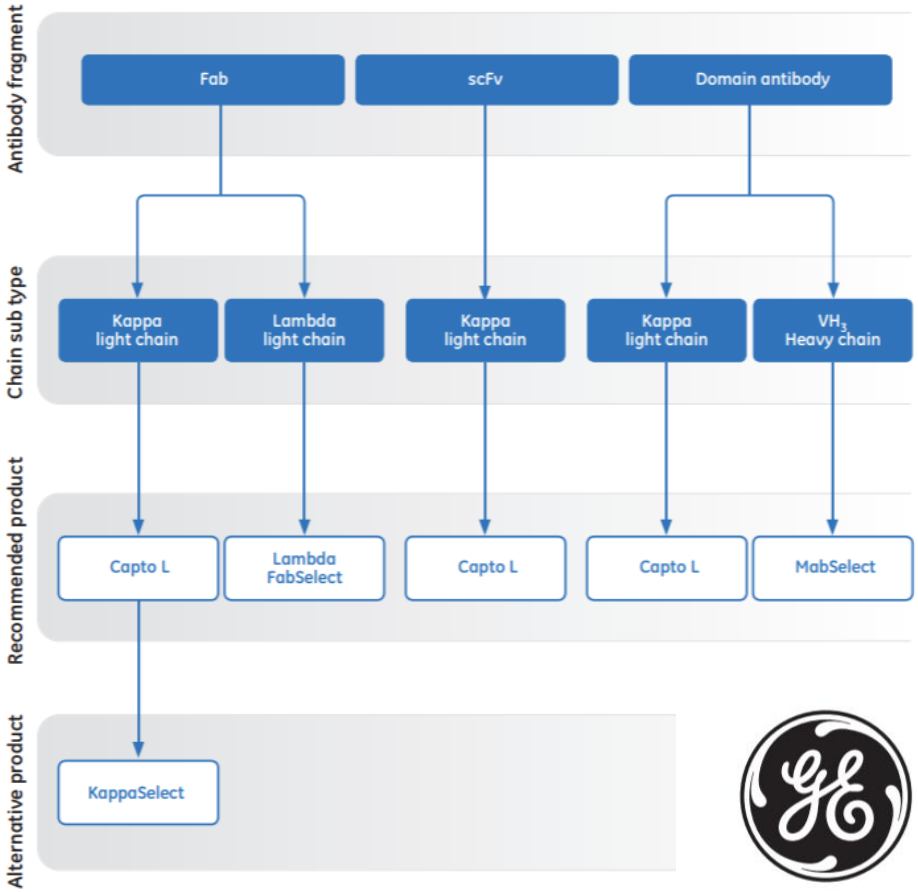


Figure 1
Protein L binds to the variable region of the kappa light chain

✓Immunoglobulins whose Fc region is not easily accessible for Protein A, such as IgM or IgA.

Protein L binding affinities

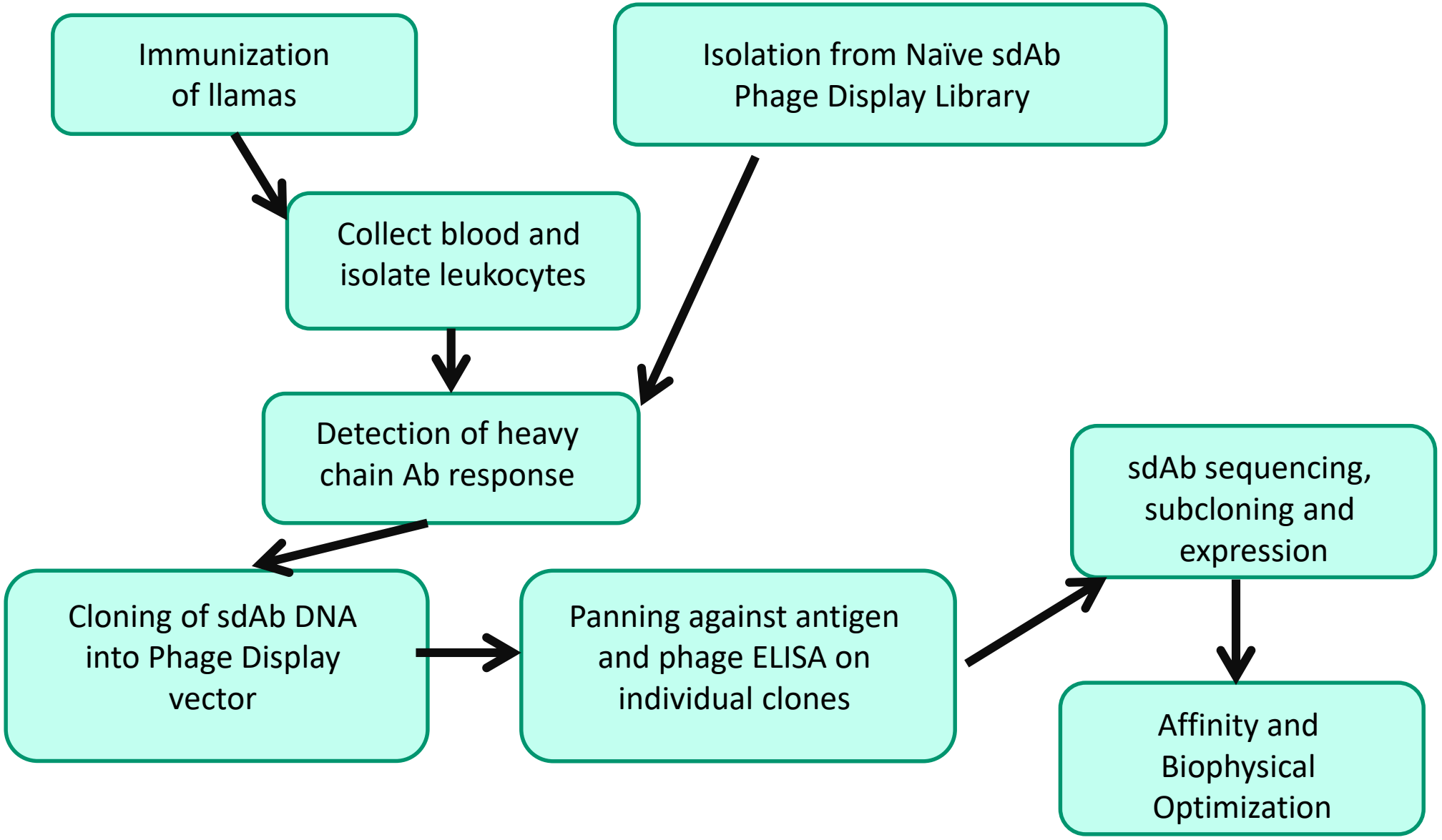
De Chateau, M. et al.
On the interaction between protein L and immunoglobulins of various mammalian species.
Scand. J. Immunol. 37, 399-405 (1993).



Species	Antibody class	Affinity†
General	Kappa light chain (subtypes 1,3,4)	Strong
	Lambda light chain	No binding
	Heavy chain	No binding
	Fab	Strong
	ScFv	Strong
	Dab	Strong
Human	IgG_1	Strong
	IgG_2	Strong
	IgG_3	Strong
	IgG_4	Strong
	IgA	Strong
	IgD	Strong
	IgE	Strong
	IgM	Strong
Mouse	IgG_1	Strong
	IgG_{2a}	Strong
	IgG_{2b}	Strong
	IgG_3	Strong
	IgM	Strong
Rat	IgG_1	Strong
	IgG_{2a}	Strong
	IgG_{2b}	Strong
	IgG_{2c}	Strong
Pig	Total IgG	Strong
Dog	Total IgG	Weak
Cow	IgG_1	No binding
	IgG_2	No binding
Goat	IgG_1	No binding
	IgG_2	No binding
Sheep	IgG_1	No binding
	IgG_2	No binding
Chicken	Total IgG	No binding

Workflow for generating an sdAb target specific

www.GenScript.com



Camelid derived single domain antibody fragments

BAC (BioAffinity Company) - Capture Select® (GE)

- Custom designed ligands (12-15kDa)
- Produced in *Saccharomyces cerevisiae*, enabling high level production with minimal purification effort as well as easy scale-up.
- Stability, affinity, and selectivity
- Reduction of process steps: higher yields, reduced costs
- Selectivity: high purity in single step / feed stock independent
- Mild elution conditions: retaining biological activity of target
- Efficient clearance of HCP, DNA, virus: high selectivity in capture step

- Capture Select human IgA
- CaptureSelect IgM directed towards a unique domain on the Fc part of IgM
- CaptureSelect Fab kappa: enable purification of human IgG, IgA, IgM, and IgE.
- CaptureSelect Fab lambda: enable purification of human IgG, IgA, IgM, and IgE.
- CaptureSelect Human IgG4 : enables purification of human IgG4's without cross reactivity with human IgG from subclasses 1, 2, and 3

DARPins (Designed Ankyrin Repeat Proteins)

An Innovative High-Throughput Platform for Next-Generation Binder Discovery

University of Zurich, Switzerland - Prof. Dr. Andreas Plückthun – Dr. Jonas Schaefer

✓Designed Ankyrin Repeat Proteins (DARPins) against different epitopes on a variety of targets

- small (~170 aa), very stable repeat proteins
- recognize structural 3D-epitopes
- work intracellularly (no Cys)
- available in flexible formats / modifications

✓**Ankyrin repeat** is a 33 residue motif in proteins consisting of two alpha helices separated by loops

✓Domains consisting of ankyrin repeats mediate protein-protein interactions and are among the most common structural motifs in known proteins

✓Provide monomeric binders that specifically recognize different, non-overlapping epitopes with high affinities

✓HT Ribosome Display (RD) enables selection against 94 targets in parallel

✓High-Throughput Screenings allow to identify best suited DARPin binders

Plückthun, A. (2015) DARPins: Binding Proteins for Research, Diagnostics, and Therapy. Annu Rev Pharmacol Toxicol. 555:489-511

Mimetic Ligand™ Affinity Chromatography

Synthetically "mimicking" and enhancing the natural molecular affinity of binding ligands toward targeted biomolecules

www.prometic.com

- Biologically inert
- very strong
- excellent liquid flow properties
- resistant to most chemical treatments
- stable in the pH range 2 - 14
- can be treated with denaturants (8M urea), detergents (Triton, SDS)
- can be sterilized by autoclaving.

➢ Capacities can exceed 50mg protein per ml of packed gel. Low Ligand Leakage

➢ Alkali-stable adsorbents - Enables cleaning, sterilization and depyrogenation with 1M sodium hydroxide, ensuring long operational lifetimes.

➢ Albumin • Proteases • Blood Proteins • Oxidases • Dehydrogenases • Ligases • Kinases • Antibodies • Nucleases • Cytokines

➢ **Custom Media Development Programs**

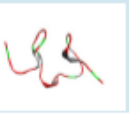
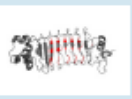
➢ Chemical Combinatorial Library™, + with a rational ligand design approach

Scil Proteins: Affilin™ Techology

- Novel human binding proteins for therapy and applications in chromatography.
- Affilin™ molecules are small non-immunoglobulin proteins which are designed for specific binding of proteins and small molecules.
- New Affilin™ molecules can be quickly selected from libraries which are based on two different human derived scaffold proteins: **Gamma crystalline**, a human structural eye lens protein and **Ubiquitin** one of the highest conserved proteins known

  - Small scaffolds
  - High temperature stability
  - Almost resistant to pH changes and denaturing agents.

- Created by engineering the protein surfaces through a locally defined randomization of exposed amino acids
- Can be easily produced in the cytoplasm of *E. coli*

Scaffold name/ technology	Scaffold structure with randomization residues	Parent protein	Origin of parent protein	Residues/ S–S bridges	Structure (PDB code)	Randomization	Selection method	Refs/Company
Atrimer/ Tetranectin		C-type lectin domain $CTLD_3$	Human	$n \times 40$/ 3 S–S	Five flexible loops (1TN3)	11 residues	Phage display	[13] Anaphore
Avimer/ Maxibody		Multimerized LDLR-A module	Human/artificial (consensus design)	$n \times$ ~43/3 S–S + Ca^{2+}	Four loops (1AJJ)	28 residues	Phage display	[12] Amgen
Centyrin		Fn3 domains of hTenascin C (Tencon)	Human	89/0	β-Sheet (3TES)	13 residues	CIS display, phage display	[84]
DARPin		Ankyrin repeat proteins	Human/artificial (consensus design)	67 + $n \times$ 33/0	α_2/β_2 repeated (4JB8	Seven residues in each *n*-repeat (original library)	Ribosome display, phage display	[67,85] Molecular Partners
DARPin		Ankyrin repeat proteins	Human/artificial (consensus design)	67 + $n \times$ 33/0	α_2/β_2 repeated (4JB8)	Eight residues in each n-repeat (reduced hydrophobicity library)	Ribosome display	[86]
DARPin		Ankyrin repeat proteins	Human/artificial (consensus design)	67 + $n \times$ 33 (57)/0	α_2/β_2 repeated (4K5C)	Additional 13 residues in elongated loop (Loop DARPin library)	Ribosome display	[87]
Fynomer		SH3 domain of the human Fyn tyrosine kinase	Human	63/0	β-Sandwich (4AFQ)	Six residues in two loops (RT- and n-Src-loop)	Phage display, DNA display	[14] Covagen
Kunitz domain		BPTI/LACI-D1/ITI-D2/APPI	Human	58/3 S–S	α-Helices, β-sheets (1ZR0)	1–2 loops	Phage display	[42] DYAX
Obody/ OB-fold		OB-fold of the aspartyl tRNA synthetase	Archaea *Pyrobaculum aerophilum*	111/0	Five-stranded β-barrel (4GLA)	17 residues	Phage display	[88]
Pronectin		14th extracellular domain of human fibronectin III	Human	90–95/0	Two β-sheets and three surface-exposed loops (3R8Q)	Three loops (BC, DE, FG loops)	Phage display	Protelica
Repebody		Leucine-rich repeat (LRR) modules of variable lymphocyte receptors (VLRs)	Jawless vertebrates artificial (consensus design)	170 + $n \times$ 20–29/0	β-Strand turn α-helix (4J4L)	Five residues in each LRR	Phage display	[89]

Non-immunoglobulin scaffolds: a focus on their targets

Katja Skrlec et al. Trends in Biotechnology July 2015, Vol. 33, No. 7

Scaffold name/ technology	Scaffold structure with randomization residues	Parent protein	Origin of parent protein	Residues/ S–S bridges	Structure (PDB code)	Randomization	Selection method	Refs/Company
ABD		Albumin-binding domain	Bacterial	46/0	Thee α-helices (1GJT)	15 surface residues	Phage display, ribosome display	[77]
Adhiron		Phytocystatin protein	Plant	~100/0	Four-strand antiparallel β-sheet core with a central helix (4N6T)	Insertion of two variable peptide regions	Phage display	[78]
Adnectin/ Monobody		10th domain of human fibronectin	Human	94/0	β-Sandwich of seven β- sheets (3RZW)	Residues in BC, DE, and FG loops (loop only library)	Phage display, mRNA display, yeast-two-hybrid	[30] Adnexus Therapeutics
Adnectin/ Monobody		10th domain of human fibronectin	Human	94/0	β-Sandwich of seven β- sheets (3UYO)	Residues in C and D β-sheets, and DE and FG loops (side and loop library)	Phage and yeast display	[79]
Affibody		Z-domain of staphylococcal protein A	Bacterial	58/0	Three α-helices (1LP1)	13 residues in two helices	Phage display, ribosome display	[45] Affibody AB
Affilin		γ-B-crystallin	Human	176/0	β-Sheet (2JDG)	Eight residues	Phage display	[80] SCIL Proteins
Affilin		Ubiquitin	Human	76/0	α/β (1UBI)	Six residues in the β-sheet	Ribosome display	[81] SCIL Proteins
Affimer		Protease inhibitor Stefin A	Human	98/0	Three-fold clustering (1NB5)	12–36 residues	Phage display, yeast-two-hybrid, CIS display	[82] Avacta Life Sciences
Affitin/ Nanofitin		DNA-binding protein Sac7d	Archaea	66/0	Five-stranded incomplete β-barrel (4CJ2)	14 residues located in the β-sheet	Ribosome display	[83]
Alphabody		Triple antiparallel helices	Artificial (*de novo* design)	70–100/0	Three α-helices (4OE8)	11 residues (A and C helix)	Phage display	[31] Complix
Anticalin		Lipocalins	Human/ insect	160–180/ 0–2 S–S	Eight-stranded β-barrel (4GH7)	Four loops (up to 24 aa)	Phage display	[28] Pieris AG
Armadillo repeat proteins		Armadillo (homologous to β-catenin)	Various/artificial (consensus design)	$n \times$ ~40/0	Three α-helices (4DB6)	Six residues in each internal repeat	Ribosome display	[79]

Charge-Variant Profiling of Biopharmaceuticals

LCGC NORTH AMERICA VOLUME 36 NUMBER 1 JANUARY 2018

Jared R. Auclair, Anurag Rathore, and Ira Krull

Y. Du, A. Walsh, R. Ehrick, W. Xu, K. May, and H. Liu, mAbs 4(5), 578–585 (2012).

The term *acidic species* refers to a negatively charged species; using cation exchange these variants are eluted before the main peak, and when using anion exchange they are eluted after the main peak

The term *basic species* refers to positively charged species; using cation exchange HPLC, these variants are eluted after the main peak, and when using anion exchange these variants are eluted before the main peak.

Acidic species and Basic species

Y. Du, A. Walsh, R. Ehrick, W. Xu, K. May, and H. Liu, mAbs 4(5), 578–585 (2012)

- Sialic acid
- Deamidation
- Nonclassical disulfide linkage
- Trisulfide bonds
- High mannose
- Thiosulfide modification
- Glycation
- Modification by maleuric acid
- Cysteinylation
- Reduced disulfide bonds
- Nonreduced species
- Fragments

- C-terminal Lys
- N-terminal Glu
- Isomerization of Asp
- Succinimide
- Met oxidation
- Amidation
- Incomplete disulfide bonds
- Incomplete removal of leader sequence
- Mutation from Ser to Arg
- Aglycosylation
- Fragments
- Aggregates

Bispecific Antibodies

Bispecific Antibodies Close in on CancerMarianne Guenot, GEN Exclusives February 27, 2017

- Bispecific antibodies are products of genetic engineering that allow one antibody-like molecule to bind several different antigens at once
- In December 2014, for the first time, a bispecific antibody, blinatumomab (Blincyto©), was approved for therapeutic use.
- Amgen's blinatumomab, is a molecule composed of the single-chain variable region (scFv) of one antibody targeting the T-cell activating molecule CD3, linked to another scFv, which binds to the B-cell antigen CD19. In the context of acute lymphoblastic leukemia (ALL), this bispecific antibody brings the T cell's potent cytotoxicity close to the malignant B cells, allowing them to be specifically targeted.
- Since then, over 120 bispecific molecules have entered the clinical pipeline
- Global market for 2024 is estimated to hit a staggering $5.8 billion annually,

  Research and Markets report ("Bispecific Antibody Therapeutics Market, 2014–2024").
- Can bring clinical benefits beyond the injection of two monospecific antibodies

What are Biosimilars?

Biosimilars. Are They Ready for Primetime? Gina Battaglia and Leala Thomas — July 12, 2017 – Bioradiations BIORAD

http://www.bioradiations.com/biosimilars-are-they-ready-for-primetime/?WT.mc_id=170712020560&mkt_tok=eyJpIjoiTUdSaE1XUXpPVE16TVdZMCIsInQiOiJ1VTh4d2RJT1wvcHY5Z0tSckdXQ201VkFmdkUySHZQUGhEY1BoT1A5YVNsbTRBQXpmZk1ZdFVSZ09xUVNadmV6S1EyNE1sUnVvamlEU1JYUEY4MmMxaWlxQUowc1l1VndNQ1hcLzhMdzRWMFZRNkdPbUdKbzROcWFGbE13S05mTmJEIn0%3D#references

✓ A biosimilar is “a product that is highly similar to the reference product despite minor differences in clinically inactive components; and there are no clinically meaningful differences between the biological product and the reference product in terms of the safety, purity, and potency of the product,”

Emanuela Lacana, PhD, Associate Director, Biosimilars and Biologics Policy, Office of Biological Products, CDER, FDA, at the PDA/FDA Joint Regulatory Conference in Washington, DC in September 2016 (Tierney 2016).

✓ More simply, it is a biologic that is almost identical to a previously approved biological product, with no clinically meaningful differences in safety or efficacy.

✓ **Market is expected to grow to US$11 billion by 2021 from US$3.4 billion in 2016**

✓ Key players in the biosimilars market include by Pfizer Inc. (U.S.), Sandoz International GmbH (Germany), Teva Pharmaceuticals Industries Ltd. (Israel), Amgen Inc. (U.S.), Biocon Ltd. (India), Dr. Reddy’s Laboratories Ltd. (India), F. Hoffmann-La Roche Ltd. (Switzerland), Celltrion, Inc. (South Korea), and Samsung Bioepis (South Korea).

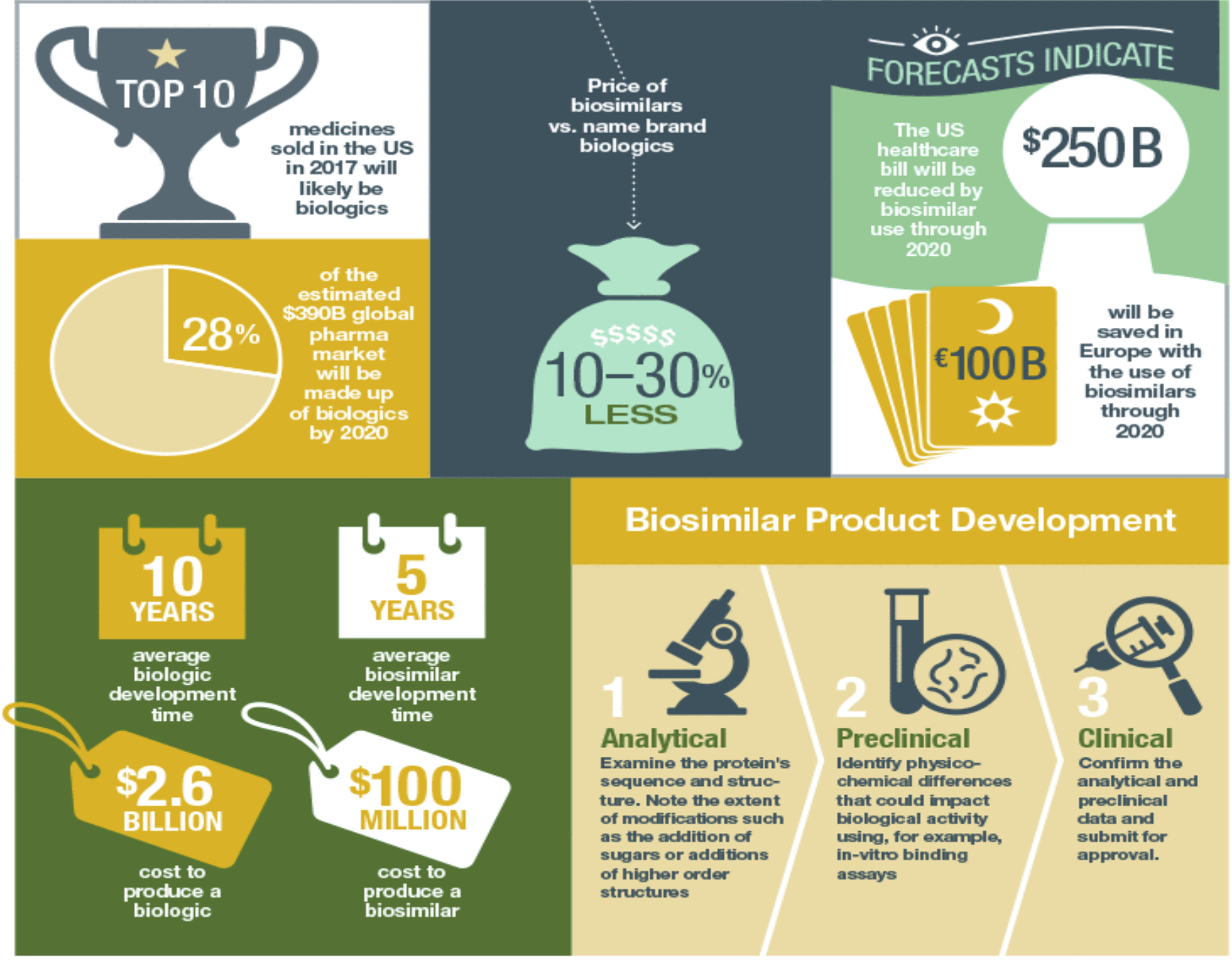
✓ **Currently account for 20% of the global pharmaceutical market.**

✓$2.6 billion to develop a new biologic, and development time of over a decade (Di Masi et al.2016).

✓The European Union approved its first biosimilar in 2006 and leads the global biosimilar market with 35 approved products. In addition, they were the first to develop product-specific development guidelines

✓Biosimilar development requires extensive physicochemical and biological characterization of the reference originator product using multiple analytical techniques to ensure similarity. Based on the results of this characterization, preclinical studies and clinical trials are undertaken to resolve uncertainty about the biosimilarity of the new biosimilar product.

✓The uncertainty about legal issues (for example, patent infringement) and the complex expertise required to synthesize biologics will likely mean that biosimilar development will be limited to large pharmaceutical companies with secure finances and extensive experience.


TOP 10
medicines sold in the US in 2017 will likely be biologics
28%
of the estimated $390B global pharma market will be made up of biologics by 2020
Price of biosimilars vs. name brand biologics
$$$$$
10–30%
LESS
FORECASTS INDICATE
The US healthcare bill will be reduced by biosimilar use through 2020
$250B
€100B
will be saved in Europe with the use of biosimilars through 2020
10
YEARS
average biologic development time
5
YEARS
average biosimilar development time
$2.6
BILLION
cost to produce a biologic
$100
MILLION
cost to produce a biosimilar
Biosimilar Product Development
1
Analytical
Examine the protein's sequence and structure. Note the extent of modifications such as the addition of sugars or additions of higher order structures
2
Preclinical
Identify physico-chemical differences that could impact biological activity using, for example, in-vitro binding assays
3
Clinical
Confirm the analytical and preclinical data and submit for approval.

$81B

ENBREL NOVOLOG LANTUS NEULASTA HUMIRA RITUXAN AVASTIN LUCENTIS REMICADE HERCEPTIN

worth of biologics will lose patent by 2020

5 IN THE USA since 2015

13 IN AUSTRALIA since 2010

7 IN THE UK since 2016

APPROVED BIOSIMILARS

9 IN JAPAN since 2009

35 IN EUROPE since 2006

7 IN SOUTH KOREA since 2012

APPROVED SIMILAR BIOLOGICS

(not subject to the same rigorous standards):

382 in China since 1995 | 66 in India since 2007

FACTS

The biosimilar market has the potential to disrupt healthcare by exponentially increasing access, providing the highest quality care at the lowest cost.

By 2021, the biosimilar market is expected to grow up to US$11B.

There are more than 280 biosimilars in the product pipeline. Approximately 32 molecules are targeted by developers.

Development Hot Spots