

"Beyond Antibodies: Novel Tools for Science and Medicine"

Louise D. Teel, Ph.D

Microbiology And Immunology Dept.



Affinity tools are needed for:

- Diagnosis/detection of target molecules
- Discrimination among like agents, organisms
- Purification/segregation of molecules
- Neutralization of toxins, viruses, tumors
- Blocking unwanted cell/receptor interactions
- Delivering drugs and toxins to the desired site of effect

Outline

- The pros and cons of native antibodies
- Monoclonal antibodies- humanization
- Single chain variable fragments
- Single domain antibody fragments
- Human protein scaffold molecules
- Aptamers
 - Structure
 - Synthesis/Selection
 - Applications
 - Limitations

Antibodies: molecular superheros

- incredible variability
- affinity maturation
- Polyclonality increases sensitivity/efficacy
- Monoclonals display single epitope specificity
- interactive component Fc maximizes other cell reactions, purification, immobilization



Limitations of natural antibodies

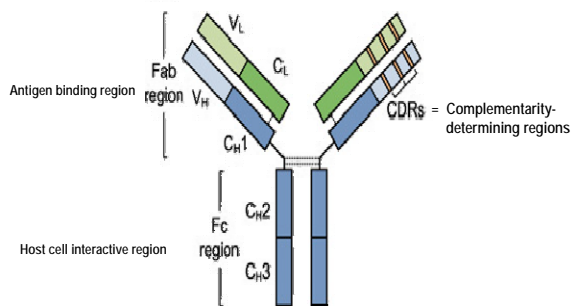
- Only suitable for non-self types of antigens
- Not useful for antigens that are toxic to the vaccinee
- Complex molecular structure is sensitive to harsh conditions use restricted to near physiologic conditions
- Disulfide bonds, glycosylation limits their production to cell culture (non-bacterial) synthesis
- Fc domain, species specificity, cellular affinity
- Large bulky molecules may be less effective than smaller binders for affinity chromatography etc.
- Long half life in the body (several weeks) may be beneficial or detrimental (toxic immunoconjugates)

Most Ab applications use IgG:



- IgG undergoes affinity maturation, somatic hypermutation within the region of antigen specificity with repeat interactions with antigen (and T cell help!)
- Production is amnesic - can be boosted
- Plentiful in serum (no assembly required)
- Access to the Fc maximizes other cell reactions, purification apps

Antibody (IgG) structure



V. Ruigrok, et al., Biochem. J. (2011) 436, 1-13.

Most polyclonal Abs are of animal origin:



Vaccination with Ag results in an adaptive immune response. Quantity is limited to what is produced in the animal host.

Polyclonal antibodies still have broad applications:

- Reagents for science
- Very limited therapeutics
 - Botulinum, Tetanus and snake venom **antitoxins**
- Cross reactivity of the Ab molecule among species is the greatest limitation in medicine
- Lack of the defined sequence and prevents production by recombinant means



Human polyclonal antibodies currently in therapeutic use

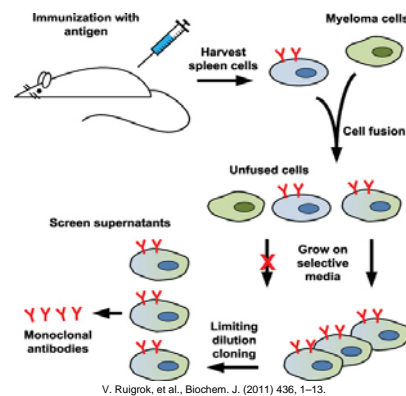
- Respigam® - Respiratory syncytial virus IG for neonates
- BayRab® - Rabies IG for suspected rabies infection
- BayTet® - Tetanus toxin IG for prophylaxis against tetanus
- BayGam® - hepatitis A IG for prophylaxis
- Cytogam® - Cytomegalovirus IG for prophylaxis in transplant recipients
- Nabi-HB® - Hepatitis B IG for transplant recipients
- BabyBIG® - Botulism IG botulism antitoxin for treatment of infant botulism types A and B.

Kohler and Milstein were awarded the Nobel Prize in 1984 for

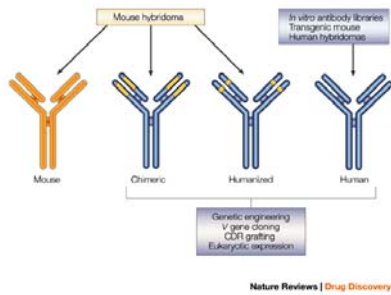


their 1975 Monoclonal Ab breakthrough!

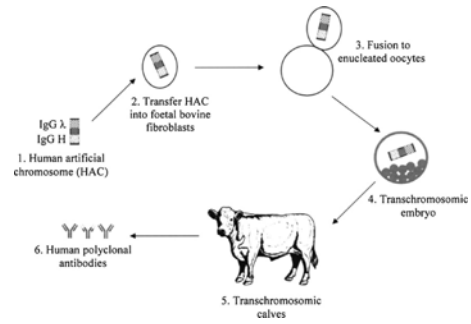
Hybridomas yield unlimited supply of antibody!



Becoming less foreign...



Human antibody production in cattle



22 FDA-approved therapeutic monoclonals to date:

Trade name	Company	Target	Source	Year	Indication
Orthoclone [®]	Ortho Biotech, Inc. (subsidiary of J&J)	CD3	all rodent	1986	Transplantation rejection
ReoPro [™]	Centocor, Inc. (subsidiary of Johnson & Johnson) and Eli Lilly	GP1Ib, IIla	chimeric	1994	High risk angioplasty
Rituxan [™]	Biogen Idec and Genentech, Inc.	CD20	chimeric	1994	Non-Hodgkin's lymphoma, rheumatoid arthritis
REMICADE [®]	Centocor, Inc. (subsidiary of Johnson & Johnson)	TNF- α	chimeric	1998	Crohn's disease
Simulect [®]	Novartis	CD25	chimeric	1998	Transplantation rejection
Synagis [™]	Medimmune	RSV F protein	humanized	1998	RSV infection
Zenapax [®]	Hoffmann-La Roche Inc., Protein Design Labs	CD25	humanized	1997	Transplantation rejection
Herceptin [®]	Genentech	HER-2	humanized	1998	Breast cancer
Myelarg [™]	UCB and Wyeth	CD33	humanized	2000	Acute Myeloid Leukemia
Campath [®]	Miltenium Pharmaceuticals, Inc. and Biex Laboratories, Inc.	CD52	humanized	2001	Chronic lymphocytic leukaemia, T-cell lymphoma
Zevalin [®]	Idex Pharmaceuticals Corporation	CD20	murine - with yttrium-90 or indium-111	2002	Non-Hodgkin's lymphoma
HUMIRA [™]	Abbott Laboratories/Cambridge Antibody Technology	TNF- α	human	2002	Inflammatory diseases - mostly autoimmune disorders like rheumatoid arthritis, psoriasis, Crohn's disease
Bevaciz [®]	Covix Corp. and GlaxoSmithKline	CD20	murine - covalently bound to iodine 131	2003	Morbus Chron, Non-Hodgkin's lymphoma

Monoclonals continued

Xolair [®]	Genentech, Tanox, Inc., Novartis Pharmaceuticals	IgE	humanized	2003	Severe (allergic) asthma
Avastin [™]	Genentech	VEGF	humanized	2004	Metastatic colorectal cancer, non-small cell lung cancer, metastatic breast cancer
TYSABRI [®]	Biogen Idec and Elan Corp.	α 4 subunit of α 4 β 1	humanized	2004	Multiple Sclerosis, Chron's disease
Erbix [™]	Merck KG aA / Bristol-Myers Squibb / ImClone Systems	EGFR	chimeric	2004	Colorectal cancer, head and neck cancer
Vectibix [™]	Amgen	EGFR	human	2006	Metastatic colorectal carcinoma
LUCENTIS [™]	Genentech	VEGF-A	humanized Fab	2006	Wet Macular Degeneration
Soliris [®]	Alexion Pharmaceuticals, Inc.	CD59	humanized	2007	Paroxysmal nocturnal hemoglobinuria
CIMZIA [®]	UCB	TNF- α	Humanized (Fab)	2008	Morbus Chron, rheumatoid arthritis
Simponi [™]	Centocor (subsidiary of Johnson & Johnson)	TNF α	human	2009	Rheumatoid & psoriatic arthritis, active ankylosing spondylitis

Priorities for new affinity tools

- Same great specificity and avidity of antibodies
- Less immunogenicity
- Genotype based
- Suitable for synthesis by recombinant techniques in an inexpensive host
- Or totally synthetic
- Capacity for non-physiological applications

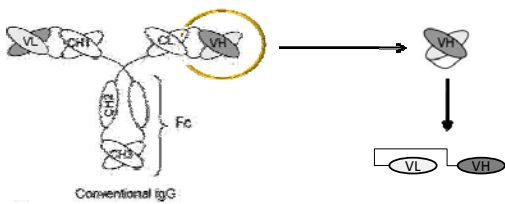
The next steps in affinity tool development, or:



Relying on recombinant DNA strategies, not animals to produce affinity tools.

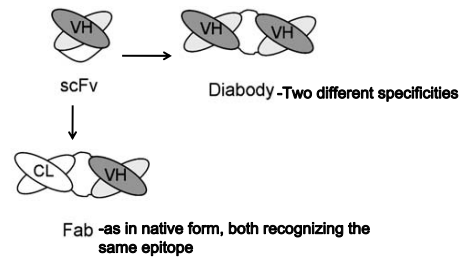
Focus on the antigen recognition fragments portions of antibodies!

scFv Single chain variable region fragment



link the variable "binding" region of the heavy and light domains recombinantly

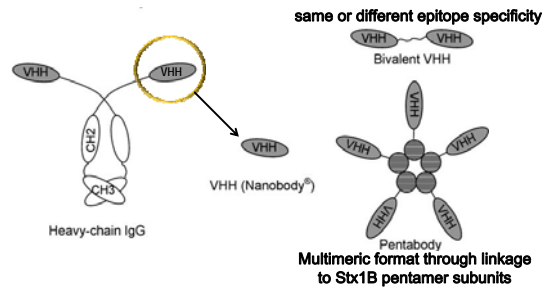
Other potential fragment arrangements:



Sources of single domain antibodies



Fragments based on camelid single heavy chain antibodies - sdAb, single domain Ab



Where are the sources of DNA sequences on which to base scFvs, sdAbs, and other fragment formats?

- Unimmunized animals
- Immunized animals
- Human B-cells

The choice depends on whether you wish to direct a particular immune response or simply sample for tools from the diverse pool of reassorted genes.

To obtain potential binding sequences

- Collect B-cells from a human/animal population
- Extract mRNA
- Synthesize cDNA
- PCR amplify the variable domain sequences
- Create a library of binding moiety genes
- Express the genes as proteins and
- Screen the library for a domain that recognizes your desired target

A strategy for a human scFv library

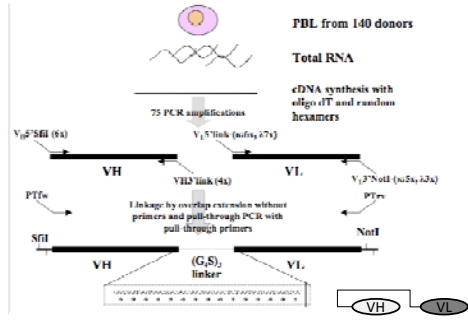
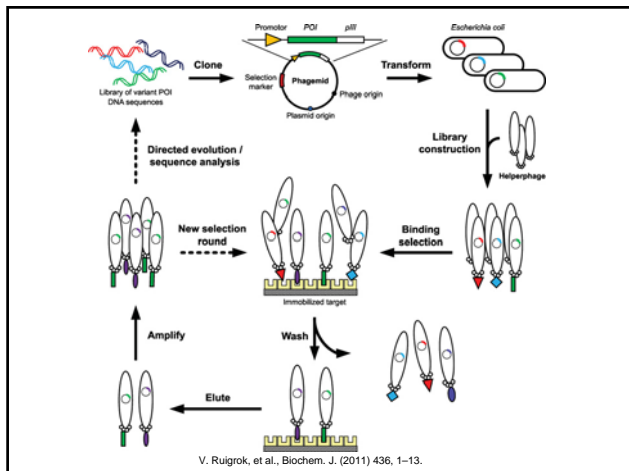


Diagram by Pansri, et al., *BMC Biotechnology* 2009, 9:6

The basics of phage display

1. Make a gene library of Ab genes from host B cells. naïve, immunized (rearranged) using RNA converted to cDNA
2. Ligate these assorted fragments into a phagemid vector
3. Transform lab *E. coli* with phagemids
4. Harvest phage that now each encode a gene from the library among the surface proteins and
5. Pan for, or selectively trap, phages that bind the antigen or ligand of choice.



V. Ruljok, et al., *Biochem. J.* (2011) 436, 1–13.

Do scFvs and sDabs meet these criteria?

- Same great specificity and avidity of antibodies **-yes**
- Less immunogenicity -probably not if animal derived
- Genotype based- **yes**
- Suitable for synthesis by recombinant techniques in an inexpensive host **-yes**
- **Additionally, they are smaller and more stable/versatile than whole Abs**
- Applicable for non-physiologic conditions- not very

So next, to address immunogenicity:



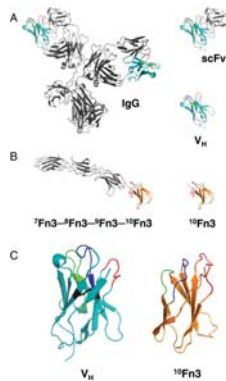
and leave the animals out of the synthesis of the next generation of affinity tools altogether!

There are antigen recognition fragments not derived from antibodies!

FN3 -Fibronectin domain III - based protein scaffold

- Fibronectin is an abundant plasma and connective tissue protein in humans
- It has 10 domains, each with a 7-beta sheet structure that maintains its conformation by virtue of hydrophobic interactions
- The terminal loops are variable and have been substituted by randomly generated sequences to form libraries for screening
- Currently licensed for VEGFr2

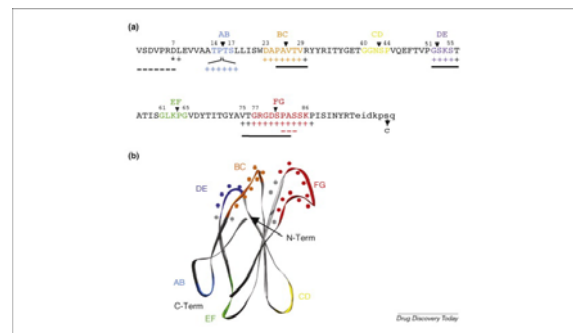
Engineered antibodies and 10Fn3-based target-binding protein



Lipovšek D Protein Engineering, Design and Selection
2011;24:3-9

peds
protein engineering design & selection

Generating FN3-based binding tools



Drug Discovery Today

DARPins



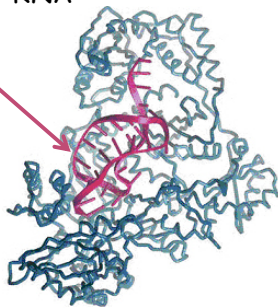
- **Designed Ankyrin Repeat Proteins**
- Ankyrin proteins are membrane components of human RBCs, and other cell types
- Each repeat unit consists of two helices and a loop (totaling 33 aas)
- Like FN3, there varying aa composition of the loops in the otherwise rigid structure creates extensive binding diversity

Do FN3 and DARPins meet these criteria?

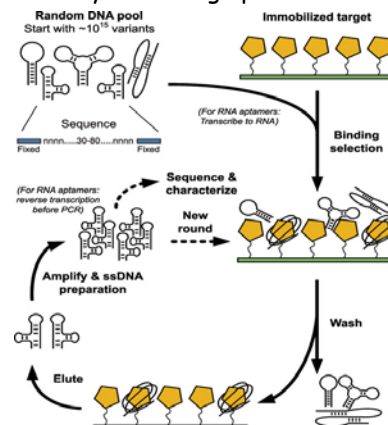
- Same great specificity and avidity of antibodies -**yes**
- Less immunogenicity -**Probably**
- Genotype based- **yes**
- Suitable for synthesis by recombinant techniques in an inexpensive host -**yes**
- **Additionally, they are smaller and more stable/versatile than whole Abs**
- Applicable for non-physiologic conditions- not very

Aptamers -nucleic acid binding tools

- ssDNA or RNA



Synthesizing aptamers:



Advantages of Aptamers

- Toxins as well as molecules that do not elicit good immune response can be used to generate high affinity aptamers
- stable to long term storage and can be transported at ambient temperature (terminal additions can prevent exonuclease degrad.)
- Selection conditions can be manipulated to obtain aptamers with properties desirable for in vitro assay e.g. non-physiological buffer/Temp.
- Aptamers are produced by chemical synthesis resulting in little or no batch to batch variation
- Aptamers are identified through an in vitro process not requiring animals
- Reporter molecules can be attached to aptamers at precise locations not involved in binding

Aptamers approved for use

- Pegaptanib is an anti-vascular endothelial growth factor RNA aptamer
- Used in the treatment of macular degeneration and diabetic macular edema

Do Aptamers meet these criteria?

- Same great specificity and avidity of antibodies **-yes**
- Less immunogenicity **-yes**
- Genotype based **-yes**
- Suitable for synthesis by recombinant techniques in an inexpensive host **-yes**
- **Additionally, they are smaller and more stable/versatile than whole Abs**
- Applicable for non-physiologic conditions **-yes**

Comparison of affinity tools

Characteristic	Antibody	Binding protein	Aptamers (ssDNA and RNA)
Size (kDa)	~150-160	<30	5-20 (15-60 nt)
Selection	<i>In vivo</i>	<i>In vitro</i>	<i>In vitro</i>
Production	Animal or recombinant	Recombinant	Synthetic
Post-selection modifications	Possible, but heterogeneous products	Possible, can be designed for homogeneous products	Wide variety of options (sugar, base or phosphate; 5', 3' or internal)
Stability	Several weeks at 4°C	Variable	DNA: years at room temperature; RNA: several months at -80°C
Binding site	Monoclonal: homogeneous; polyclonal: heterogeneous	Homogeneous	Homogeneous
Target molecules	Mainly immunogenic macromolecules	Macromolecules and low-molecular-mass molecules	Low-molecular-mass molecules, macromolecules and cells
<i>In vivo</i> half-lives	Days to weeks	n.a.	Untreated: seconds to minutes; treated: days
Application conditions	Physiological	Physiological and non-physiological	Physiological, non-physiological and organic solvents (to some extent)

Applications for affinity tools

+, reported in literature; ++, commercially available.

Application	Antibodies	Engineered binding proteins	Aptamers
Therapeutics/treatment	++	+	++
Targeted drug delivery	++	+	+
Molecular imaging	++	+	+
Drug discovery			
Diagnostics	++	+	++
Affinity purification	+	++	+
Biosensors		+	+

Summary

- Animal derived polyclonal and monoclonal antibodies are still among our best laboratory tools and serve as the only therapeutics for some diseases
- Enhanced specificity, stability, and flexibility is afforded by less complex formats based on antibody design (scFvs, camelid sdAbs)
- Advances in binding technology has yielded totally synthetic affinity molecules (FN3, Darpins, aptamers) that provide greater flexibility in applications

Questions?



Antibodies still an awesome tool! Gaston Mélingue, "The Injection", 1894
Depicts the first vaccinia virus immunization by Edward Jenner in 1797 a century earlier!