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Cellular Synthesis of Antibody Fusion Proteins

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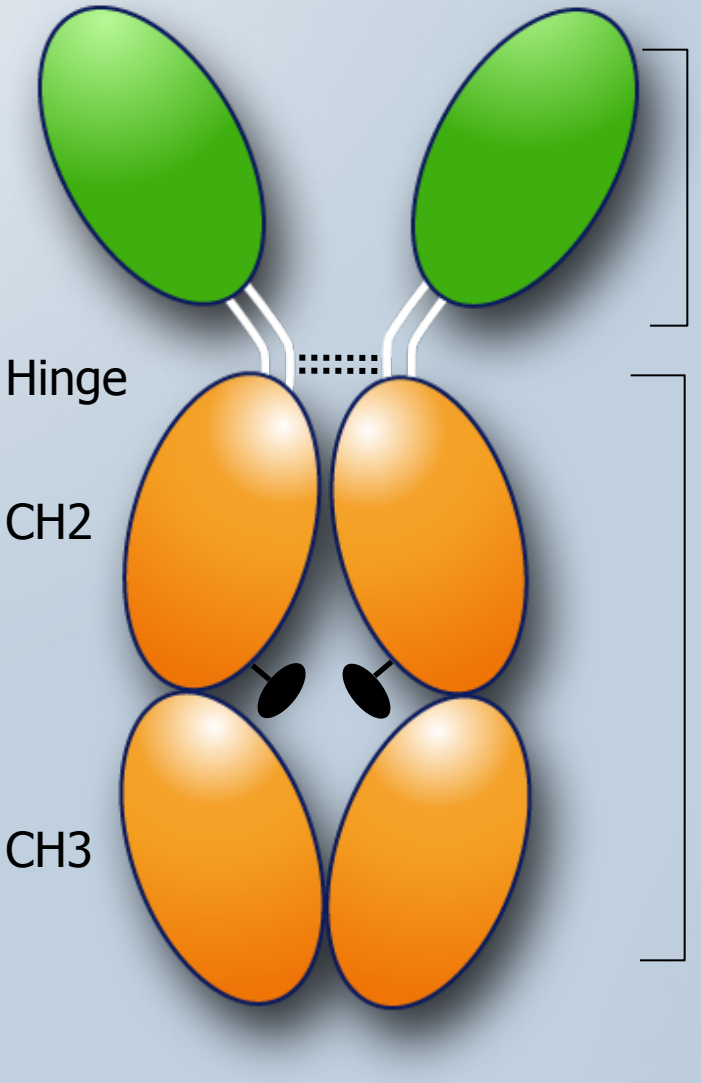
Outline

- Antibody Fc-fusion proteins as designer drugs
  - Structure/function
  - First use as a therapeutic
  - Approved products today
- Production
  - Challenges
- Case studies
  - Glycosylation of rhuEpo-Fc
  - Binding capacity of Fc-fusion proteins on Protein A

Antibody Fc-fusion proteins as designer drugs

Fc-fusion protein (immunoadhesin)



Protein of interest

- Receptor
- Cytokine
- Enzyme
- Peptide

Fc region

FcγR binding	→	ADCC
C1q binding	→	CDC
FcRn binding	→	Half-life

1989 Report describing CD4IgG, the first therapeutic Fc-fusion protein

Designing CD4 immunoadhesins for AIDS therapy

Daniel J. Capon, Steven M. Chamow*, Joyce Mordenti†, Scot A. Marsters, Timothy Gregory*, Hiroaki Mitsuya†, Randal A. Byrn§, Catherine Lucas‖, Florian M. Wurm¶, Jerome E. Groopman§, Samuel Broder† & Douglas H. Smith

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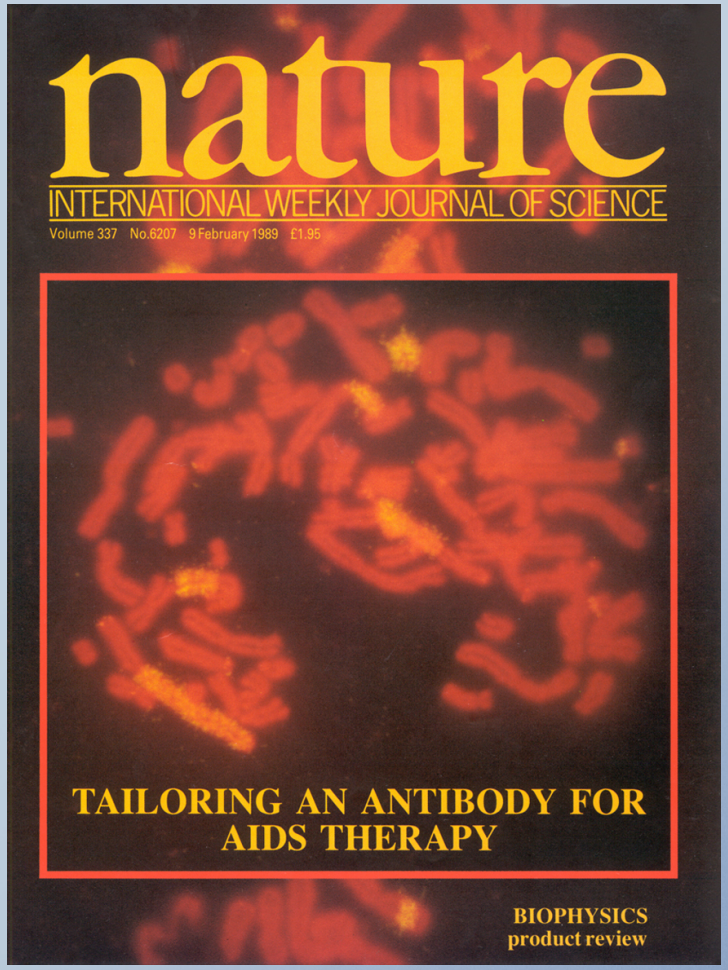


Capon *et al., Nature* **337**, 525-531 (1989)

Design of CD4IgG

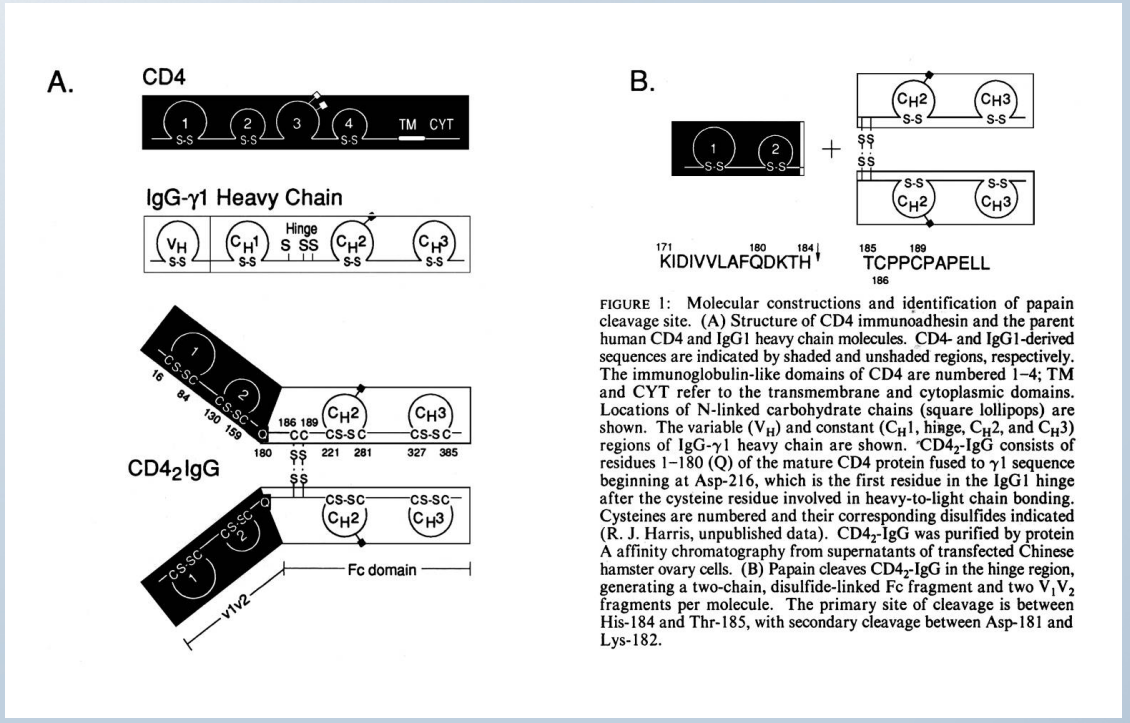


FIGURE 1: Molecular constructions and identification of papain cleavage site. (A) Structure of CD4 immunoadhesin and the parent human CD4 and IgG1 heavy chain molecules. CD4- and IgG1-derived sequences are indicated by shaded and unshaded regions, respectively. The immunoglobulin-like domains of CD4 are numbered 1–4; TM and CYT refer to the transmembrane and cytoplasmic domains. Locations of N-linked carbohydrate chains (square lollipops) are shown. The variable (V_H) and constant (C_H1, hinge, C_H2, and C_H3) regions of IgG-γ1 heavy chain are shown. "$CD4_2$-IgG consists of residues 1–180 (Q) of the mature CD4 protein fused to γ1 sequence beginning at Asp-216, which is the first residue in the IgG1 hinge after the cysteine residue involved in heavy-to-light chain bonding. Cysteines are numbered and their corresponding disulfides indicated (R. J. Harris, unpublished data). $CD4_2$-IgG was purified by protein A affinity chromatography from supernatants of transfected Chinese hamster ovary cells. (B) Papain cleaves $CD4_2$-IgG in the hinge region, generating a two-chain, disulfide-linked Fc fragment and two V_1V_2 fragments per molecule. The primary site of cleavage is between His-184 and Thr-185, with secondary cleavage between Asp-181 and Lys-182.

Chamow *et al., Biochemistry* **29**, 9885-9891 (1990)

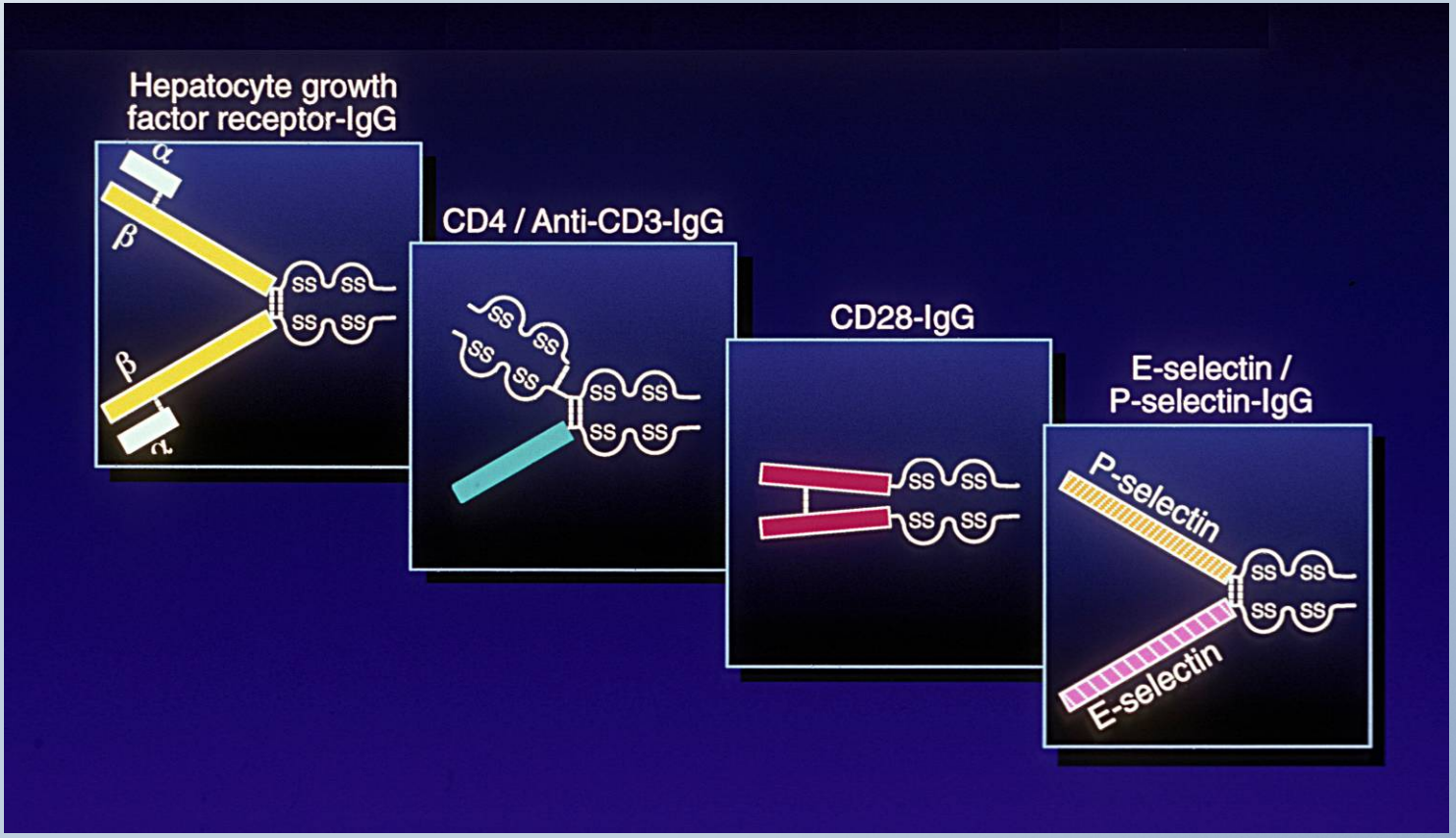
- Assembles into a homodimer
- Retains fidelity of papain cleavage site in hinge
- Binds C1q
- Binds Protein A
- Is transported across placenta

Clinical testing of CD4IgG confirms non-toxicity and increased *in vivo* half-life

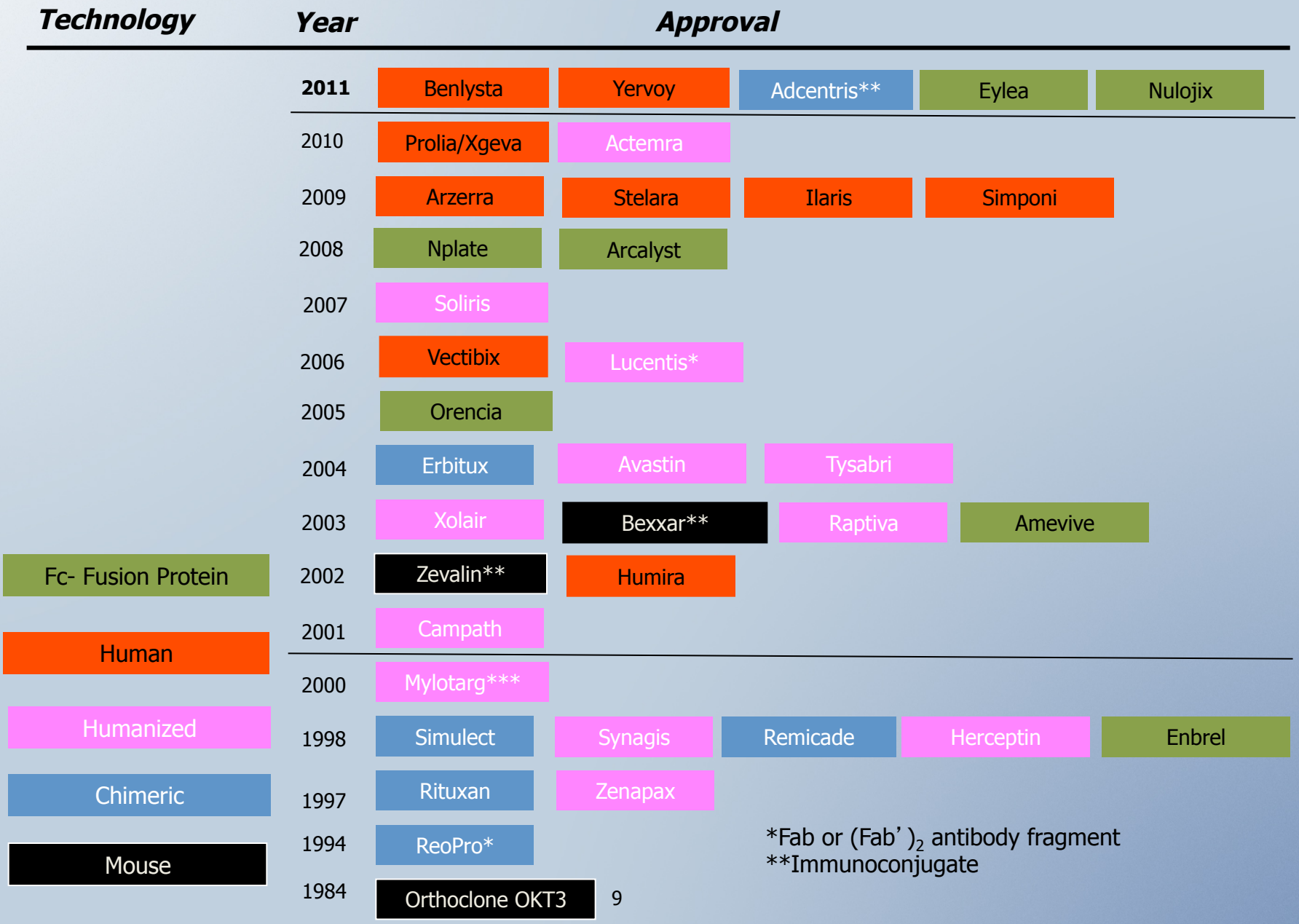
- Produced for clinical testing
- Filed IND with FDA to evaluate in humans
- Tested for safety, pharmacokinetics in small clinical trial (*Int'l Conf. AIDS* 1990)
  - METHODS: Dose-escalating Phase I study of 30, 100, or 300 ug/kg of rCD4-IgG given weekly by intravenous bolus for up to 12 weeks.
  - RESULTS: Twelve homosexual men (mean age 43, mean CD4 count 89+/-120/mm3) with AIDS (N=7) or ARC (N=5) have entered to date. **rCD4-IgG was well tolerated with no observable clinical, hematologic or blood chemistry toxicity**. The maximum serum concentrations at 30, 100 and 300 ug/kg were 426, 996, and 3880 ng/ml. The volumes of distribution for rCD4-IgG were (central compartment) 85.2+/-12.8 ml/kg and (steady state) 125.8+/-32.2 ml/kg. **Clearance was 3.7+/-0.9 ml/hr/kg, 15 times slower than for soluble rCD4**. In a multi-compartmental model, the terminal elimination half-lives ranged from 18-51 hours (median 33 hours). To date, CD4, CD8 and total lymphocyte counts have remained stable over the course of the study. None of the first 6 subjects have developed CD4 antibodies. Nine of 9 subjects tested had HIV (p24) antigenemia; median levels were 120 pg/ml at entry and 94 pg/ml at week 4. Among the first 6 subjects, HIV plasma viremia was detected in all at entry. In 2 subjects whose undiluted plasma cultures at day 14 or 21 were negative, cultures diluted to 10(-1) were positive, suggesting that the inhibition of plasma viremia was reversible. CONCLUSIONS: Intravenous rCD4-IgG therapy is well tolerated and has a much more favorable pharmacokinetic profile than soluble rCD4. These data suggest that higher and more frequent dosing regimens will be able to provide sustained blood levels of greater than or equal to 5 ug/ml. Additional study of the long-term safety of current and higher dosage regimens and antiviral efficacy of rCD4-IgG is warranted and underway.
- Clinical testing abandoned in 1992 due to lack of efficacy
- Additional receptors for HIV discovered
  - CCR5 and CXCR4 (1996)—chemokine co-receptors that HIV uses to enter target cells
  - Integrin $\alpha 4 \beta 7$ (2008)--another potentially important receptor for HIV

The Fc-fusion protein platform is versatile

At least 40 Fc-fusion cytokine constructs described
Jazayeri and Carroll, *Biodrugs* **22**, 11-26 (2008)



Among the 37 therapeutic monoclonal antibody approvals (USA) are 7 Fc-fusion proteins



USA-approved Fc-fusion proteins

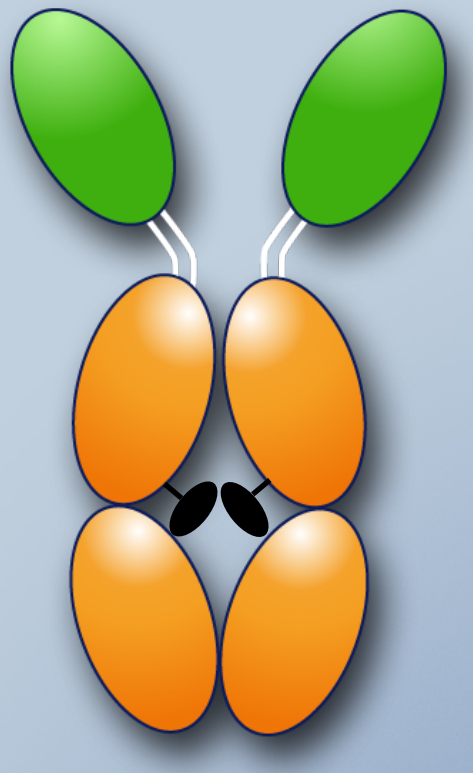
Brand name	Generic name	Sponsor	Year approved	Construct		MW (kDa)	Expression system	Target	Clinical indication
				Target binding domain	Ig domain				
Enbrel	etanercept	Immunex/ Amgen	1998	TNFR2	ϒ1 Fc	150	CHO	TNF α	Rheumatoid arthritis; juvenile idiopathic arthritis; psoriasis
Amevive	alefacept	Biogen-Idec	2003	LFA3	ϒ1 Fc	92	CHO	CD2	Psoriasis
Orencia	abatacept	BMS	2005	CTLA4	ϒ1 Fc	92	CHO	CD28 (indirect)	Rheumatoid arthritis; juvenile idiopathic arthritis
Arcalyst	rilonacept	Regeneron	2008	IL1-RI➔IL 1RAcP	ϒ1 Fc	251	CHO	IL1	Cryopyrin-associated periodic syndromes

USA-approved Fc-fusion proteins (cont'd.)

Brand name	Generic name	Sponsor	Year approved	Construct		MW (kDa)	Expression system	Target	Clinical indication
				Target binding domain	Ig domain				
Nplate	romiplostim	Amgen	2008	Peptide mimetic of TPO	ϒ1 Fc, fusion at C term	59	*E. coli*	TPOR	Chronic idiopathic thrombocytopenic purpura
Eylea	afliberacept	Regeneron /Sanofi-Aventis	2011	VEGFR 1➔VEGFR2	ϒ1 Fc	110	CHO	VEGF	Wet macular degeneration
Nulojix	belatacept	BMS	2011	CTLA4	ϒ1 Fc	92	CHO	CD28	Tissue graft survival

Fc-fusion proteins in development

- Cytokine Fc-fusion
  - *Erythropoietin-Fc*
    - Pulmonary delivery of Epo [Bitoni *et al. PNAS* **101**, 9763-9768 (2004)]
  - *CEA-Fc*
    - Enables adoptive designer T-cell immunotherapy [Ma *et al. Cancer Gene Ther.* **11**, 297-306 (2004)]
- Peptide Fc-fusion ("peptibody")
  - *AMG 386*
    - Peptide inhibitor of TIE2/ANG2:Fc, I-SPY 2 TRIAL adaptive design in breast cancer, Amgen [Reichert, *Mabs* **3**, 76-99 (2011)]
- Enzyme Fc-fusion [Reichert, *Mabs* **3**, 76-99 (2011)]
  - *Factor VIII-Fc*
    - Biogen-Idec
  - *Factor IX-Fc*
    - Biogen-Idec
- Bispecific Fc-fusion
  - *Iduronate-2-sulfatase/anti-hu insulin recpetor*
    - Crosses BBB [Lu *et al., Bioconjugate Chem.* **21**, 151-156 (2010)]



Production

Considerations in constructing Fc-fusion proteins

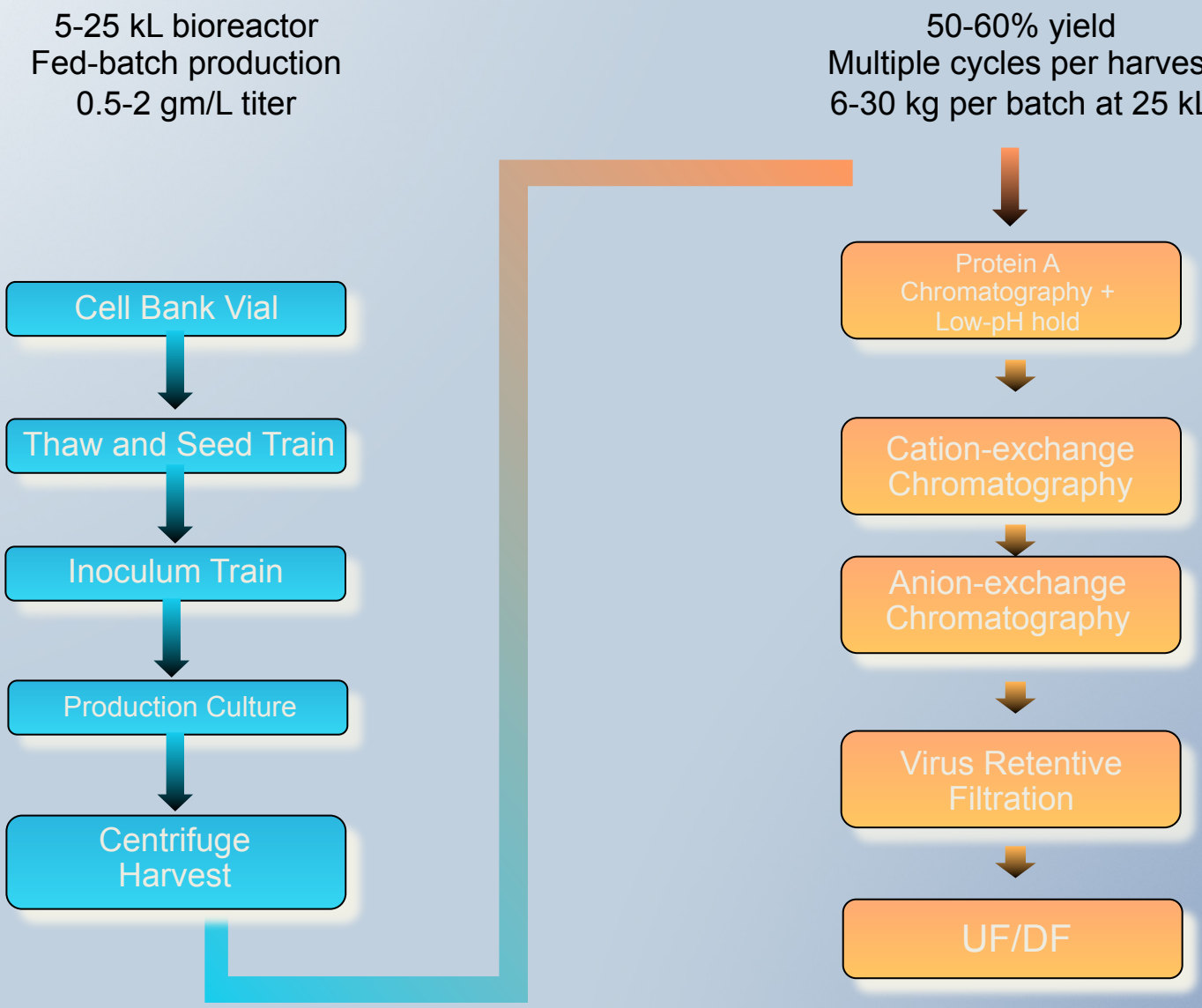
- Selection of Fc Ig subclass
  - hIgG1 most common
- Fc region attached to N- or C-terminus of protein
  - Biological activity dependent on free end
- Where to truncate proteins for attachment?
  - Maintain structural domains for proper folding, biological activity
  - Minimize unpaired cys
- Glycosylation
  - Can be problematic
  - If not required, microbial expression is an option, e.g., Nplate
- Junction spacer peptides (optional)

  Jazayeri and Carroll, *Biodrugs* **22**, 11-26 (2008)
  - Ser-gly rich—limits unwanted interactions
  - Leu-val-pro-arg↓gly-ser--thrombin cleavage site
- Plasmid
  - pFUSE-Fc (InvivoGen, San Diego, CA)

Potential production issues with Fc-fusion proteins

- Upstream
  - Expression of Fc fusion proteins tends to be lower than for mAbs
    - Folding and secretion
    - Disulfide bond formation
  - Stability in fed batch culture
    - Link *et al. J. Biotechnol.* **110**, 51-62 (2004)
  - Glycosylation
    - Beck *et al. Curr. Pharmaceut. Biotechnol.* **9**, 482-501 (2008)
  - H-chain processing (Lys cleavage at C-term)
- Downstream
  - Acid lability (e.g., SpA elution)
    - High pH [Rea *et al., BioPharm Int'l*, Mar supplement (2008)]
  - Reduced SpA chromatographic capacity
    - Ghose *et al., Biotechnol. Bioeng.* **96**, 768-779 (2006)
  - Aggregation
    - Fast *et al. Biochemistry* **48**, 11724-11736 (2009)
    - Hydrophobic interaction chromatography
  - Virus inactivation
    - Solvent/detergent [Rea *et al., BioPharm Int'l*, Mar supplement (2008)]
  - Glycoform heterogeneity
    - Highly sialylated forms often desirable

mAb Production platform applies to Fc-fusion proteins



Case Study 1:
Glycosylation of rhuEpo-Fc

rhuEpo-Fc

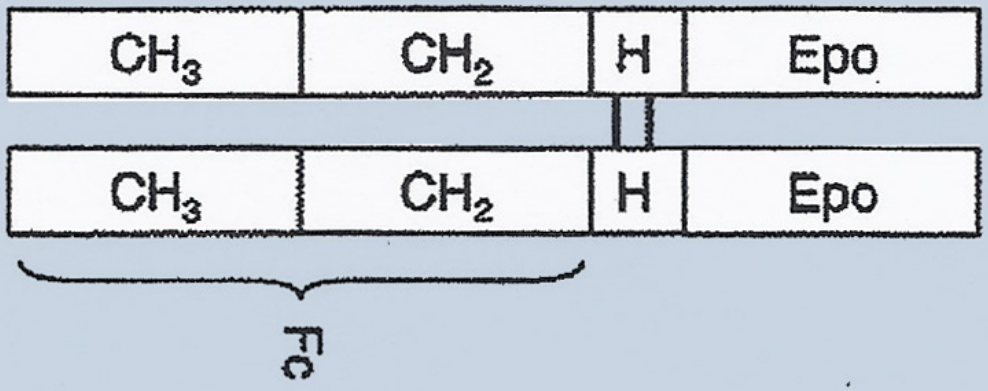


Fig. 1. Structure of N-terminally linked rhEpo with the Fc part of a human IgG_1 molecule.

Erythropoietin (Epo)

- 30 kDa human glycoprotein hormone
- 165 amino acids, single polypeptide, 2 disulfide bonds
- 3 N-linked, 1 O-linked glycan

rhEpo-Fc expressed in CHO

Schriebl *et al., Protein Exp. Purif.* **49**, 265-275 (2006)

rhuEpo-Fc is a glycosylated homodimer

Table 1
Purification of rhEpo-Fc fusion protein

	Protein concentration (pmol/ml)	Total volume (ml)	Total protein (pmol)	Yield (%)
Crude protein	242	41.8	10116	100
Protein A Sepharose FF	6957	1.4	9740	96

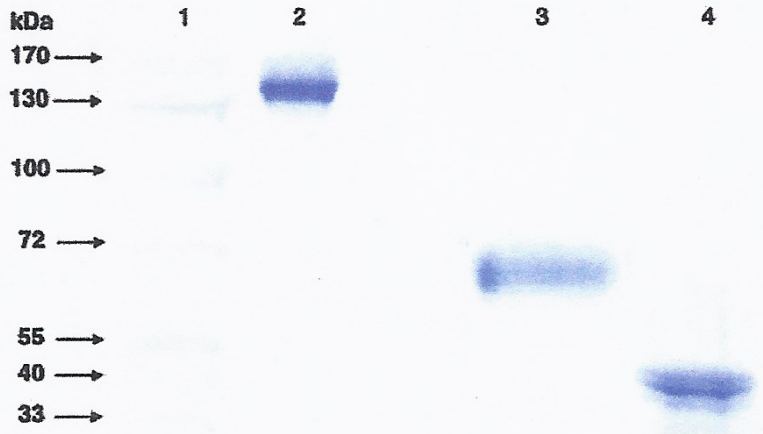


Fig. 2. Separation of rhEpo-Fc by SDS–PAGE (Tris–Glycine 4–20%) followed by Coomassie brilliant blue staining. Lane 1, molecular weight marker; lane 2, non-reduced rhEpo-Fc; lane 3, reduced rhEpo-Fc; lane 4, reduced deglycosylated rhEpo-Fc.

Schriebl *et al.*, *Protein Exp. Purif.* **49**, 265-275 (2006)

Aggregates of rhuEpo-Fc complicate purification

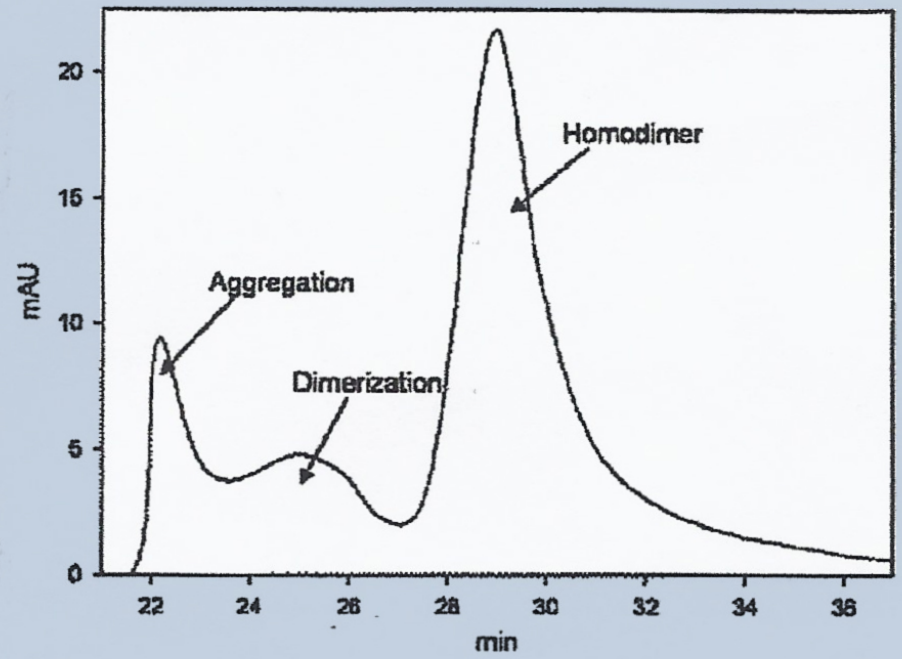


Fig. 3. Size exclusion chromatography (SEC) fractionation profiles of rhEpo-Fc. As the main peak the homodimer rhEpo-Fc was detected, followed by dimers and aggregates in the range of about 20%.

Schriebl *et al., Protein Exp. Purif.* **49**, 265-275 (2006)

N-glycans of Epo and Fc domains are structurally distinct

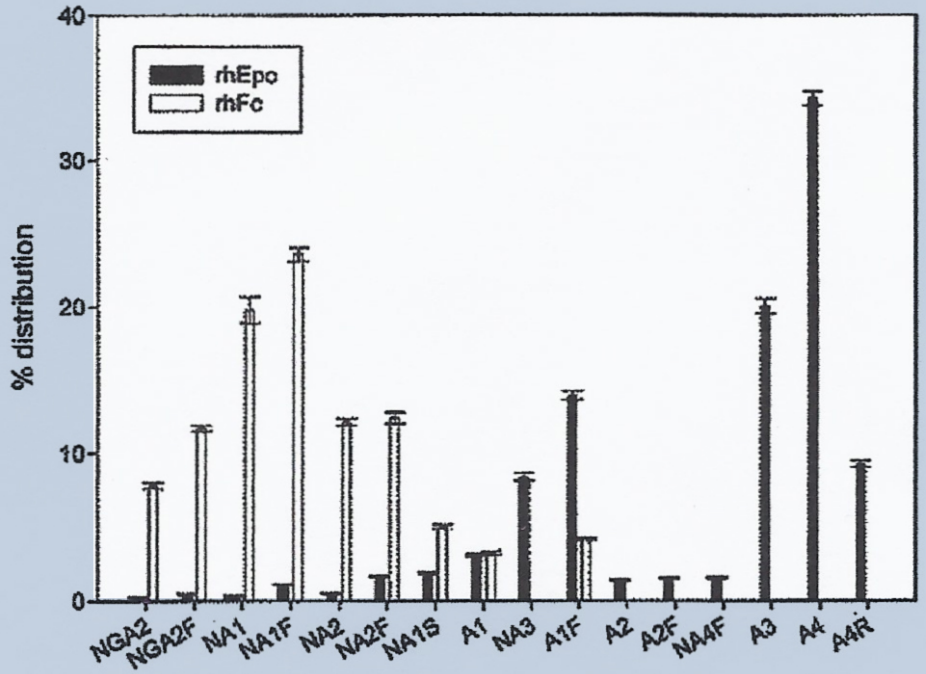


Fig. 7. The relative distribution was calculated from the normal-phase HPLC profiles of 4-ABA labeled rhEpo and rhFc *N*-glycans, the relative distribution was calculated. The rhEpo-Fc concentration before papain digestion was 2.2 nmol. The *N*-glycans were identified by comparison to oligosaccharide standards. The names of the different glycans are listed in Fig. 5. The maximum SD of three independent analyses of the *N*-glycans amounted ±0.92%.

rhEpo and Fc domains isolated by papain cleavage

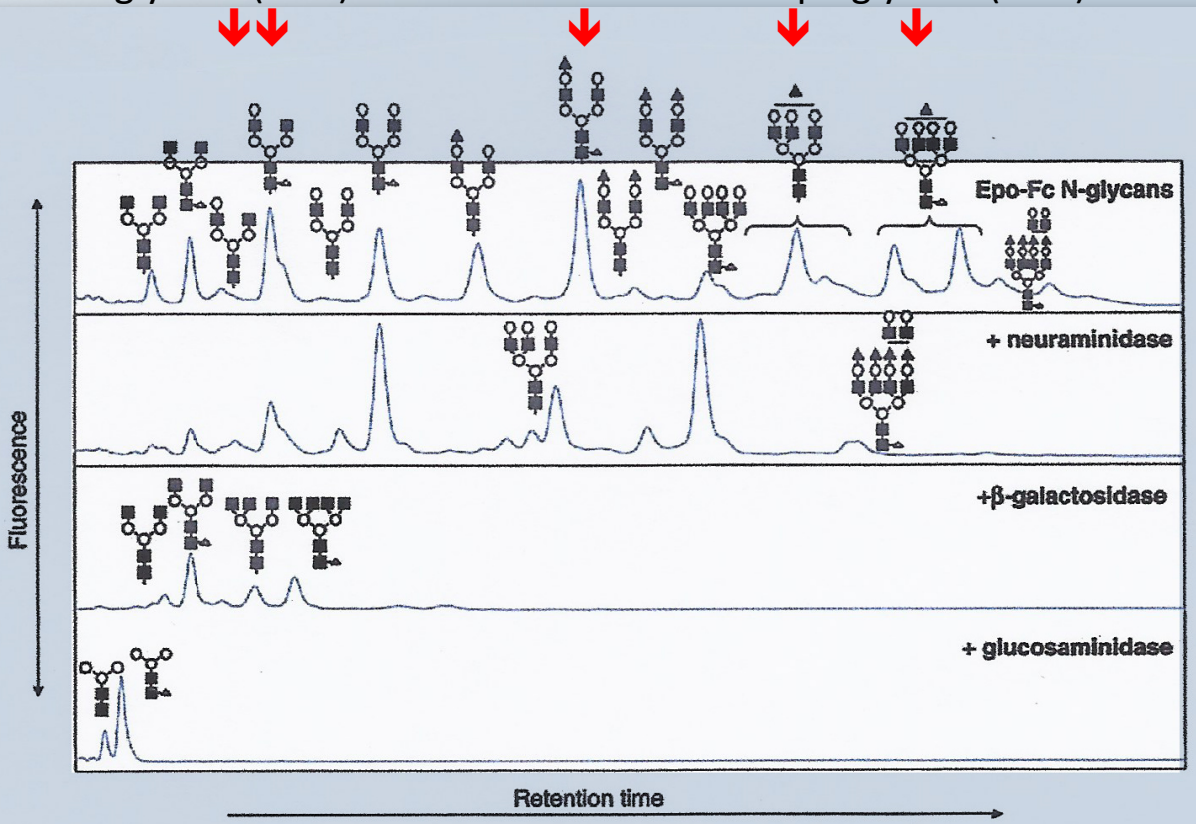
Glycans analyzed by HPLC

Schriebl *et al., Protein Exp. Purif.* **49**, 265-275 (2006)

Epo-domain glycans are sialylated

Predominant Fc glycans (44%)

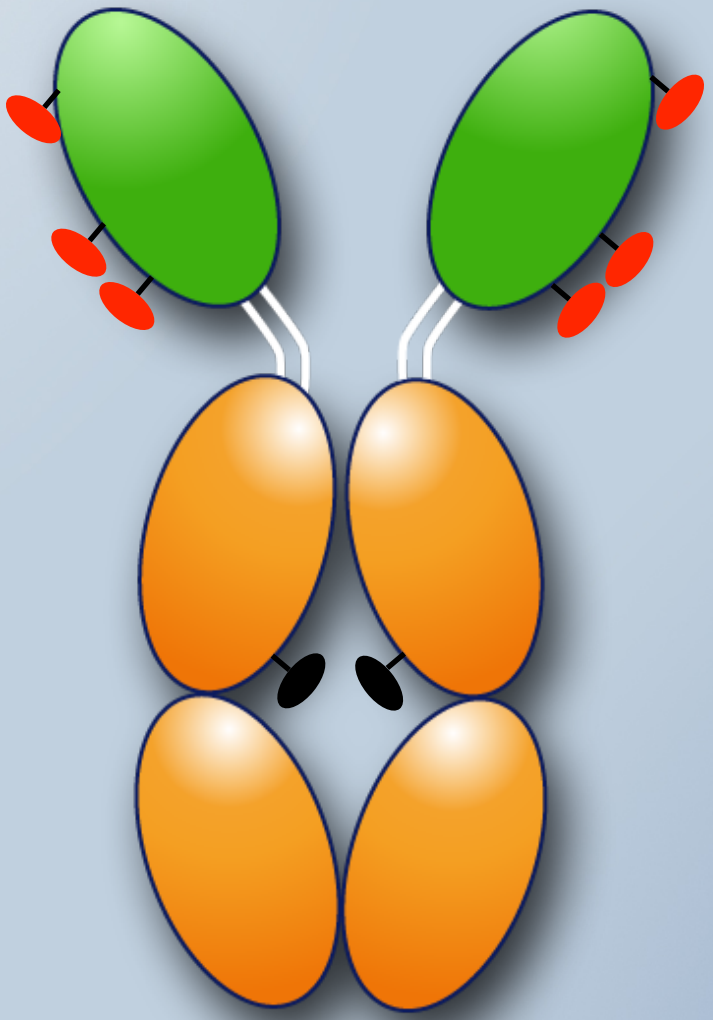
Predominant rhuEpo glycans (68%)



Schriebl *et al., Protein Exp. Purif.* **49**, 265-275 (2006)

Fig. 8. Sequential exoglycosidase digestions of 4-ABA labeled *N*-glycans released from rhEpo-Fc separated on normal-phase HPLC. The *N*-glycan concentration was approximately 440 pmol before cleavage. The chromatograms show the results of the sequential digestion, where the complexity of the *N*-glycan pattern is successively reduced.

Domains of Epo-Fc fusion protein have distinct N-glycans



N-linked, complex bi-, tri- and tetra-antennary containing terminal NANA

N-linked, complex bi-antennary containing terminal galactose

Schriebl *et al., Protein Exp. Purif.* **49**, 265-275 (2006)

Case Study 1: Summary

- Epo-Fc is a glycosylated homodimer
- The fusion protein is prone to aggregate
- The fusion protein can be cleaved by papain
- Glycans are microheterogeneous
- N-glycans of Epo and Fc domains are structurally distinct
- Epo domain N-glycans are sialylated while Fc domain glycans are not

Case Study 2:

Binding capacity of Fc-fusion proteins on Protein A

mAbs and Fc-fusion proteins were compared for binding to Protein A

Table I. Properties of molecules used in this study.

Molecule name	MW (kDa)	Type of molecule
A	144.3	Antibody (IgG2)
B	102.4	Fc-fusion of IgG1
C	92.1	Fc-fusion of IgG1
D	152.2	Fc-fusion of IgG1
E	146.6	Antibody (IgG1)
F	102.4	Fc-fusion of IgG1
G	144.0	Antibody (IgG2)

Ghose *et al., Biotechnol. Bioeng.* **96**, 768-779 (2007)

Breakthrough curves MabSelect® Protein A

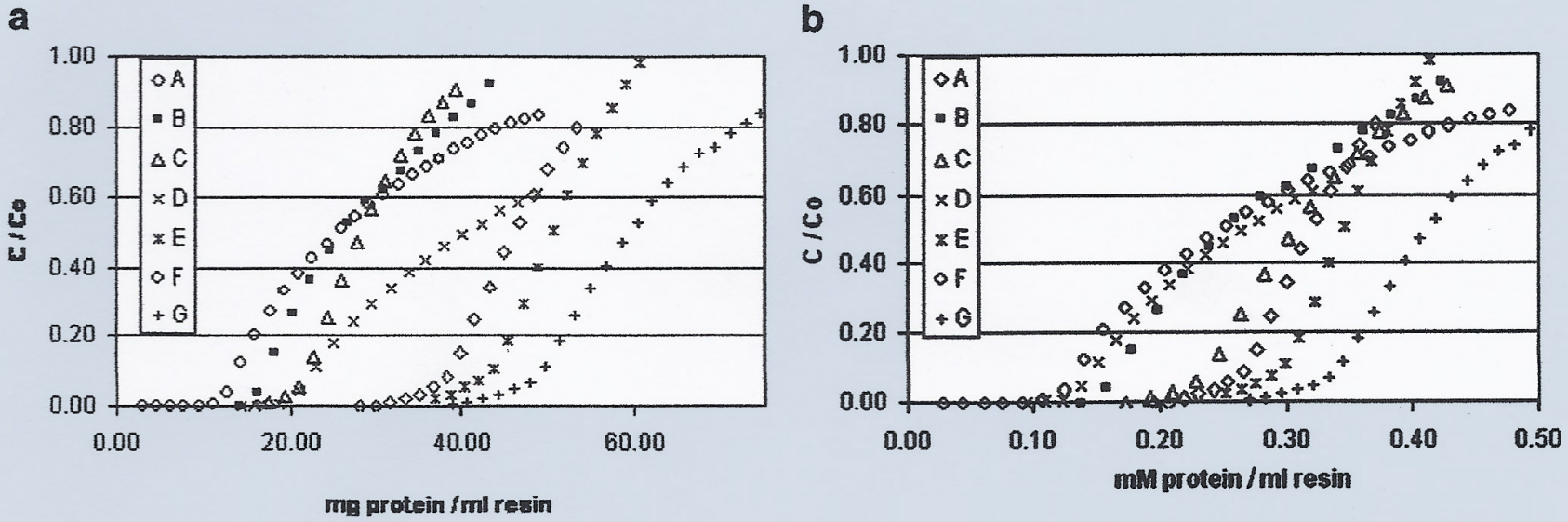


Figure 1. Breakthrough curves on MAbSelect® Protein A media at 5 min residence time. Column loading expressed in terms of (a) mg antibody/mL of resin (b) mM antibody/mL of resin.

Ghose *et al., Biotechnol. Bioeng.* **96**, 768-779 (2007)

Breakthrough curves: ProsepA HiCap® Protein A

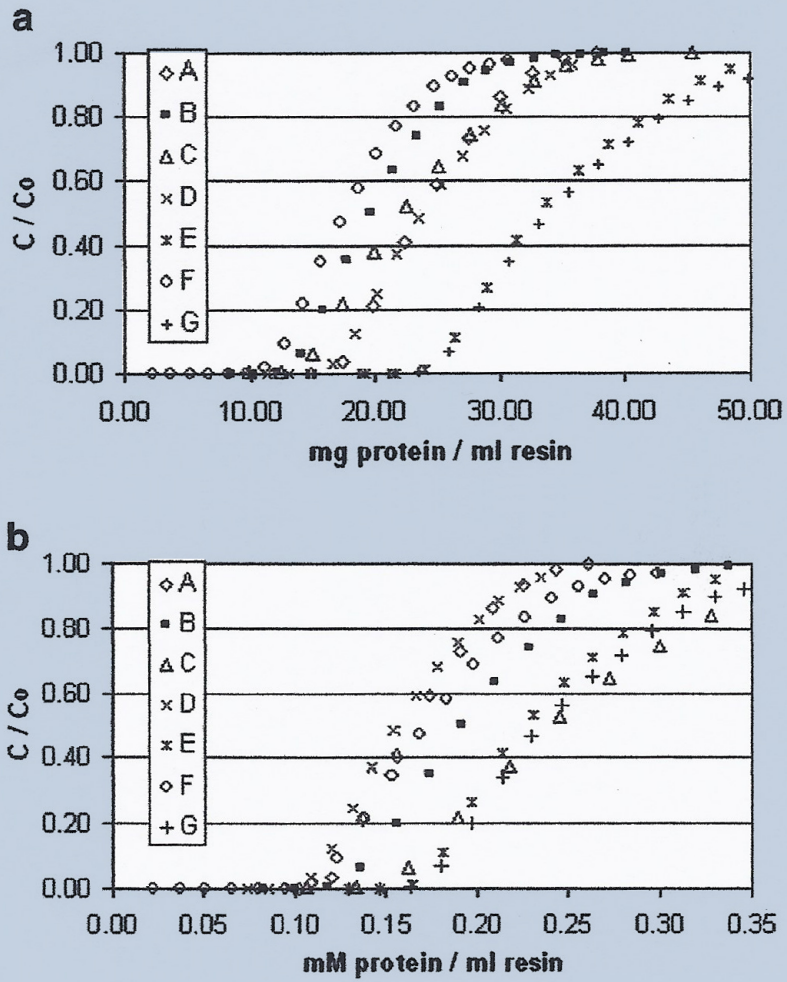


Figure 2. Breakthrough curves on ProsepA High Cap® Protein A media at 5 min residence time. Column loading expressed in terms of (a) mg antibody/mL of resin (b) mM antibody/mL of resin.

Ghose *et al.*, *Biotechnol. Bioeng.* **96**, 768-779 (2007)

Conclusion from breakthrough curves
Order of binding capacity

E=G > C=A > F=D=B

A	mAb
B	Fc fusion
C	Fc fusion
D	Fc fusion
E	mAb
F	Fc fusion
G	mAb

Dynamic binding capacity correlates inversely with molecular size

Fc fusion proteins B, D, and F have largest Stokes (hydrodynamic) radius

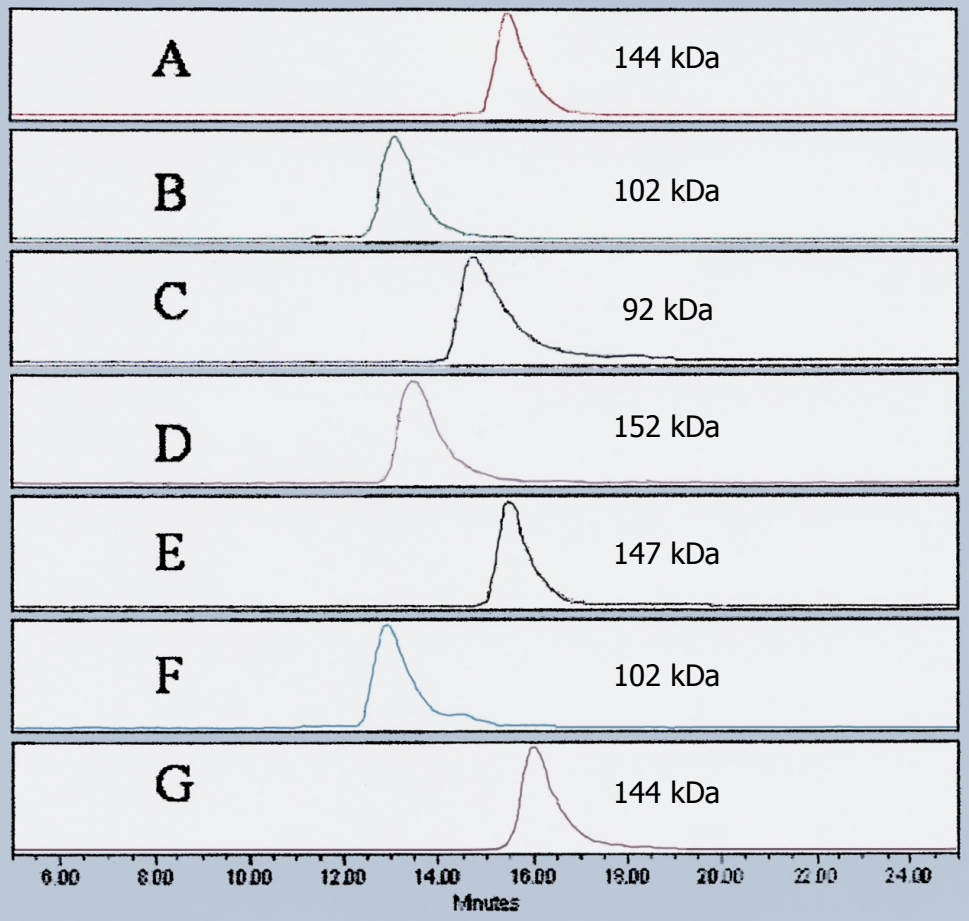


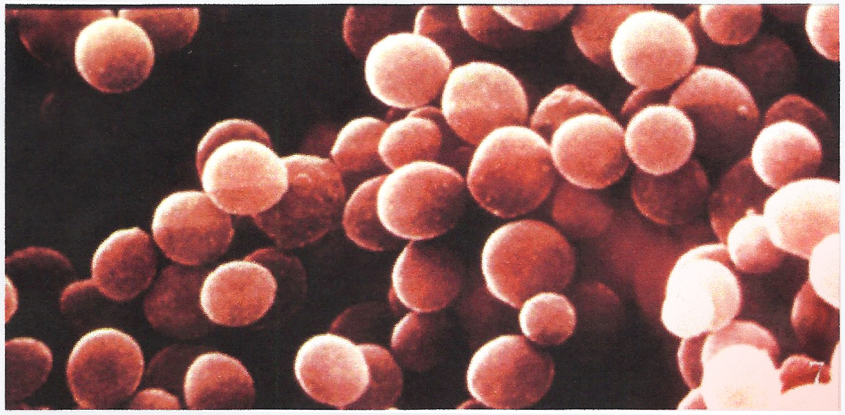
Figure 3. SEC chromatograms.

Ghose *et al.*, *Biotechnol. Bioeng.* **96**, 768-779 (2007)

Staphylococcus aureus Protein A

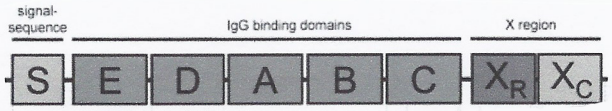
E, D, A, B, C = Ig-binding domains (58 residues ea)
X = C-terminal cell wall anchor
S = signal sequence

A

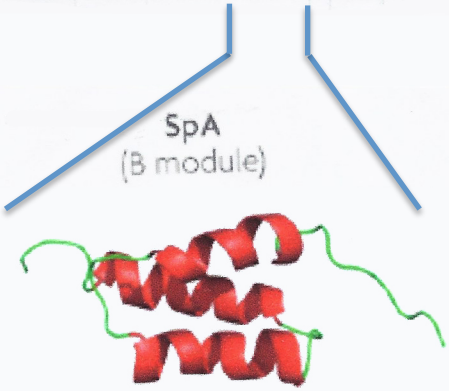


Staphylococcus aureus Electron micrograph from Visuals Unlimited, with permission.

B



C



Tashiro and Montelione, *Curr. Opin. Struct. Biol.* **5**, 471-481 (1995)

Potential stoichiometry = 5:1 molar ratio Fc:SpA

Dynamic binding capacity correlates with stoichiometry of binding to Protein A

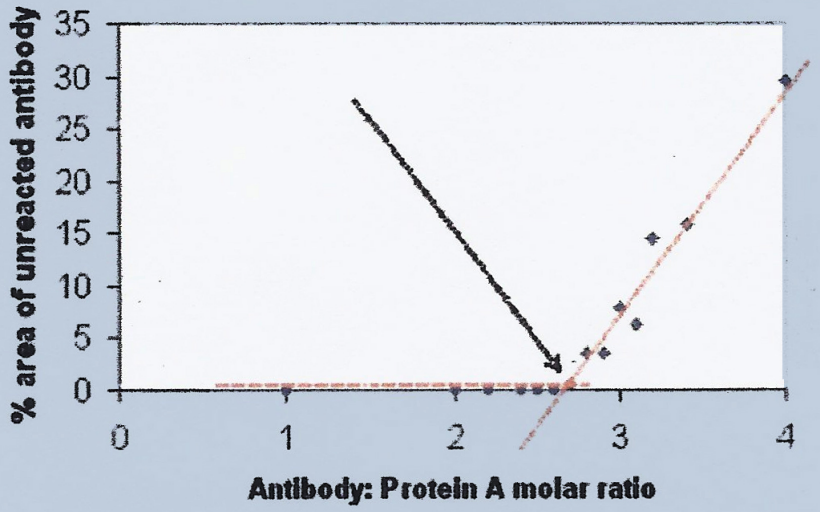


Figure 4. Calculation of solution binding stoichiometry between Protein A and a monoclonal antibody or Fc fusion protein.

Table IV. Solution binding stoichiometry data for the various molecules with Protein A.

Molecule name	Number of molecules bound/molecule of Protein A
A	2.54 ± 0.08
B	2.62 ± 0.04
C	2.4 ± 0.04
D	2.82 ± 0
E	3.05 ± 0.05
F	2.6 ± 0.09
G	3.1 ± 0.05

Ghose *et al.*, *Biotechnol. Bioeng.* **96**, 768-779 (2007)

Summary of data

ID	Type	Binding capacity	MW (kDa)	Size (Stokes radius) from SEC	Stoichiometry from solution binding
A	mAb	Intermediate	144	Small	Low
B	Fc fusion	Low	102	Large	Low
C	Fc fusion	Intermediate	92	Intermediate	Low
D	Fc fusion	Low	152	Large	Intermediate
E	mAb	High	146	Small	High
F	Fc fusion	Low	102	Large	Low
G	mAb	High	144	Small	High

Case Study 2: Summary

- A total of seven Ig molecules were tested for binding capacity on Protein A
  - 3 mAbs
  - 4 Fc fusion proteins
- Not all Ig-binding domains of Protein A are available for use
  - Total of five Ig-binding domains
  - 2-3 of these are used
- MW does not necessarily correlate with Stokes radius (hydrodynamic size in solution) for Fc fusion proteins
- Binding capacity was found to correlate with size, stoichiometry
  - Inverse correlation with Stokes radius
  - Direct correlation with stoichiometry of binding to Protein A

Conclusions

- Fc fusion proteins are an important and growing therapeutic drug class
- They are a versatile means of converting a wide variety of biomolecules into practical therapeutics
  - Extended *in vivo* half-life
  - Link to immune effector functions
- Currently 7 approved Fc fusion proteins in USA

Thank you!