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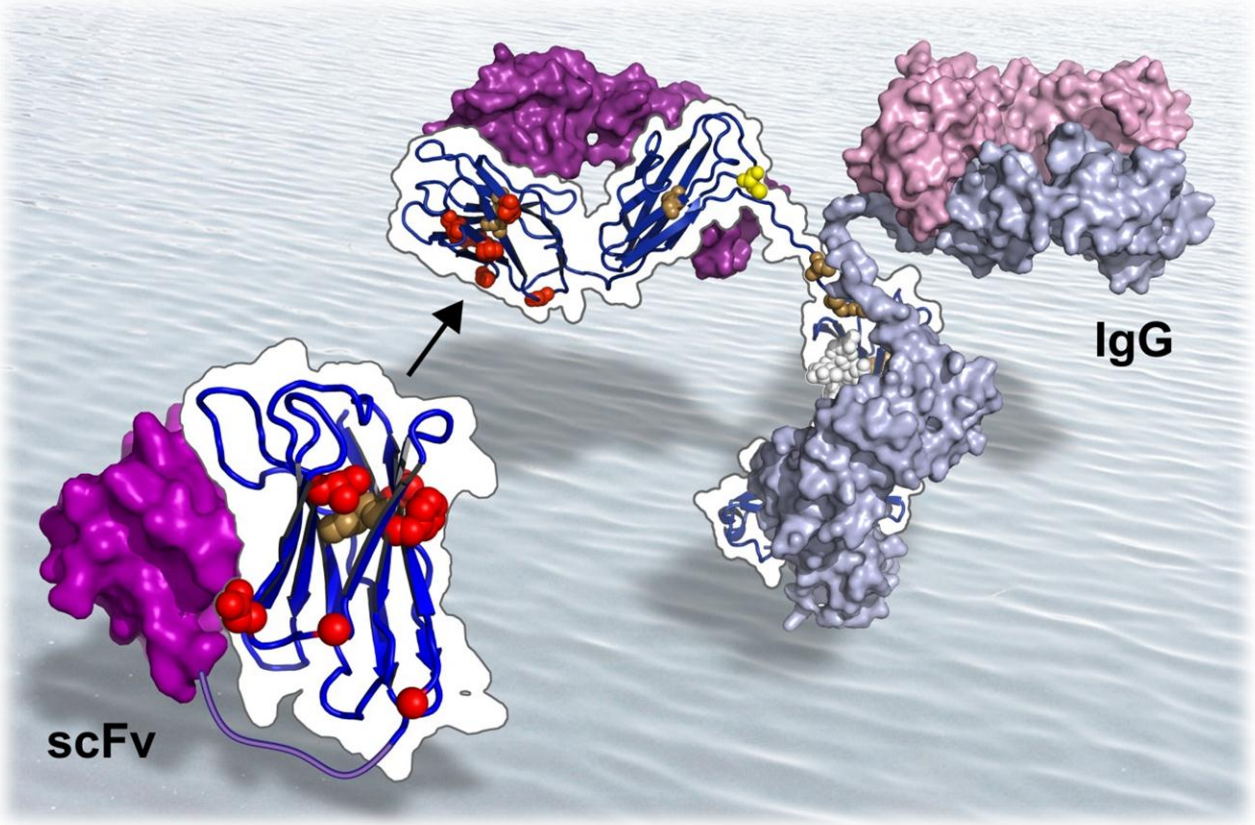
Optimize 2013
ANTIBODY ENGINEERING & DISCOVERY

February 27th, 2013

Troubleshooting and Engineering of Antibody Constructs – part II



Jonas V. Schaefer, PhD Biochemistry Department, University of Zurich

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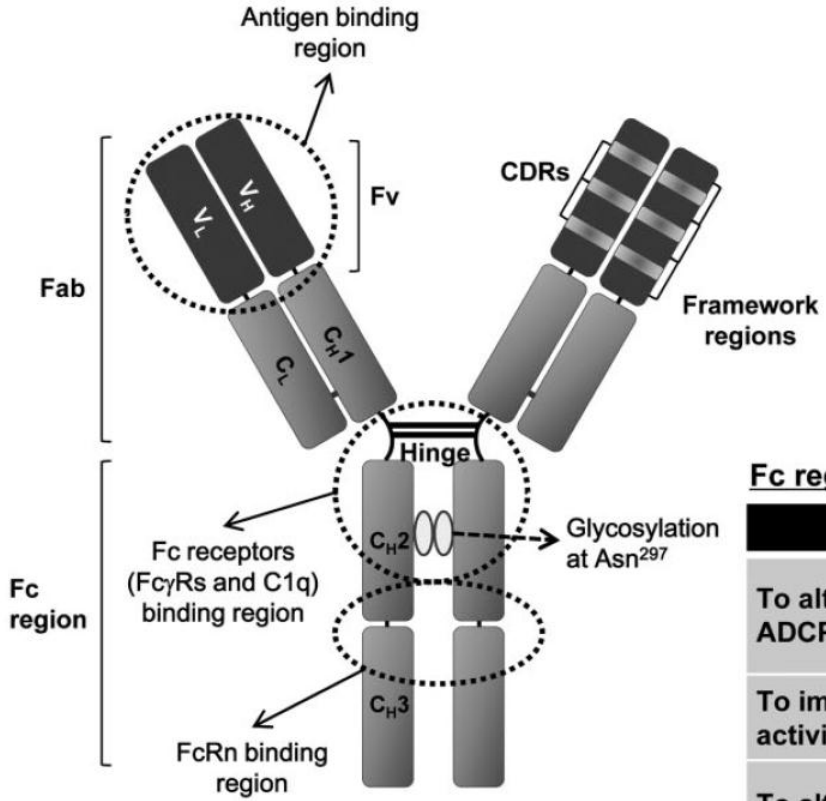
Engineering of full-length IgG



Variable region

Objectives of Engineering	Strategies of Engineering
To modulate Antigen binding affinity or specificity	• Random mutation in CDRs and high throughput screening • Rationale design in CDRs
To reduce immunogenicity	• Humanization or dehumanization of framework regions
To decrease elimination of IgG	• Mutation of variable region to lower the isoelectric point

Fc region

Objectives of Engineering	Strategies of Engineering
To alter effector functions (ADCC, ADCP and CDC)	• Sequence alteration in Fc region • Modified glycosylation (non fucosylation, Aglycosylation)
To improve an anti-inflammatory activity	• Introduction of sialylated glycans
To alter half-life (pharmacokinetic)	• Sequence alteration in Fc regions (FcRn binding region)
To construct antibody based drugs or toxins To label a radioisotope	• Fc fusion to target molecules • Introduction of Cys residue

Jeong *et al.*, *Biotechnol J.*(2011)

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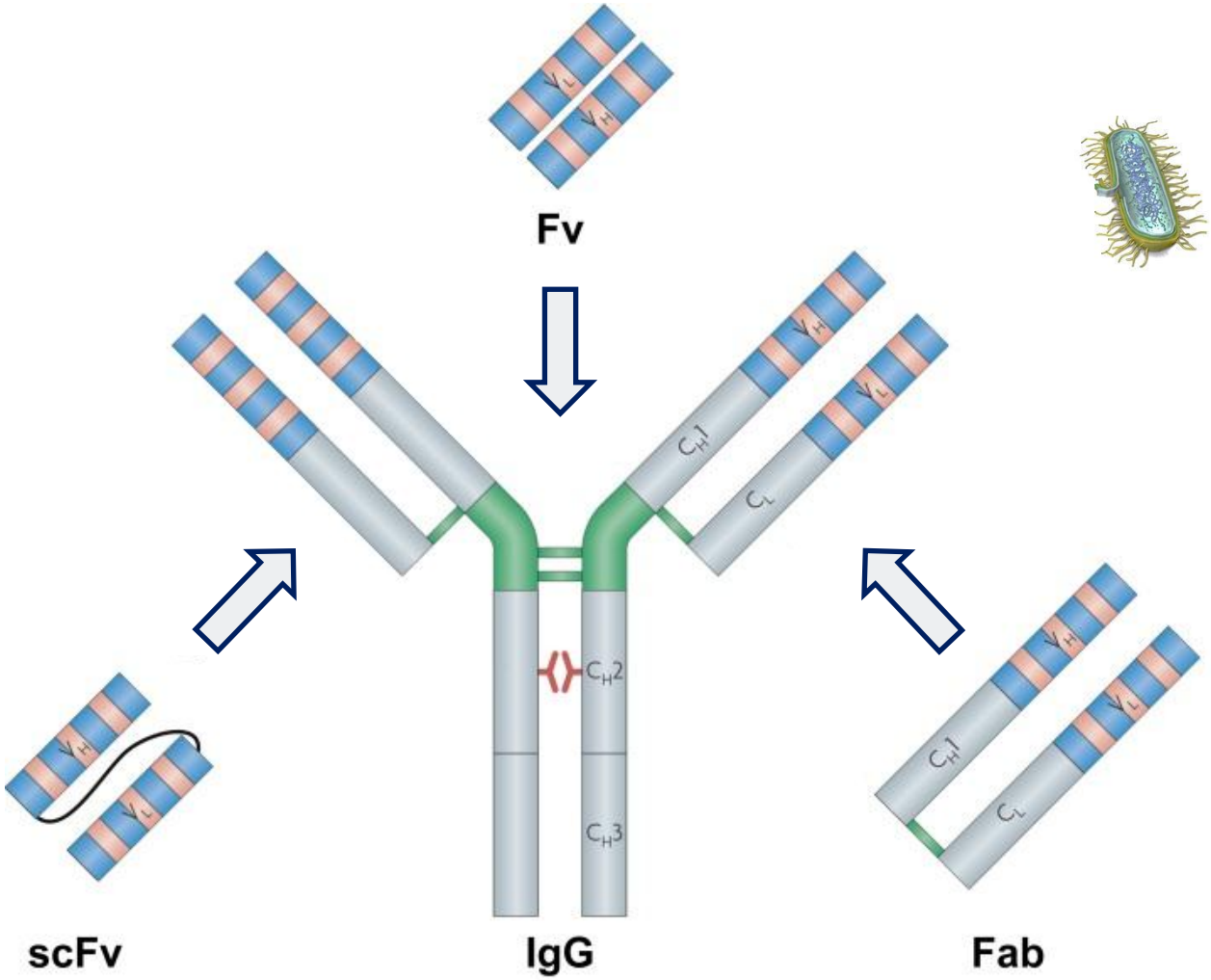
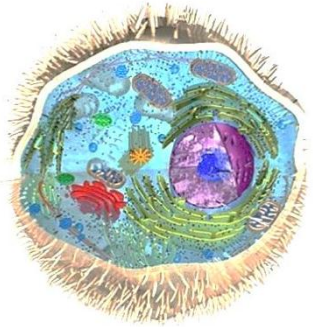
Are previous findings transferable?





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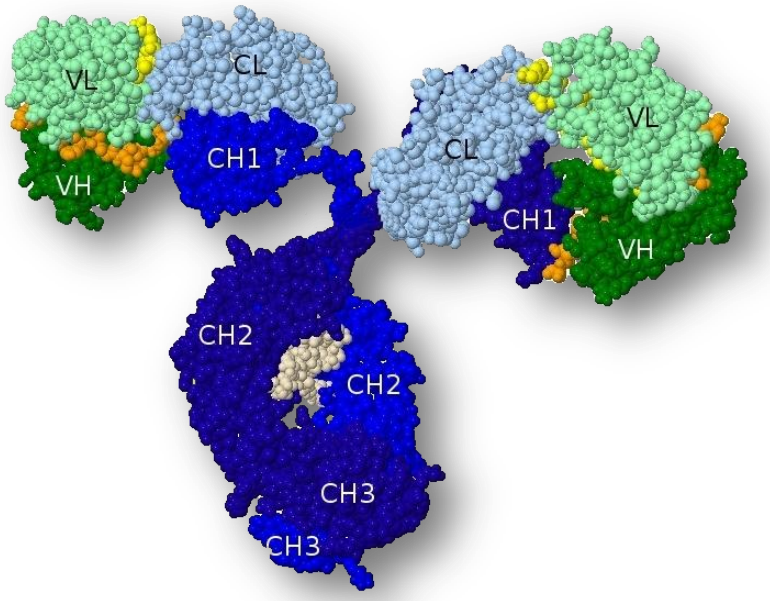
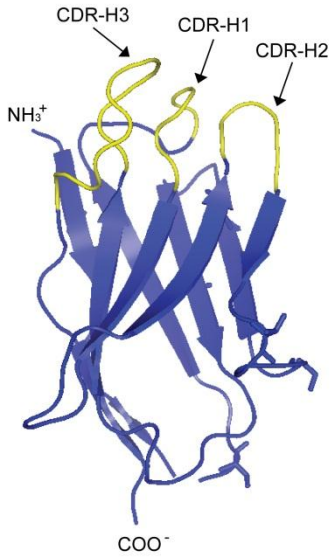
Analytical challenge: Multidomains

- IgGs consist of **six individual domains (each in duplicates)**, all having similar folds





- with most experimental setups, only **overall average** of biophysical features will be analyzed

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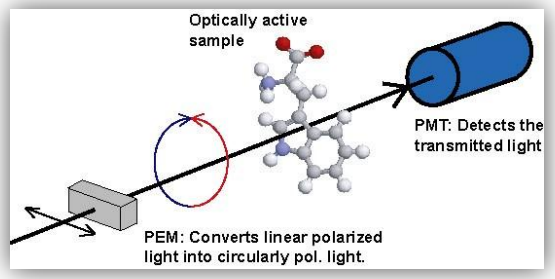
Biophysical analyses (methodology)

Circular Dichroism (CD)

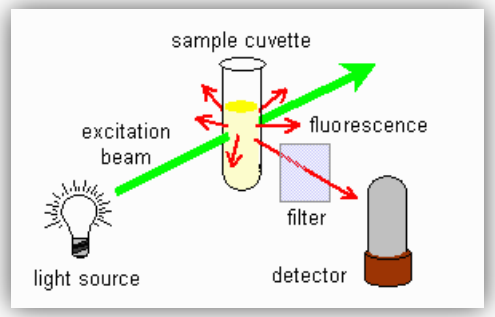


(2ry structure composition)

thermal denaturation

(aggregation analysis)

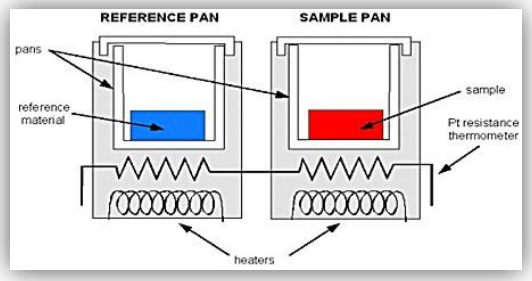
Intrinsic Tryptophan Fluorescence (ITF)

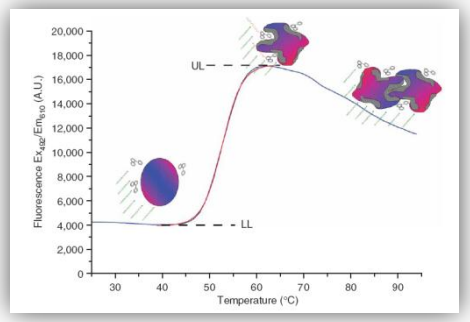


thermal denaturation

chemical denaturation

Differential scanning calorimetry / fluorimetry





analysis of individual domains

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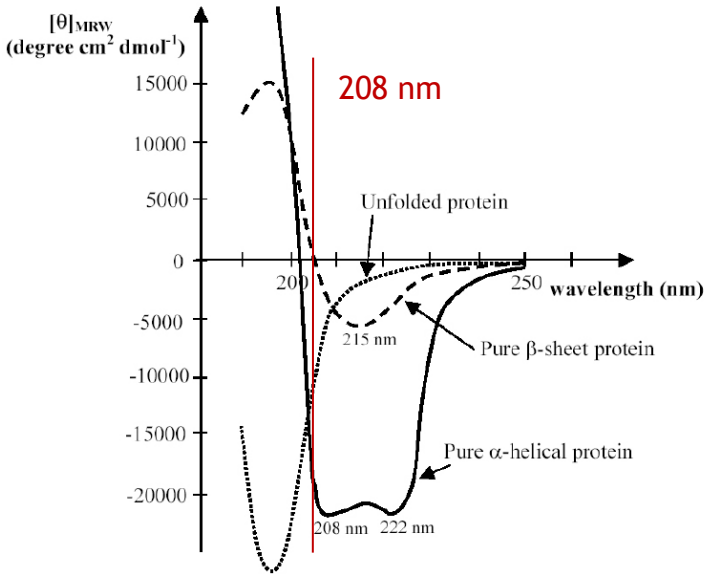
Circular Dichroism (CD)

Lambert-Beer derivative: $\Delta A = A_L - A_R = \varepsilon_L \times l \times C - \varepsilon_R \times l \times C = \Delta\varepsilon \times l \times C$

elipticity: $\theta = \frac{2.303\ (A_L - A_R)}{4l}$

MRE: $[\theta] = \frac{\theta \times 100 \times M}{C \times l \times n}$

amide chromophore of peptide bond has 2 electronic transitions of low energy: **n → π*** (signals at 222 nm and 215 nm) and **π →π*** (signals at 208 nm and 198 nm)



- at ~ **208 nm** intensity due to ß-sheets is essentially zero
- measuring ellipticity at 208 nm monitors **changes in structure** (negative shift caused by random coil formation)

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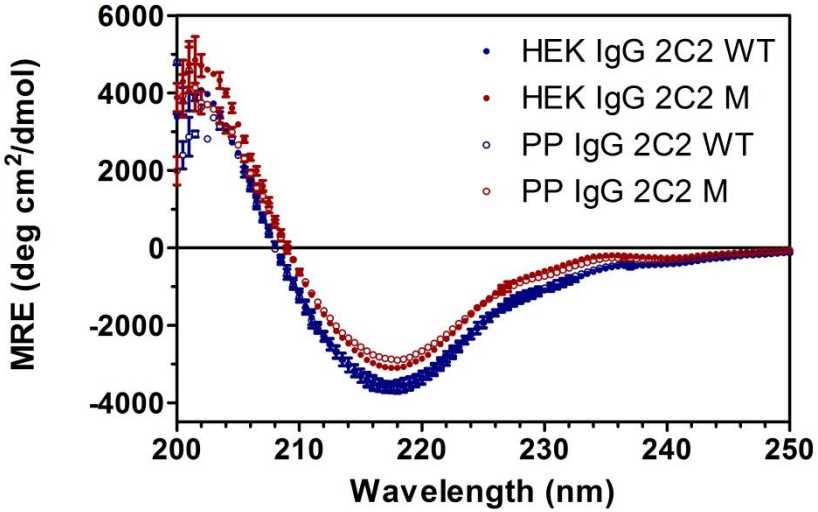
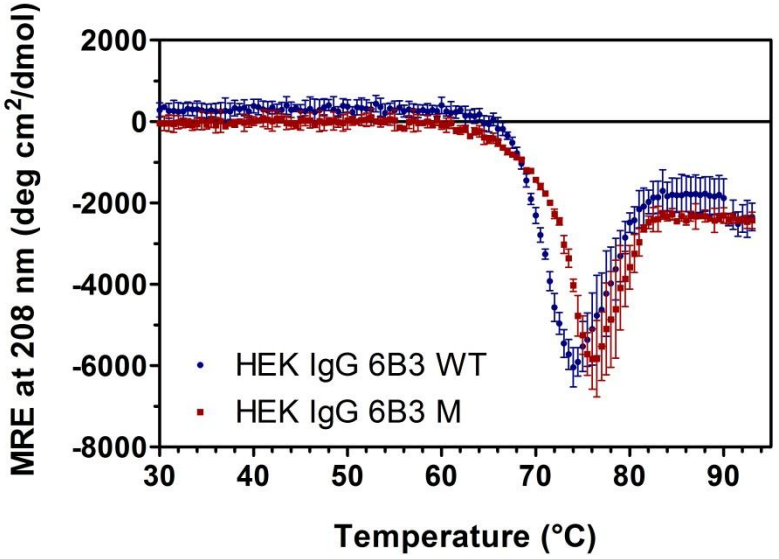
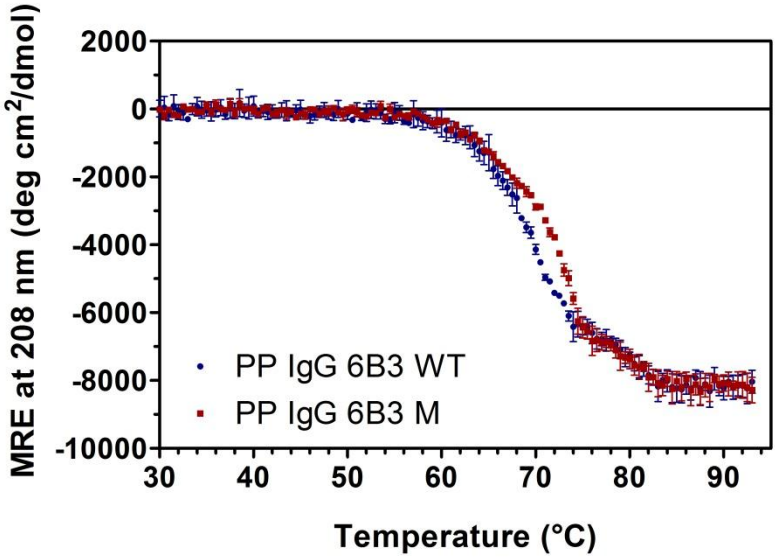
CD: real examples



➡ unfolding detectable, however sheaded by aggregation





Schaefer and Plückthun, *J. Mol. Biol.* (2012)

Schaefer and Plückthun, *Protein Eng. Sel. Des* (2012)

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Intrinsic Tryptophan Fluorescence (ITF)

Trp fluorescence is very sensitive to local conformation and environment

Quantum yields:

Phe – 0.02
Tyr – 0.13
Trp – 0.12

IgG 2C2: 24 Trp per IgG

IgG 6B3: 26 Trp per IgG

IgG 6B3

Domain	# of Trp	% of all Trp
V_H	5	38.5
CH_1	1	7.7
CH_2	2	15.4
CH_3	2	15.4
V_L	1	7.7
CL	2	15.4

IgG 2C2

Domain	# of Trp	% of all Trp
V_H	5	41.7
CH_1	1	8.3
CH_2	2	16.7
CH_3	2	16.7
V_L	1	8.3
CL	1	8.3

➡ **majority of Trp residues are located within V_H domain**

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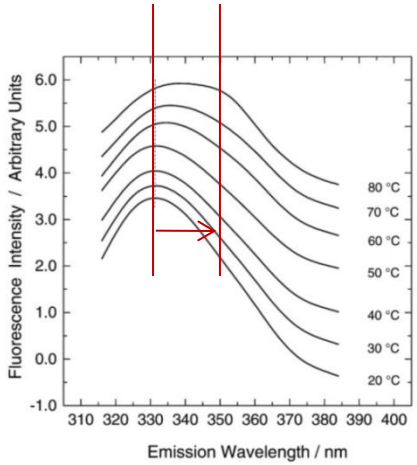
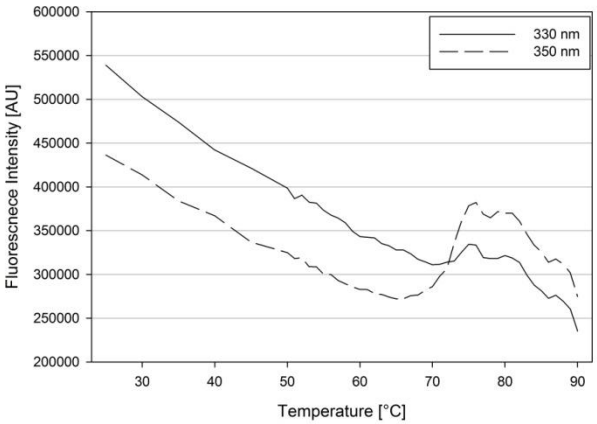
Intrinsic Tryptophan Fluorescence (ITF)

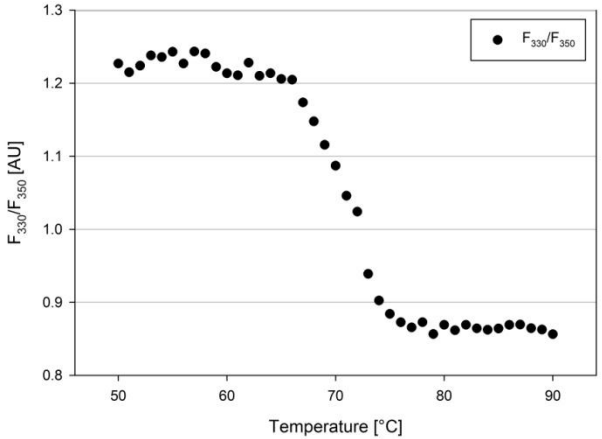
Trp fluorescence is very sensitive to local conformation and environment

wavelength maximum shifts upon heating due to changes of polarity in vicinity of Trp (**red-shift** of Trp emission spectrum)

red shift can be monitored by **ratio of intensities at 330 and 350 nm**







➡ **<u>benefit over other methods</u>:**
- aggregation doesn't cover unfolding reaction
- can easily be performed in plate reader

Method described in Garidel *et al.*, *Biotechnol. J.*(2008) **3**, 1201-11

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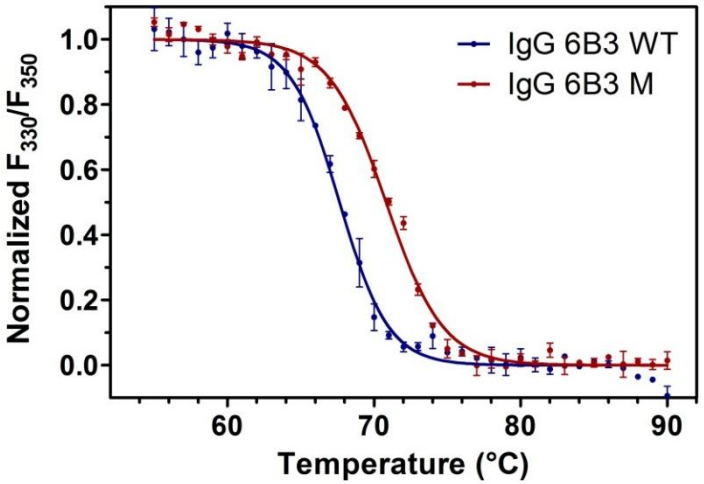
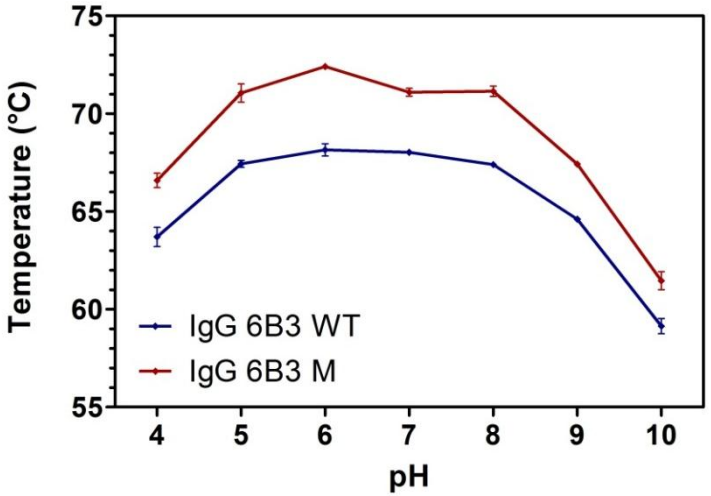
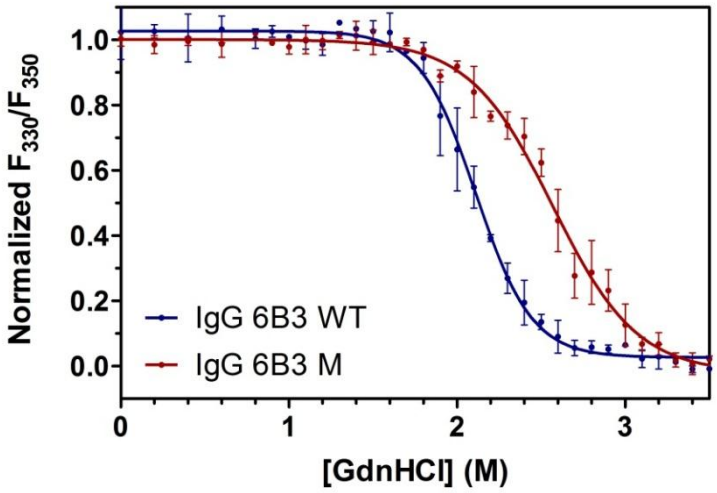
ITF: real examples

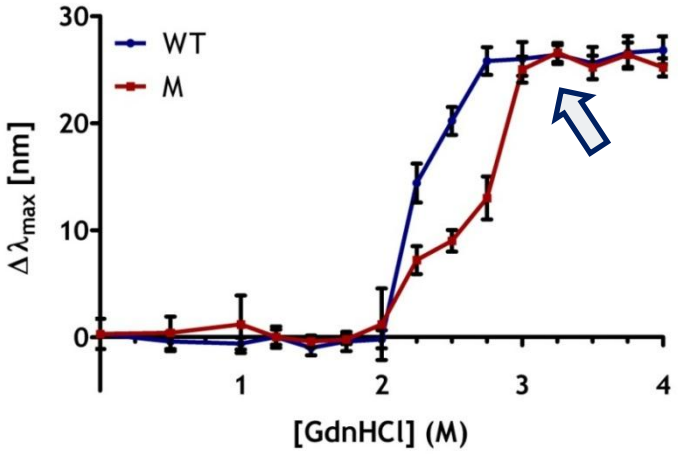
Temperature-unfolding





GdnHCl-unfolding





Schaefer and Plückthun, *Protein Eng. Sel. Des* (2012)

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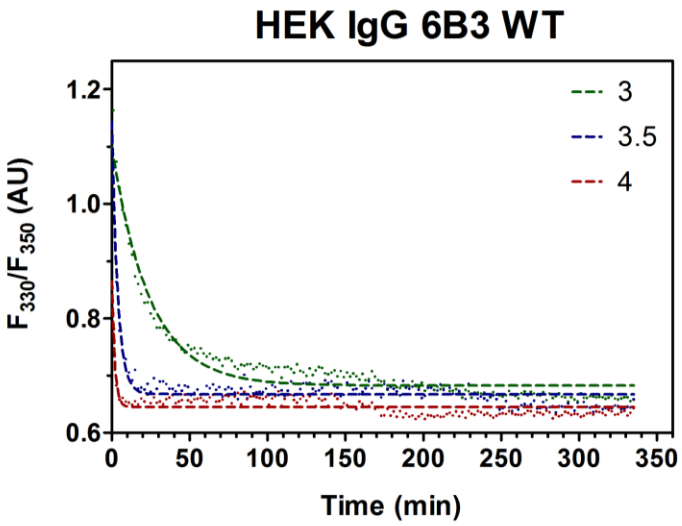
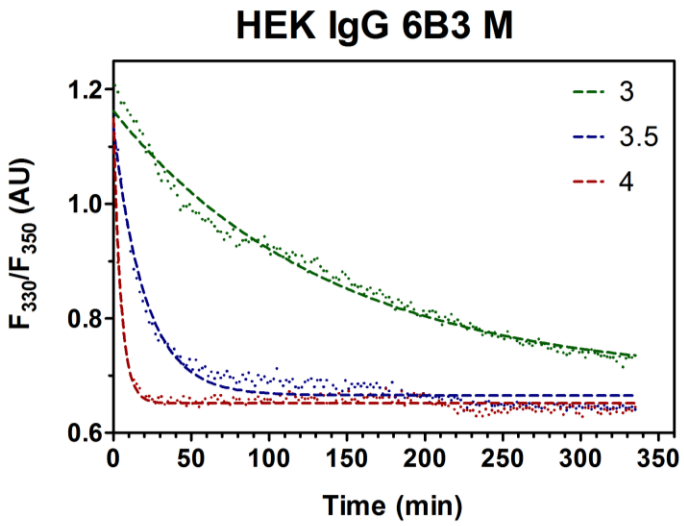
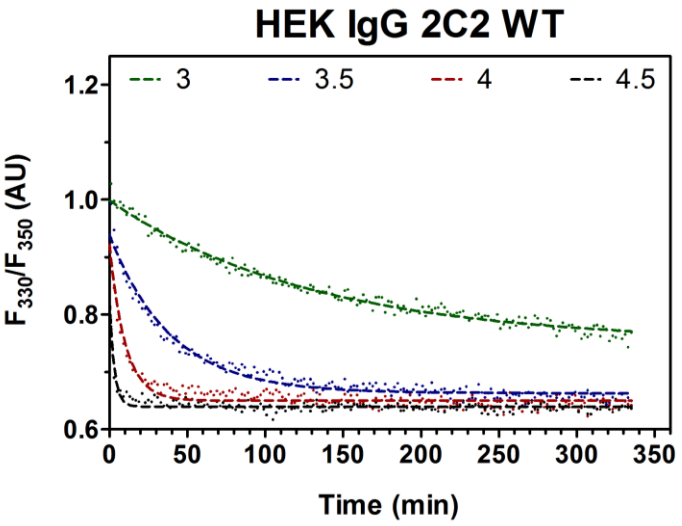
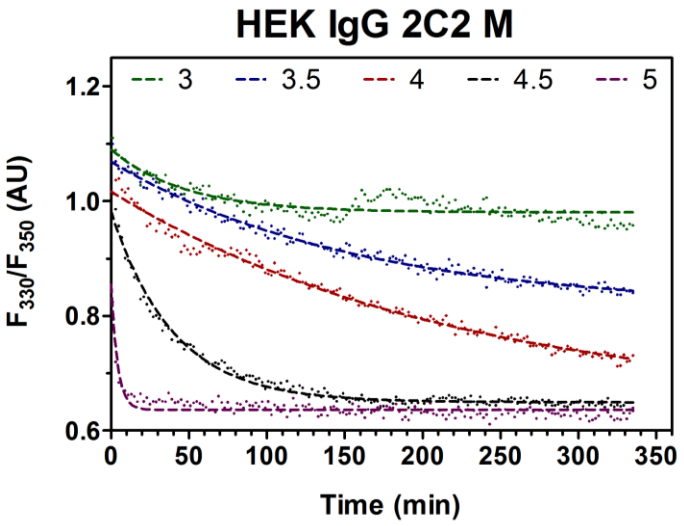
Real-time GdnHCl denaturation



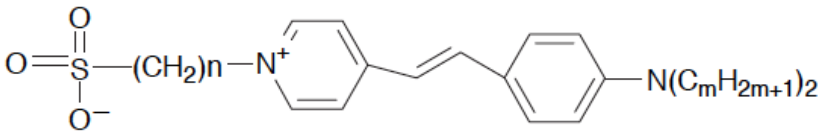




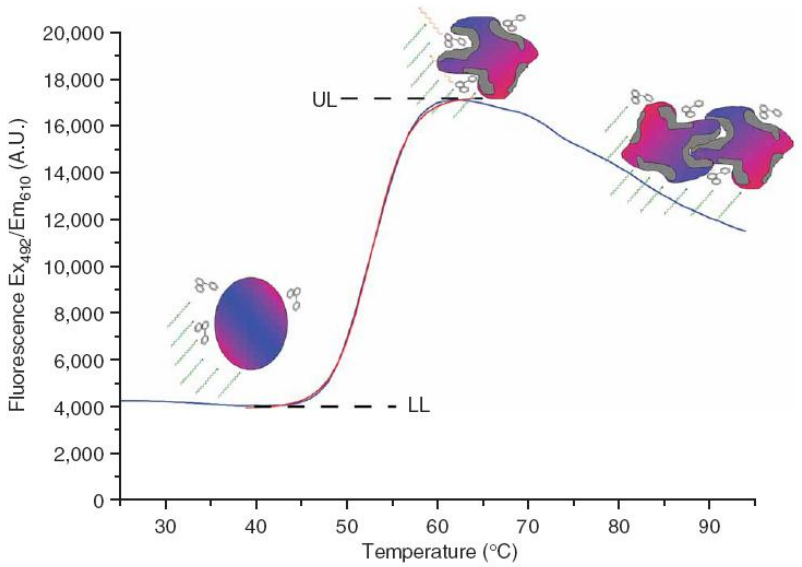


Differential Scanning Fluorimetry (DSF)

melting temperature detected by increased fluorescence of dye with **affinity for hydrophobic parts** of the protein

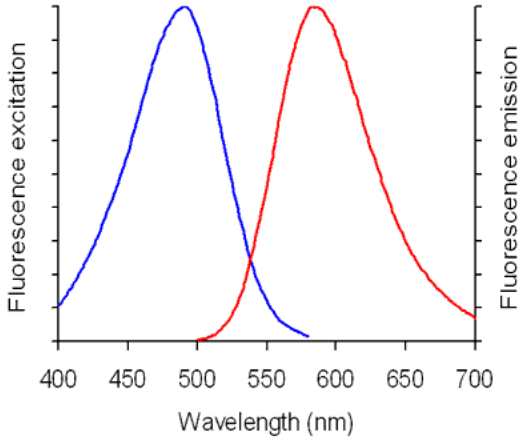


Sypro-Orange (Molecular Probes)



in aqueous solution: quenched fluorescence; highly fluorescent in non-polar environment

relatively **high excitation wavelength** decreases likelihood of small molecules interfering with optical properties of dye, causing quenching of fluorescence intensity



Method described in Niesen *et al.*, *Nature Protocols* (2007)

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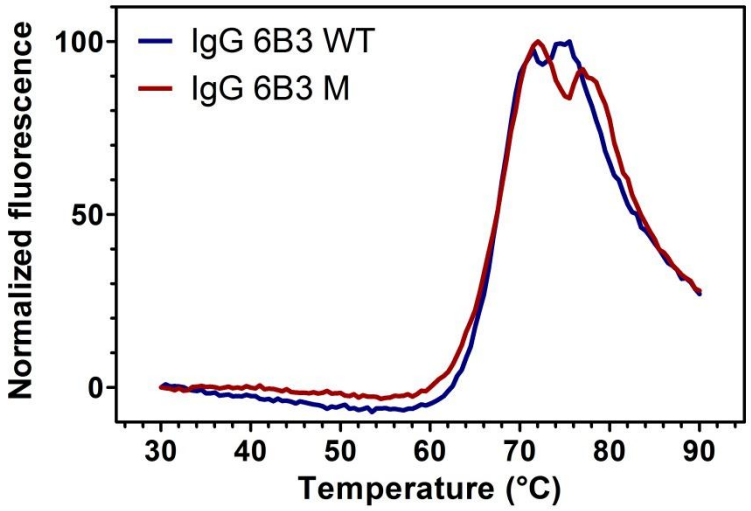
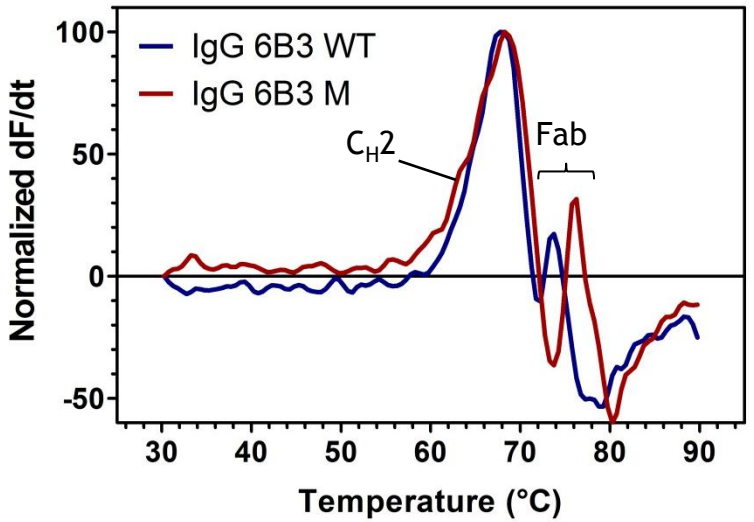
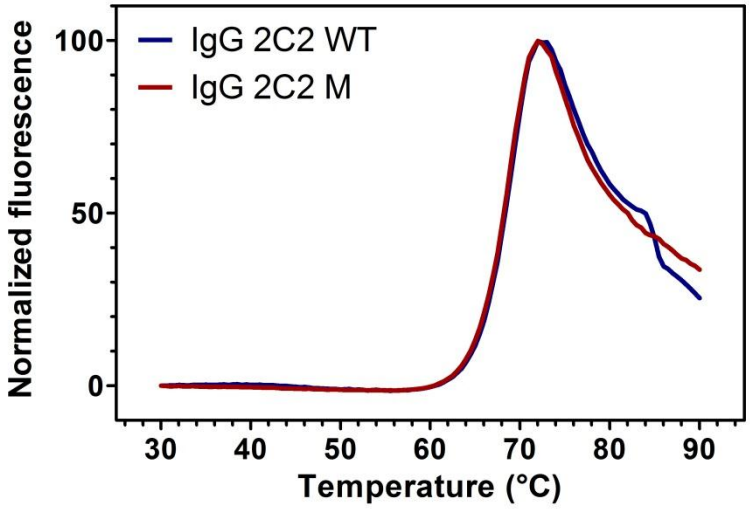
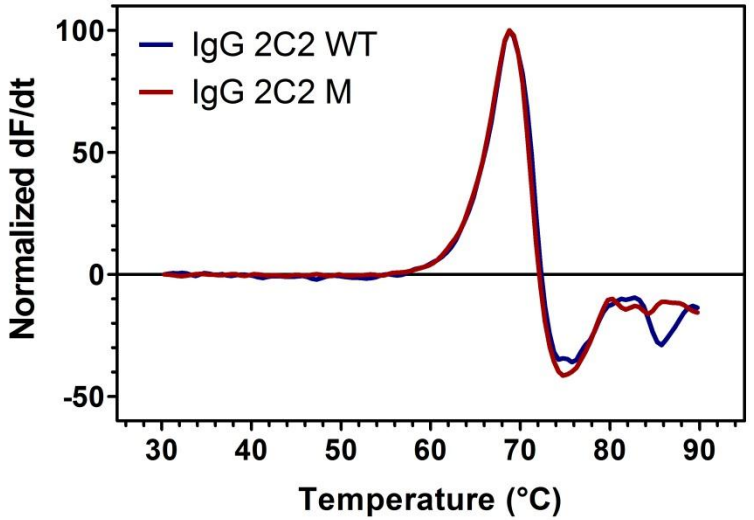
DSF: real examples





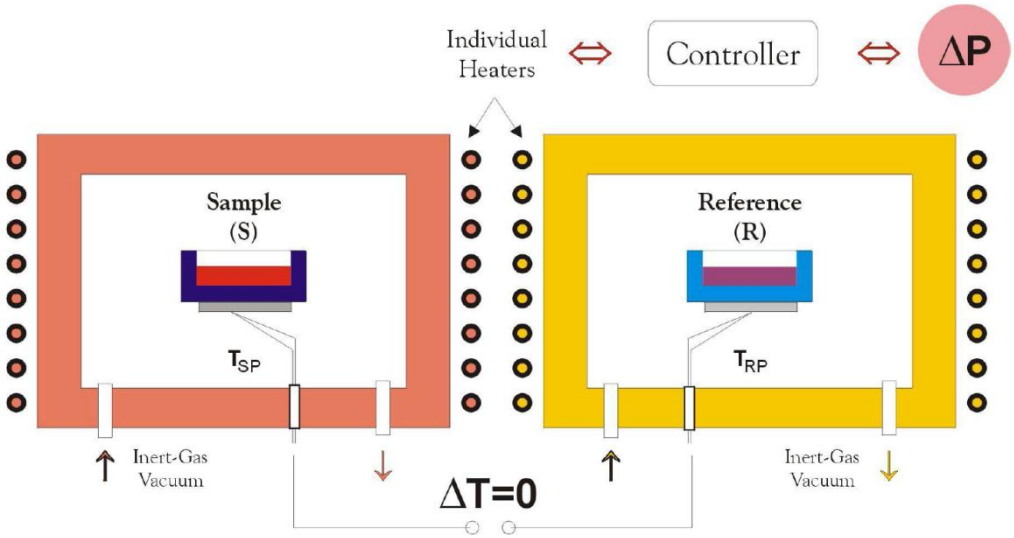




Schaefer and Plückthun, *Protein Eng. Sel. Des* (2012)

Differential Scanning Calorimetry (DSC)

Power-compensation DSC (not Heat-flux DSC)



continuously self-adjustment of heating power for keeping sample and reference at same temperature

difference of required **power [J/sec]** divided by the **scan rate [°C/sec]** leads to **heat capacity [J/°C]**

Integration of heat capacity *vs.* temperature yields the **enthalpy (ΔH)**

$$\Delta H = \int_{T1}^{T2} C_p \, dT$$

$$\Delta G = \Delta H - T \cdot \Delta S$$

(Gibbs Free Energy equation)

Methods described in Ionescu *et al.*, *J. Pharm. Sci.* (2008)

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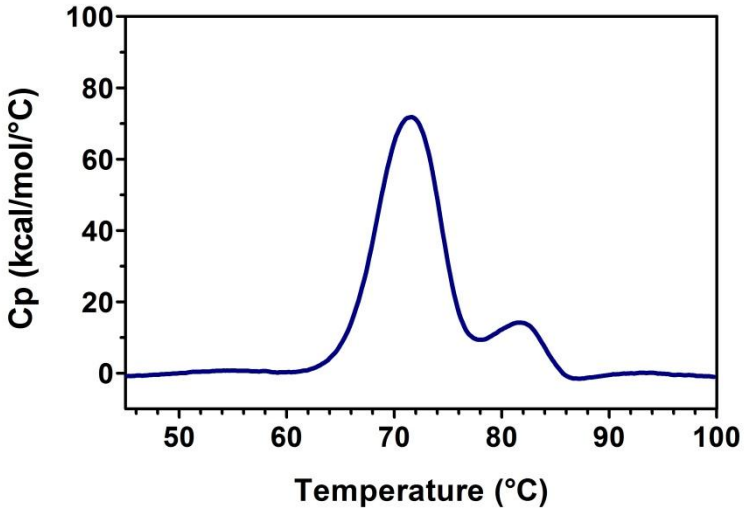
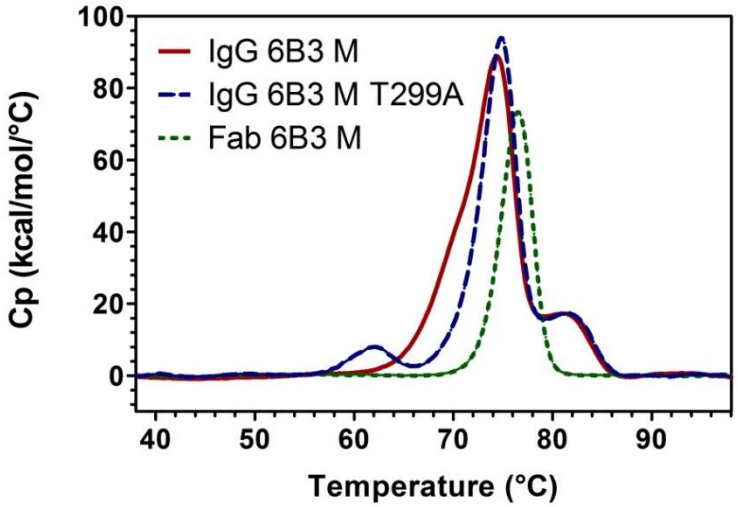
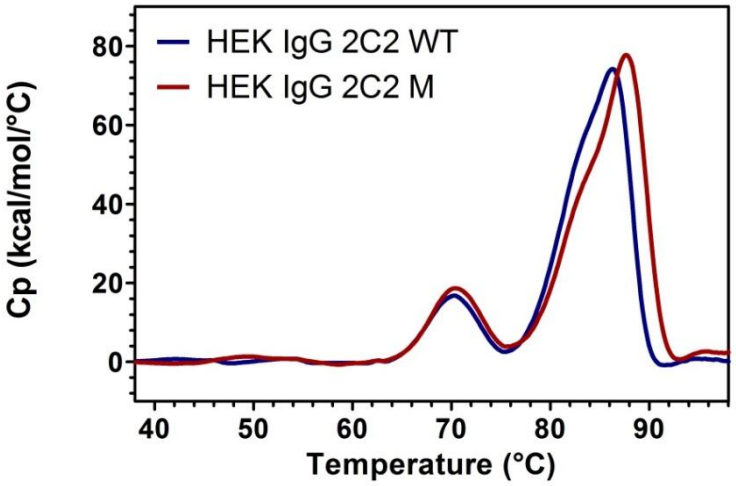
DSC: real examples







➡ DSC is only setup detecting small differences of very stable transition

Schaefer and Plückthun, *Protein Eng. Sel. Des* (2012)

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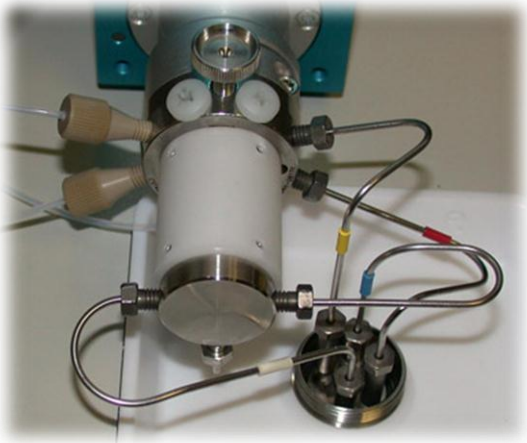
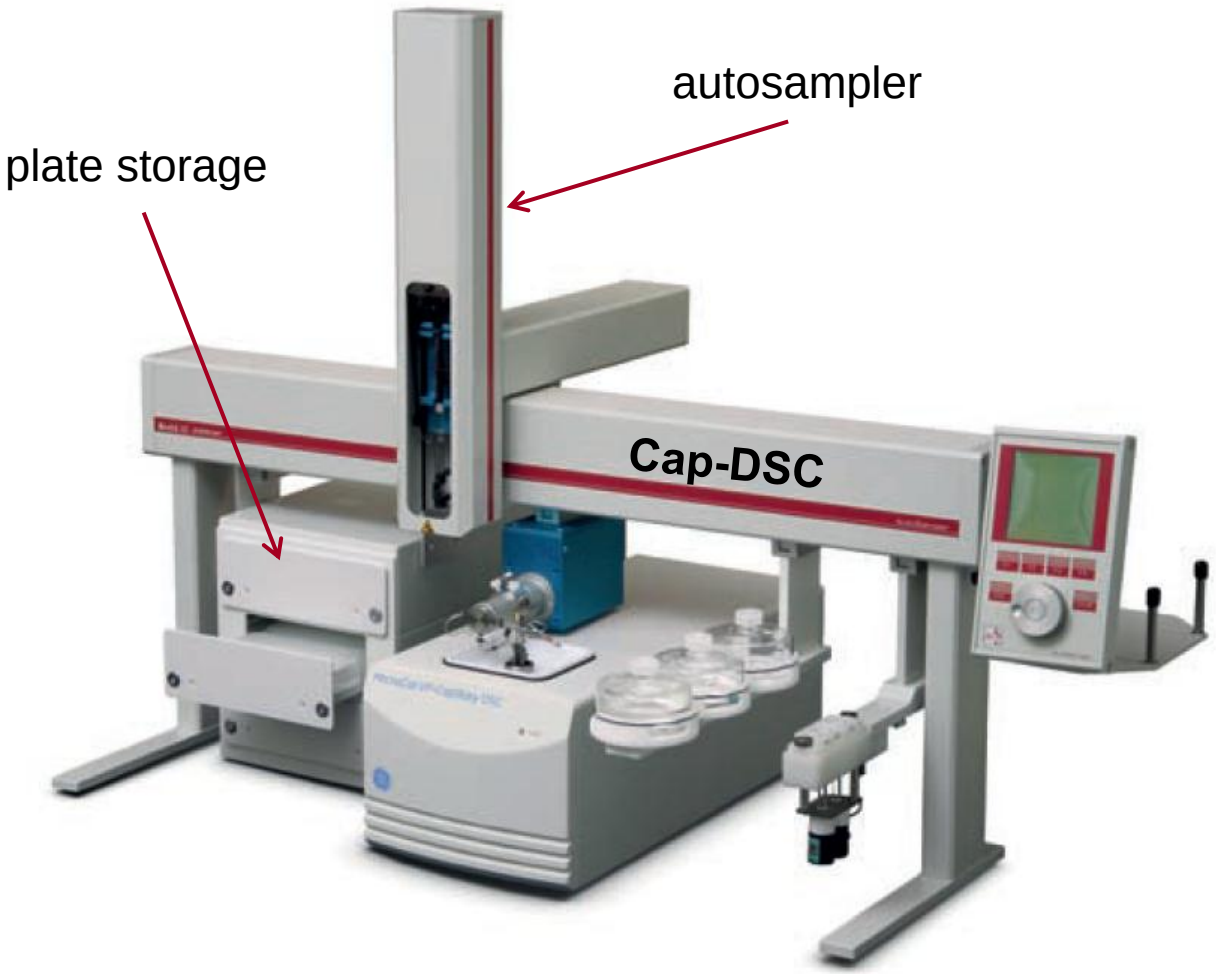
VP-DSC vs. VP-Capillary DSC





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VP-DSC vs. VP-Capillary DSC

	VP-DSC	VP-Capillary DSC
analyzed volume	510 µl	130 µl
sample volume	1´200 µl	400 µl
scan rates	0.5 - 1.5 °C/min	0.16 - 4°C/min
sample cell	coin shaped	capillary
samples	1	up to 288
measuring time	1 day	4 hrs
cleaning	manual	automatic

major advances: sensitivity, throughput, reproducibility, stability and ease of use (smaller sample requirements)

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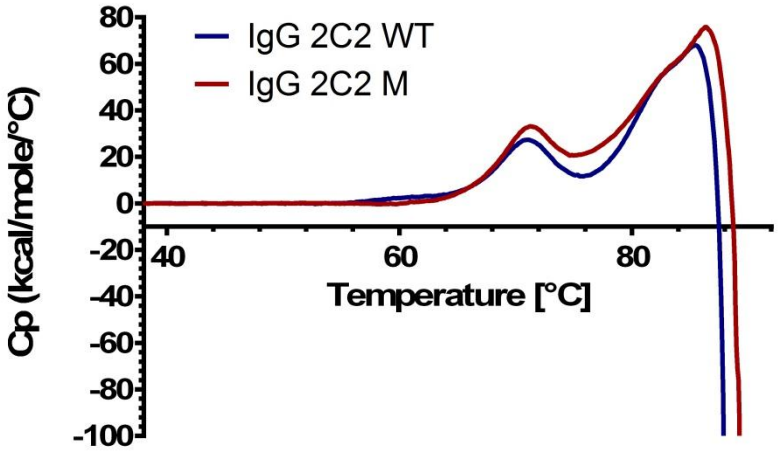
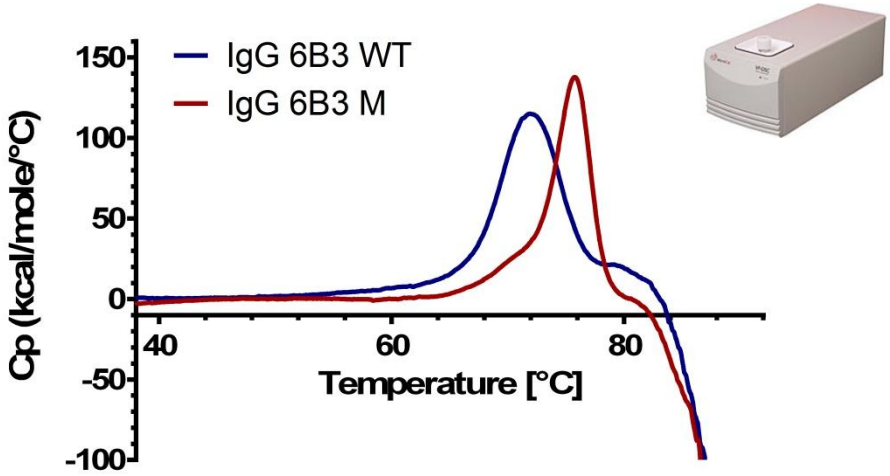
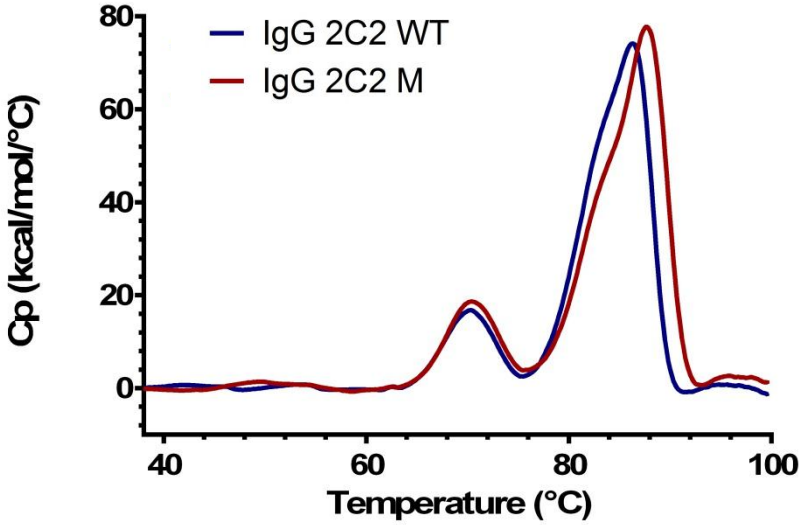
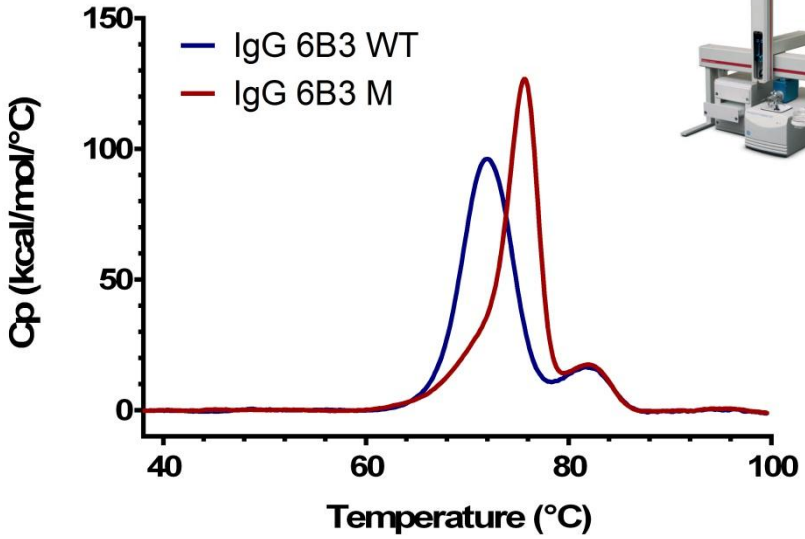
VP-DSC vs. VP-Capillary DSC









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Convection at aggregation

protein aggregation: heat signal detected by DSC is sum of both **endothermic unfolding** and **exothermic aggregation**

convection appears

once sample aggregates, interferance and baseline drop

molecules are located in small confined space

very little convection

due to small diameter of capillaries

molecules are separated with enough space (aggregation delayed)

➡ signals derived from Capillary-DSC are **less sensitive to aggregation**

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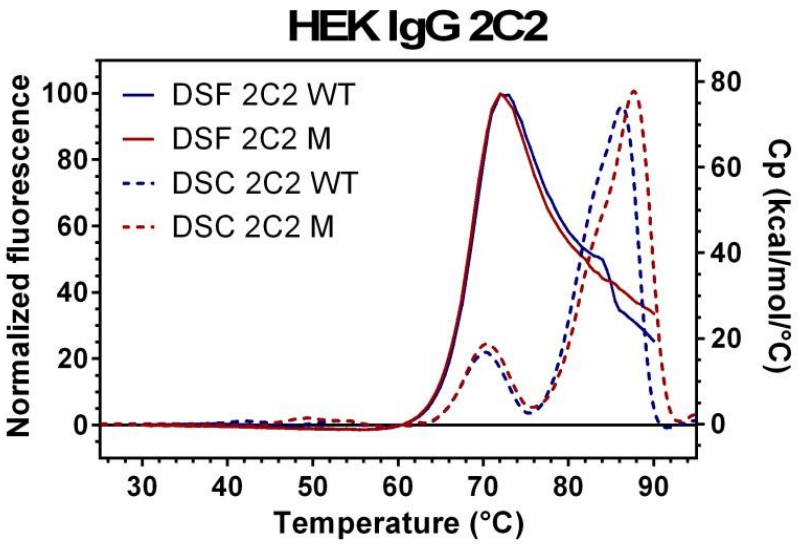
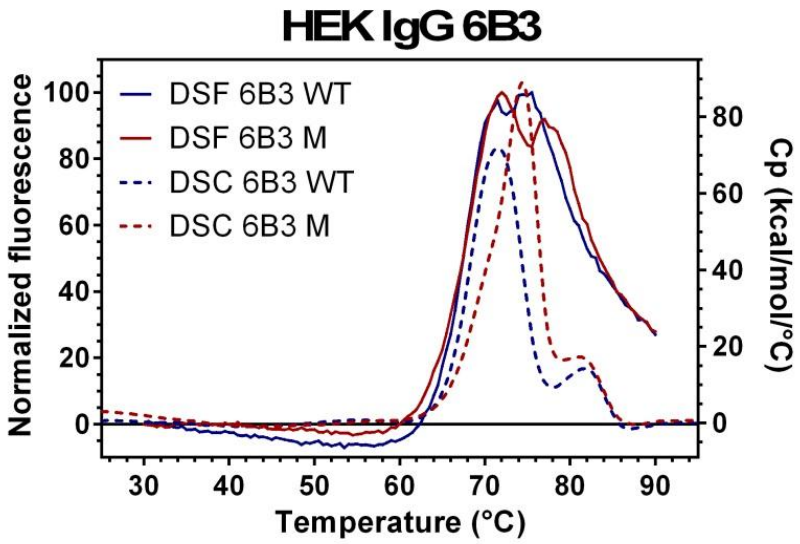
Comparison DSF vs. DSC





compared to DSC, DSF lacks "resolution" of individual domains, however is **much faster** (2-3 hrs vs. 48-72 hrs), can be **performed in parallel** and **requires much less protein** (20 µg vs. ~1 mg)

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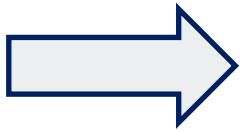
Stability overview

			ITF	GdnHCl	DSF	DSC
IgG 2C2	WT		70.4°C*	2.5 M	n.d.	86.0°C
	M		71.8°C*	3.8 M	n.d.	87.8°C
		Δ =	1.4°C	1.3 M	-	1.8°C
IgG 6B3	WT		67.6°C	2.0 M	74.5°C	72.1°C
	M		70.8°C	2.6 M	77.0°C	74.3°C
		Δ =	3.2°C	0.6 M	2.5°C	2.2°C

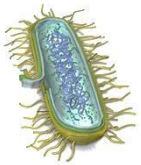
* – determined in presence of 1 M GdnHCl n.d. – not determined

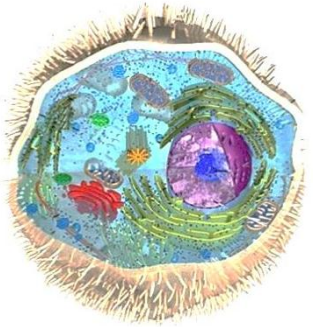
Schaefer and Plückthun, *Protein Eng. Sel. Des* (2012)

IgG stability analyses



IgG expression systems





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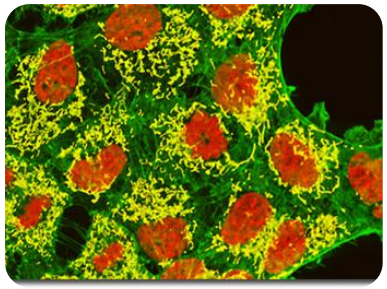
Eukaryotic expression systems

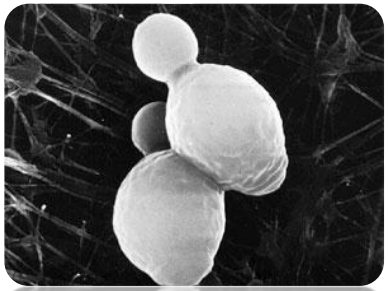
Mammalian cell culture



stable HEK293 (Flp-In)

CMV promoters (**constitutive**)

Yeast *Pichia pastoris*



stable SMD1163 (his4 pep4 prb1)

GAP promoters (**constitutive**)

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Expression system *Pichia pastoris*

Expression of full-length IgGs in **methylotrophic yeast *Pichia pastoris***

➡ **<u>advantages of expression system:</u>**

- disulfide bond formation / isomerization
- posttranslational modification (glycosylation)
- very high cell densities
- high expression levels (up to 30%)

➡ **<u>different promoters available:</u>**

- MeOH-inducible AOX1 (alcohol oxidase 1)
- constitutive GAP (glyceraldehyde-3-phosphate dehydrogenase)

➡ **only low-level secretion of endogenous proteins**, being advantageous for protein purification and downstream processing

➡ **> 50 reports describing antibody expression**
(mainly scFvs, several Fabs, only handful full-length IgG)

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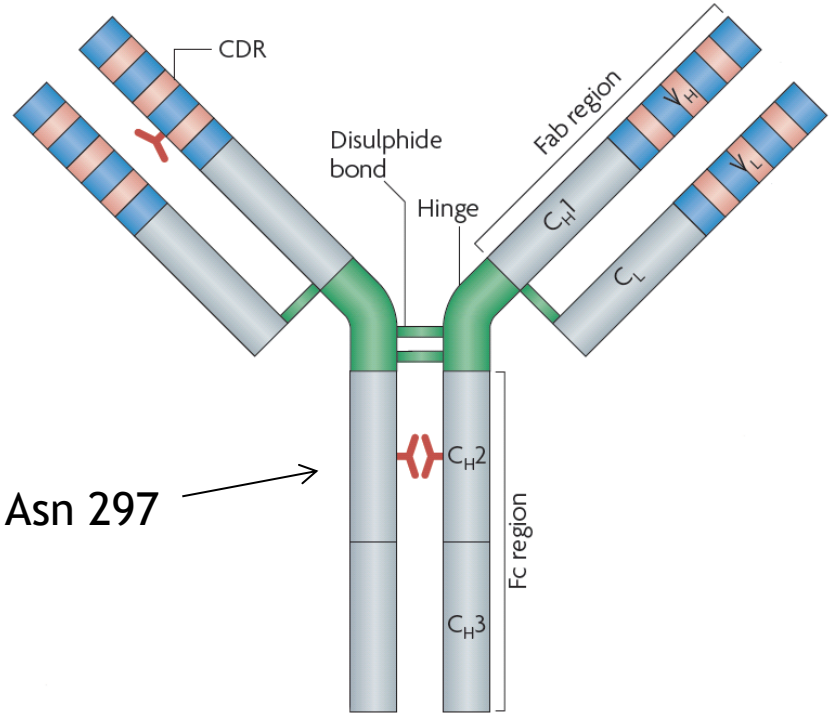
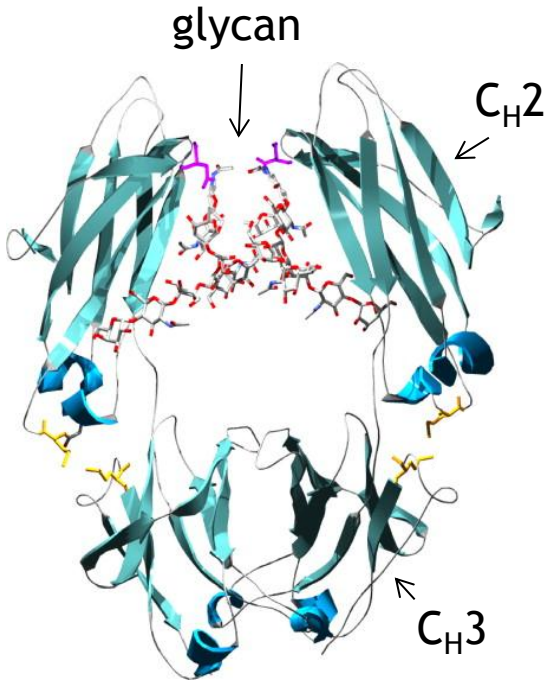
Difference in expression systems

mayor difference in expression systems: glycosylation

- yeast system processes same sugar precursor differently (in Golgi complex), resulting in a **different glycan**





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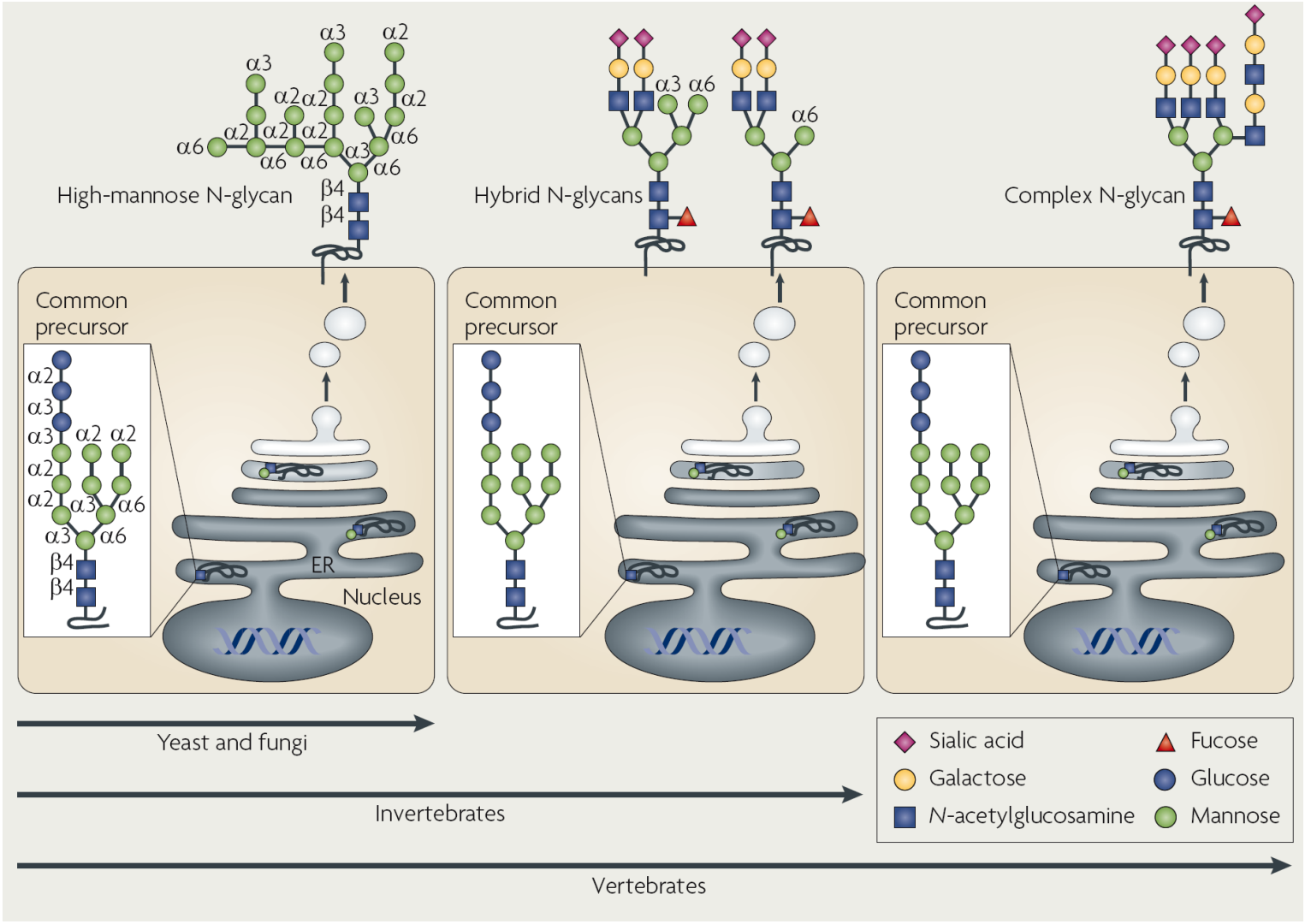
N-linked glycosylation



Marth and Grewal, *Nat Rev Immunol*. (2008)

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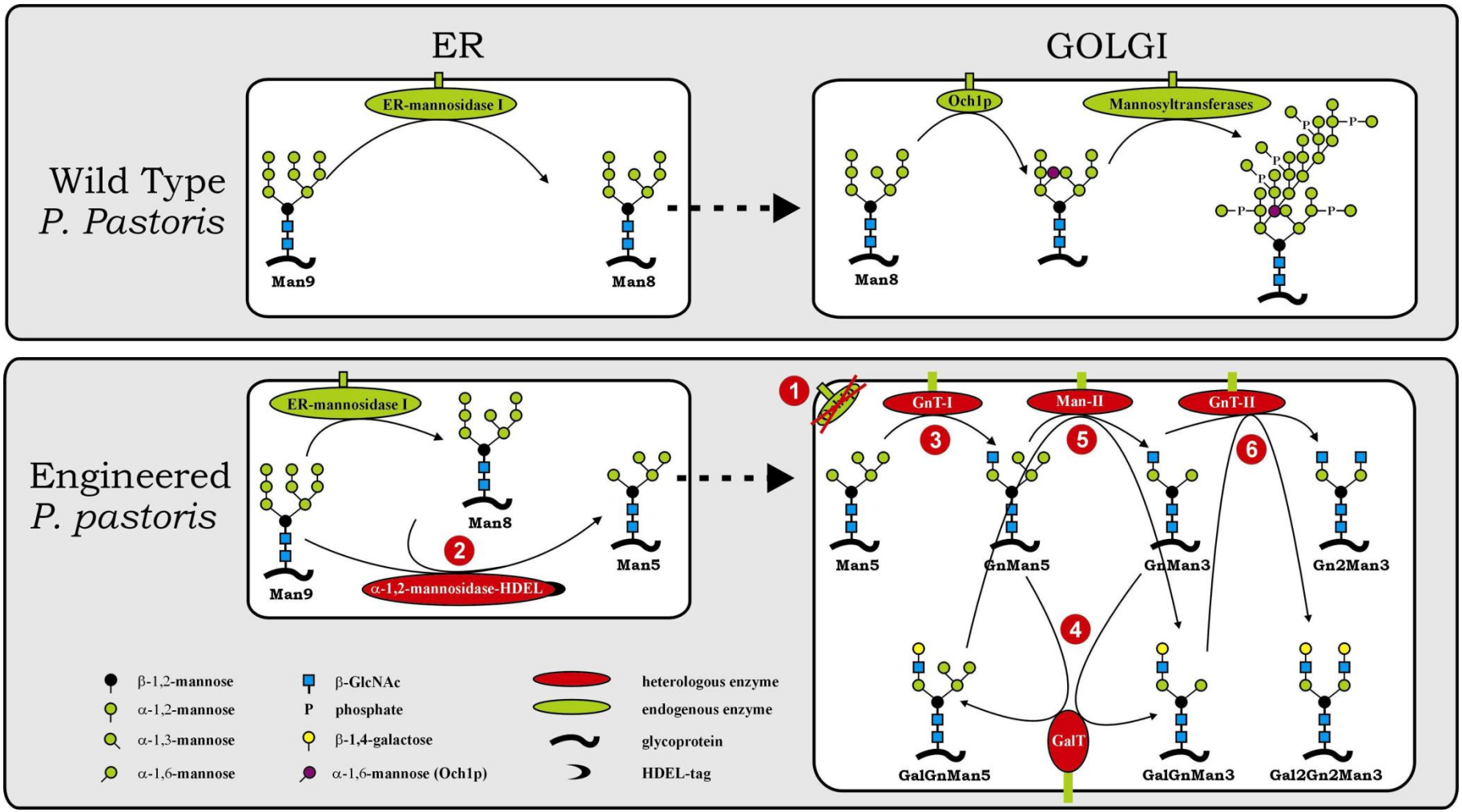
Glyco-engineering of *Pichia*

Pichia GlycoSwitch®: introducing complex, human-like glycosylation



Jacobs, Geysens, Vervecken, Contreras and Callewaert, *Nat Protoc*. 2009

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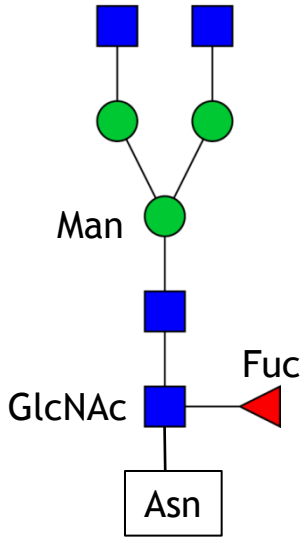
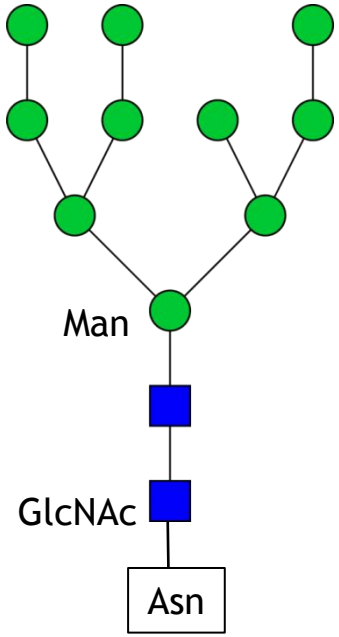
N-linked glycan processing

HEK293 cells

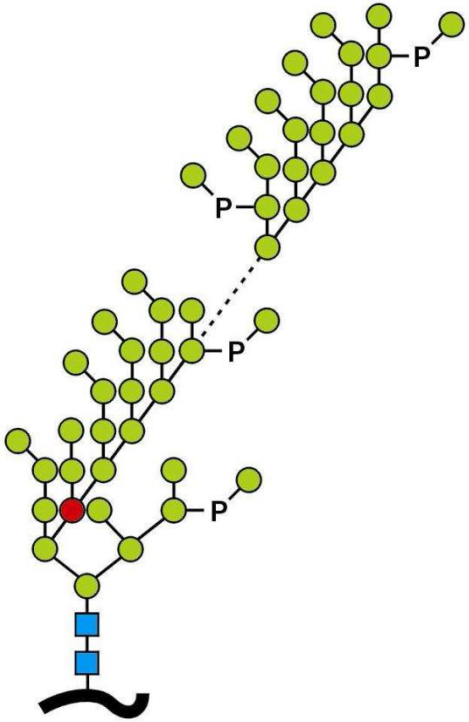


$Gal(GlcNAc)_2(Man)_3(GlcNAc)_2Fuc$

Pichia pastoris



$(Man)_{9\text{-}10\text{-}18}(GlcNAc)_2$



➡ *Pichia* glycan cause difficulties interacting with **Fcγ receptors (FcγR)** important for effector functions

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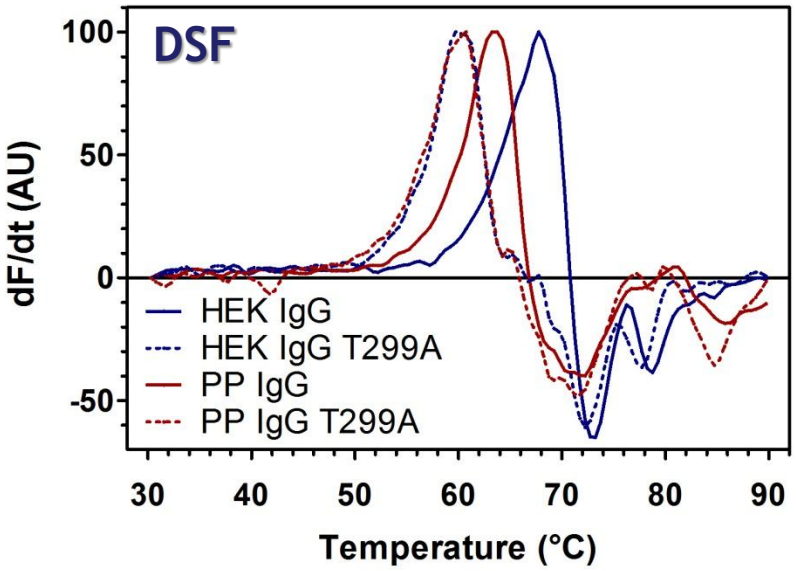
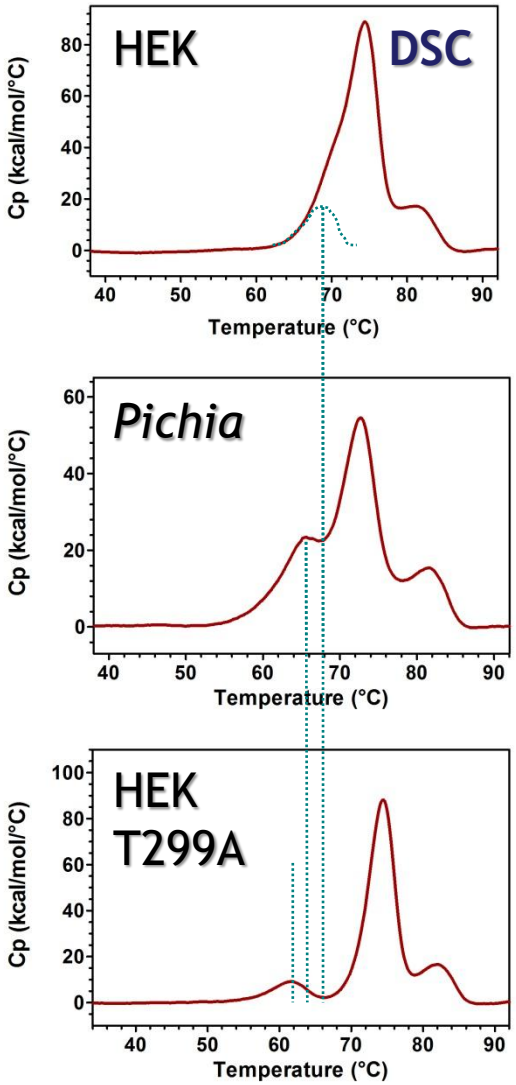
Influence of glycosylation on stability

- *Pichia* produced IgGs have decreased **C_H2 stability**, compared to mammalian expression
- different C_H2 stabilities are caused by **different glycan moieties**





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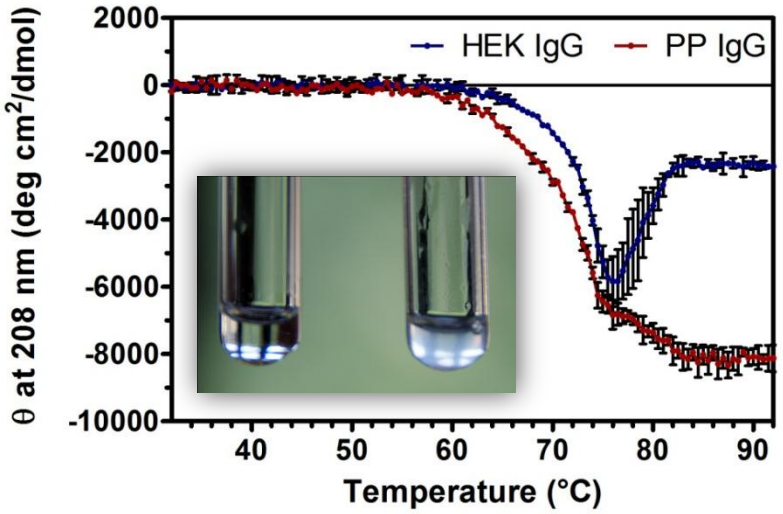
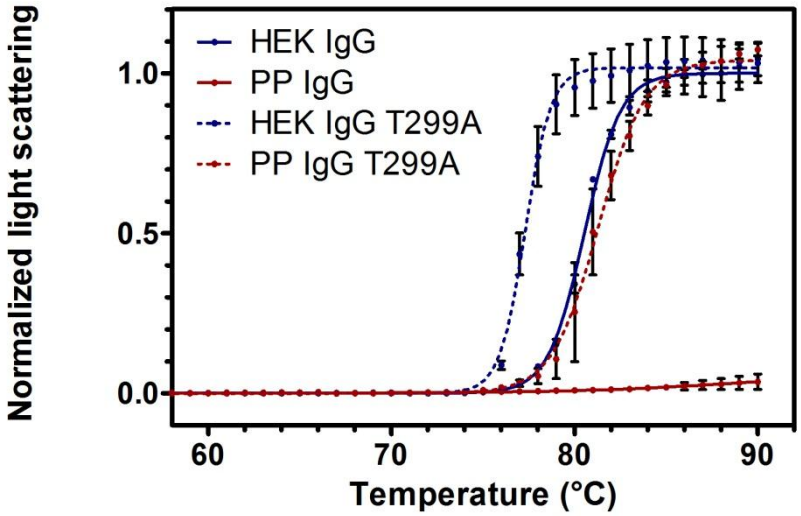
Influence of glycosylation on aggregation





- ***Pichia*-derived glycans** reduce aggregation tendency
- **EAEA-peptide** (originating from yeast signal sequence) decreases aggregation susceptibility of HEK-IgG upon N-terminal addition

Schaefer and Plückthun, *J. Mol. Biol.* (2012)

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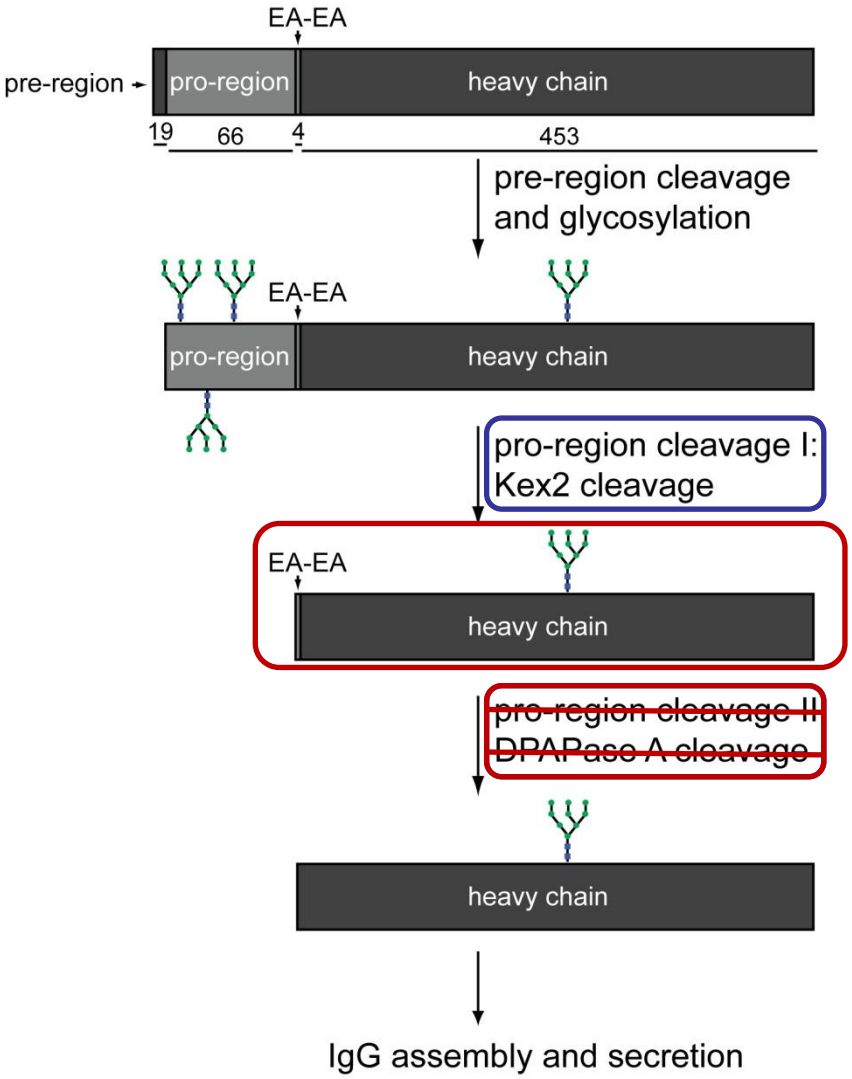
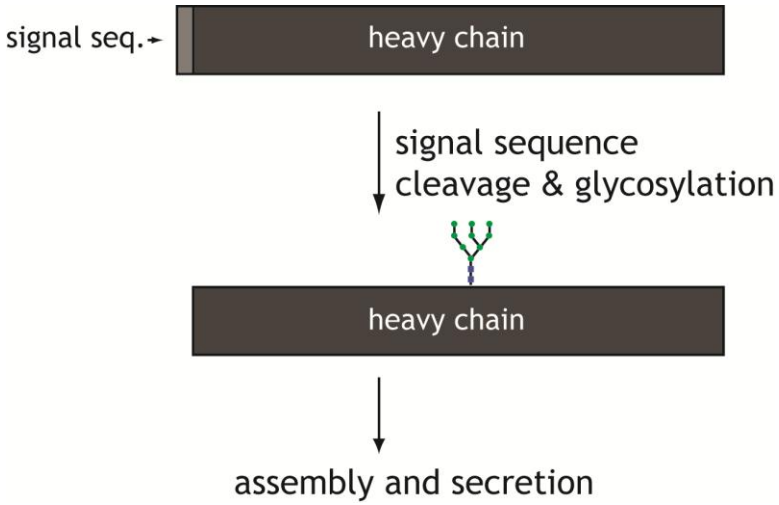
Signal sequence processing pathways

P. pastoris (PP)



mammalian cells (HEK)



➡ overexpression often results in **incomplete proteolytic processing**

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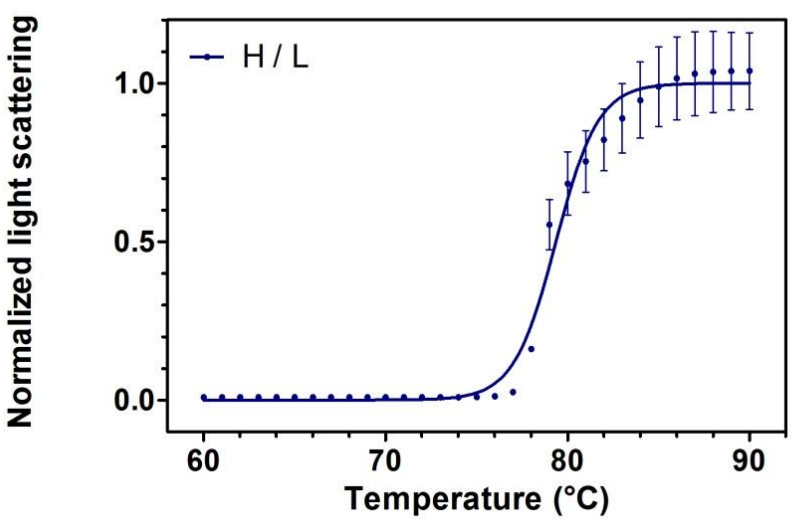
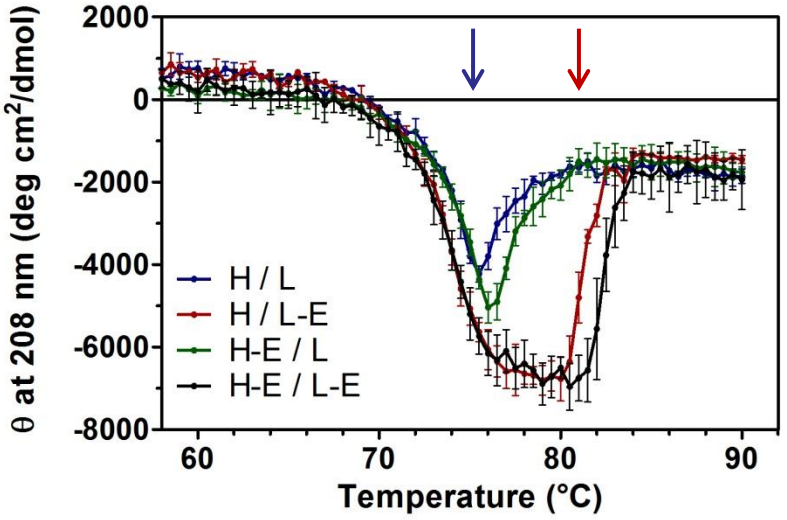
EAEA protects against aggregation

IgG (HEK)

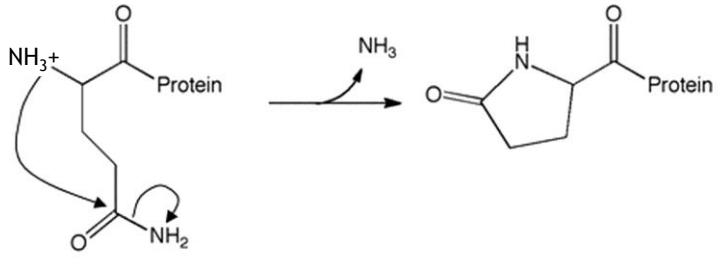




➡ **addition of EAEA decreases aggregation propensity**

➡ **position of EAEA matters:**

larger effect on LC than on HC



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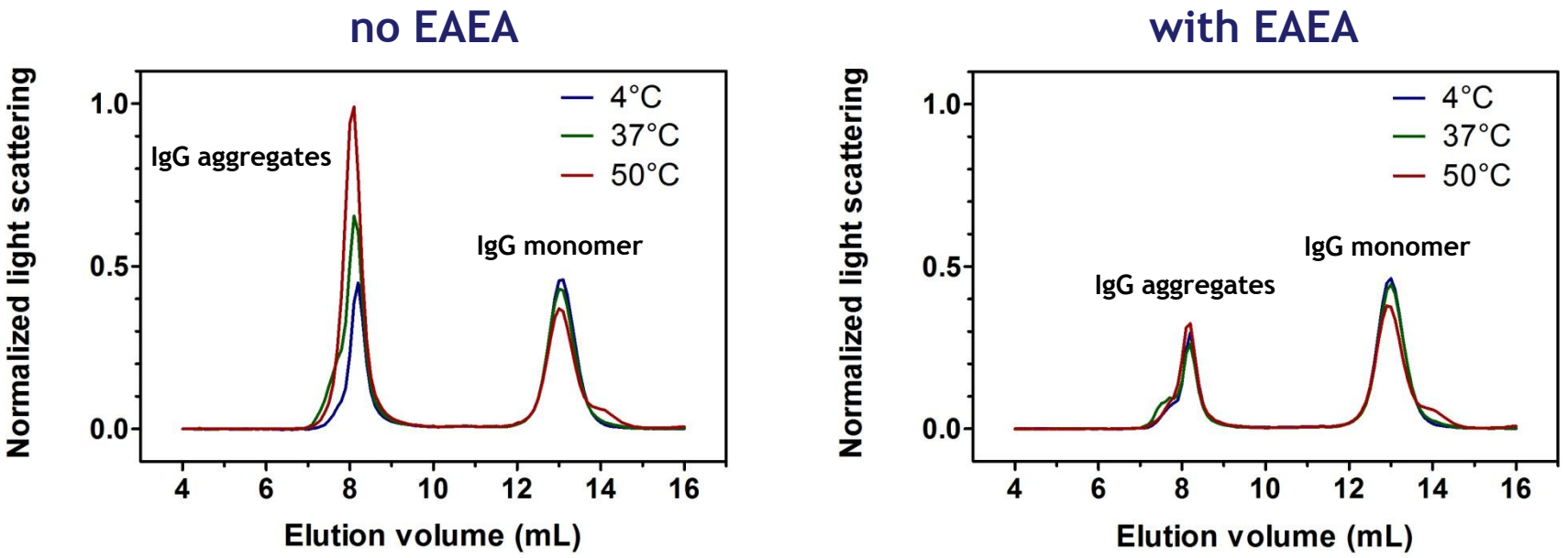
Less aggregation upon long-term storage

accelerated stress conditions (MALS analyses after 5 days)



➡ **decreased aggregation susceptibility** also at lower temperatures and at very low IgG concentrations (1 mg/ml)

Schaefer and Plückthun, *J. Mol. Biol.* (2012)

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Comparison with published results

- solubility of proteins can be enhanced by introducing charged residues (altering the overall charge)

  Lawrence *et al.*, *JACS* (2007); Arbabi-Ghahroudi *et al.*, *Protein Eng. Des. Sel.* (2009)

- aggregation-resistant V_H domains / V_{HH} possess greater negative net charge

  Jespers *et al.*, *Nat. Biotechnol.* (2004); Perchiacca *et al.*, *Proteins* (2011)

- introduction of negatively charged residues into CDR1 loop reduces aggregation suseptibility (however, other mutations effectless)

  Perchiacca *et al.*, *Proteins* (2011)

- our approach of **N-terminal addition of negative charges** does not influence antigen recognition and can easily be performed by cloning

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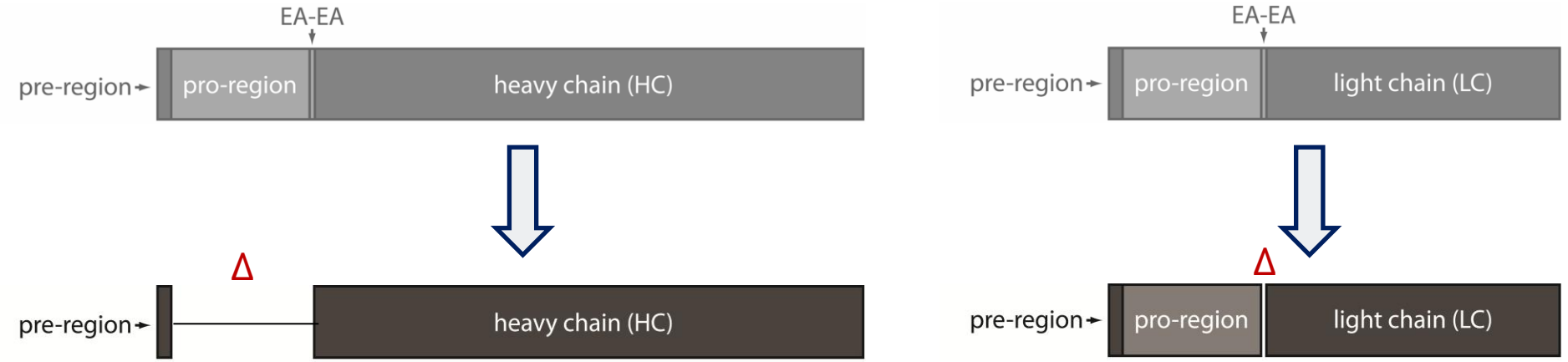
Producing correctly processed IgG



best combination of **yield and aggregation resistance**:

- for HC: without pro-region and without EAEA
- for LC: with pro-region and with EAEA

native-like IgG can be made:

- for HC: without pro-region and without EAEA
- for LC: with pro-region and without EAEA

Schaefer and Plückthun, *J. Mol. Biol.* (2012)

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Conclusion full-length IgGs

- advanced stabilities both with respect to thermal and denaturant-induced unfolding can be transferred to other formats, independent of expression system
- increase in structural integrity and homogeneity
- *Pichia pastoris* is an interesting expression system with several benefits (ease of handling, costs, …)
- optimal sequence composition for either aggregation-resistant or correctly processed IgGs available

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Margaret Jones (Welcome Trust, Oxford)

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FNSNF

FCI FONDS DER CHEMISCHEN INDUSTRIE

SSB Swiss Society for Biochemistry

p3

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Stefan Duhr (NanoTemper)

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Questions & Answers

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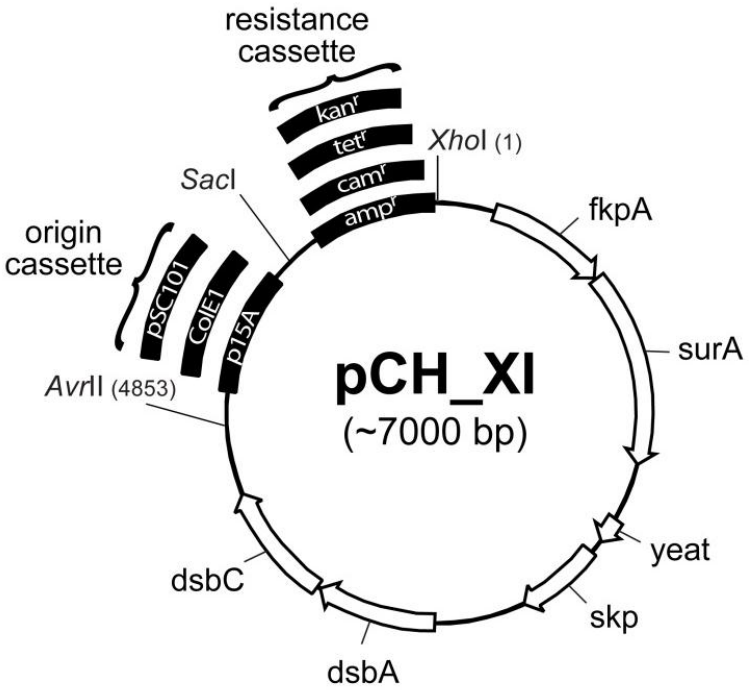
Modular co-expression of chaperons



peptidyl-prolyl cis/trans-isomerases (PPIs) with chaperone activity, **FkpA** and **SurA**

chaperone protein **Skp** precursor

thiol-disulfide oxidoreductases **DsbA** and **DsbC**

Schaefer, J. V., and Plückthun, A. (2010) in **Antibody Engineering: Improving expression of scFv fragments by co-expression of periplasmic chaperones** (Kontermann, R., and Dübel, S., eds) Vol. 2, 2nd edit., pp. 345-361, Springer Verlag,

➡ **different origin of replication:** **ColE1 (E)**, **p15A (A)** and **pSC101 (S)**

copy numbers: 50-70 20-30 ~10

➡ **modular system:**

compatibility with virtually all expression vectors; level of chaperone co-expression can be controlled; safeguards against plasmid incompatibility

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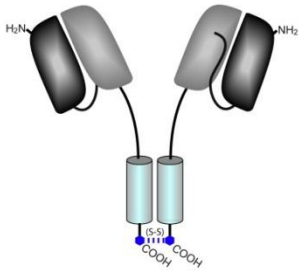
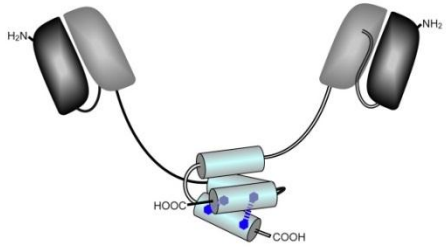
Miniantibodies: construct overview

Dimeric miniantibodies



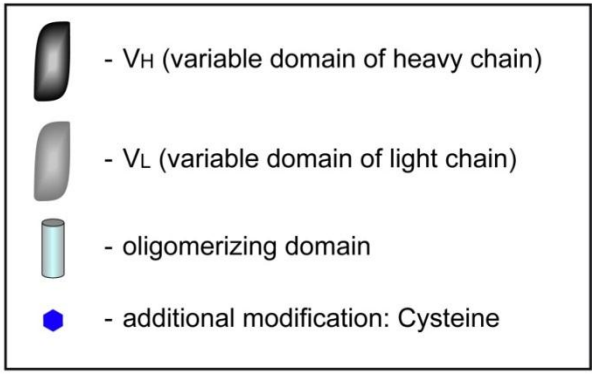
(A) scFv-ZIP(c)

(GTCN4 leucin zipper)

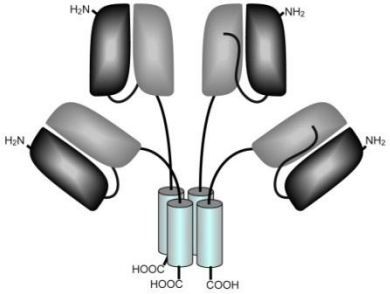


(B) scFv-dHLX (-SS)

(Helix1-turn-Helix2)

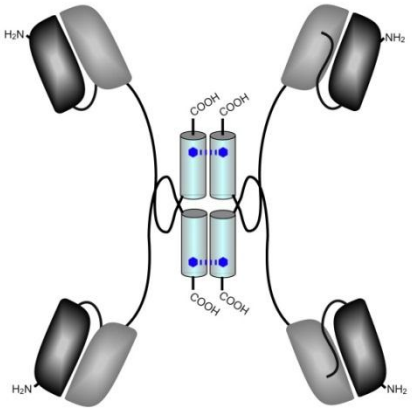


Tetrameric miniantibodies



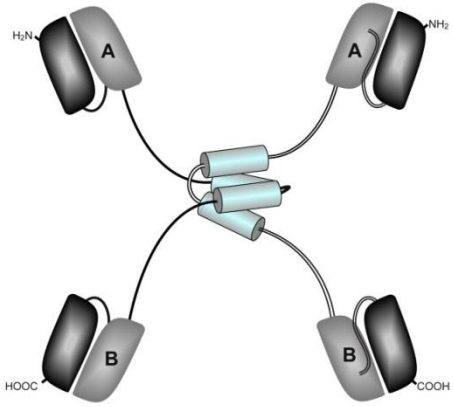
(C) scFv-TETRAZIP

(modified GTCN4: 9 mutations)



(D) scFv-p53 (-SS)

(p53 oligomerization domain)



(E) di-bi-miniantibody

(bispecificity & bivalency)

Schaefer, Lindner, and Plückthun (2010) in **Antibody Engineering: Miniantibodies** Vol. 2, 2nd edit., pp. 85-99, Springer Verlag

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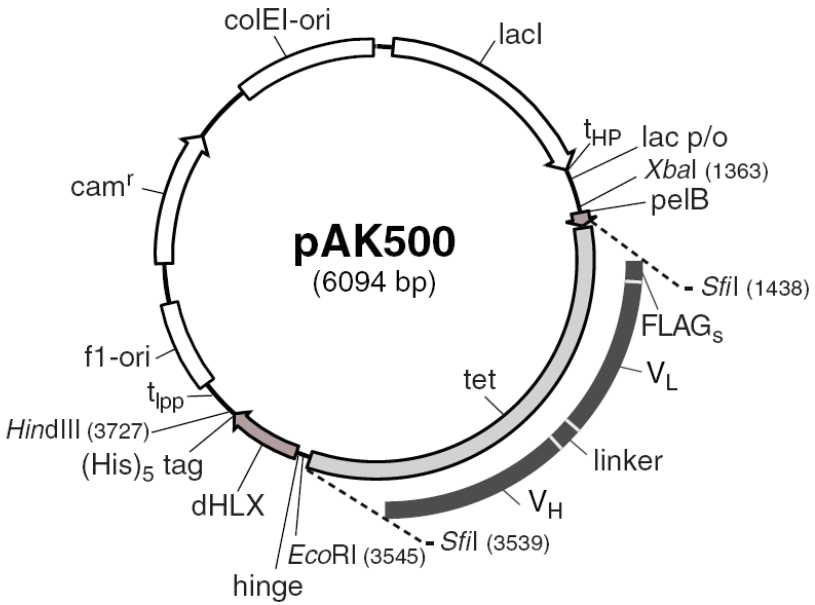
Miniantibodies: construct overview

Construct	Upper hinge	Self-associating peptide	Modifications
Bivalent			
scFv-ZIP	Murine IgG3	GCN4 leucine zipper	–
scFv-ZIPc	Murine IgG3	GCN4 leucine zipper	C-terminal Cys
scFv-dHLX	Murine IgG3	Helix1-turn-Helix2	–
scFv-dHLX-SS	Murine IgG3	Helix1-turn-Helix2	Internal Cys
Bispecific			
scFv-JUN	Murine IgG3	JUN leucine zipper	–
scFv-FOS	Murine IgG3	FOS leucine zipper	–
CH1-CL	Murine IgG3	CH1 and CL from IgG	–
Tetravalent			
scFv-TETRAZIP	Murine IgG3	GCN4 leucine zipper, modified	–
scFv-p53	Human IgG3	Oligomerization domain of human p53	–
scFv-p53-SS	Human IgG3	Oligomerization domain of human p53	Internal Cys
Tetravalent/bispecific			
di-bi	Murine IgG3	Helix1-turn-Helix2	–



TETRAZIP – exchange of all 9 hydrophobic contact positions a and d of the GCN4 zipper