“Recombinant Protein Therapeutics-Fc Fusion Proteins and Beyond”
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Making proteins “druggable”: Fc fusion proteins as a therapeutic class

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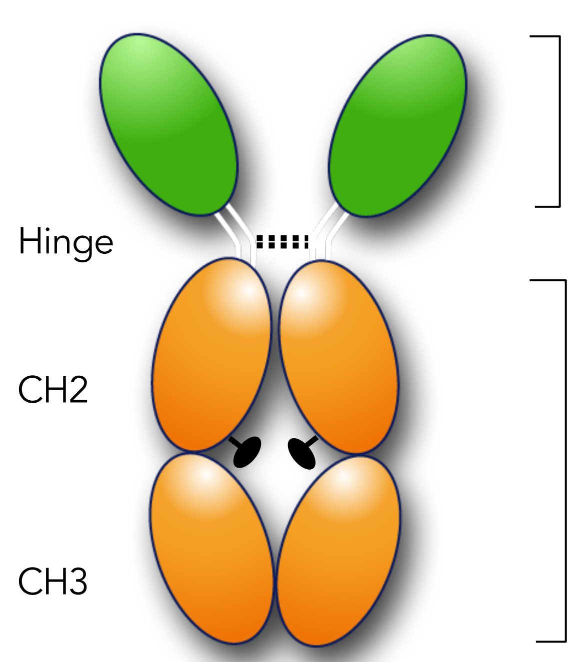
Overview

- Fc fusion protein design and structure
- Instability upon purification
  - Aggregation at low pH
  - First order kinetics
  - Rate constants are directly correlated
  - Additives to minimize aggregation
- Predicting problems
  - A screening method to reveal aggregation issues early
- Summary

Fc-fusion protein design and structure

Chamow S.M., *et al. (eds.) Therapeutic Fc Fusion Proteins* (Wiley-Blackwell) 2014

Fc-fusion format: Many proteins become "druggable"



Protein (ligand binding domain)

- Receptor extracellular domain
- Cytokine
- Peptide
- Enzyme

Fc region

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

Examples:
Enbrel, Amiveve, Orencia, Zaltrap

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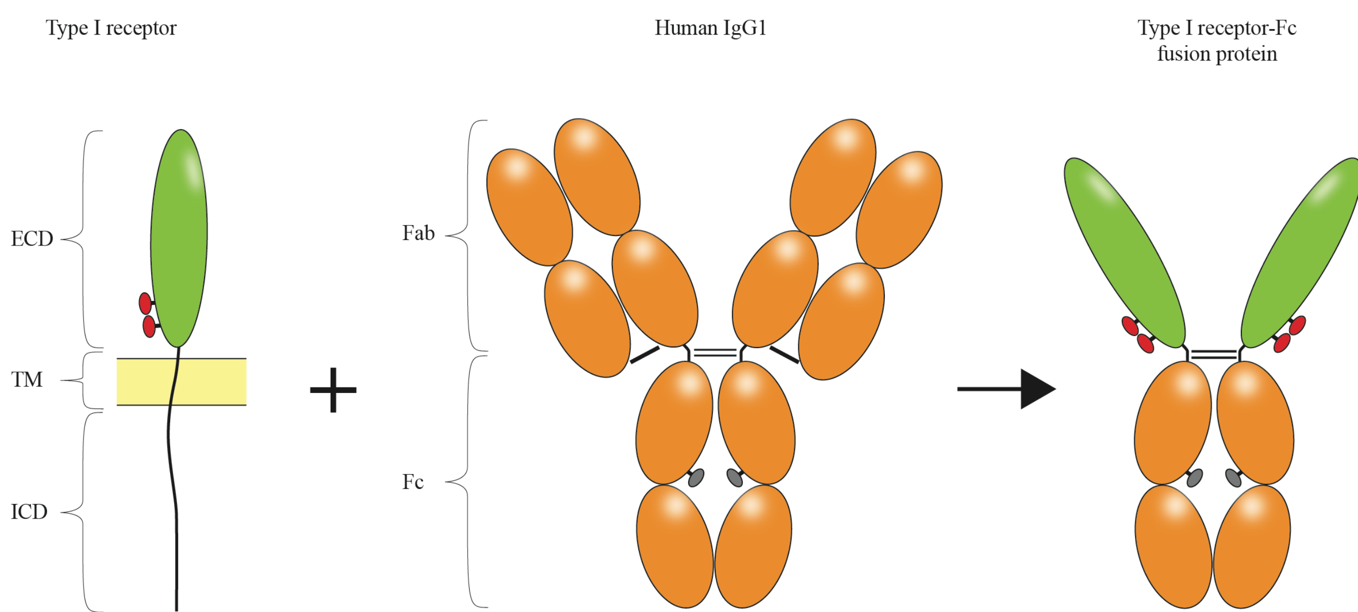
Constructing an Fc fusion protein



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Fc fusion protein: Key structural features

- Homodimer
  - Can contain two copies of ligand binding domain
    - Receptor ECD
    - Cytokine
    - Peptide
    - Enzyme (FVIII and FXI excepted)
- Protein (ligand binding domain)
  - Replaces Fab (VL-CL, VH1-CH1)
  - High affinity for target
    - Cytokine traps (Eylea/Zaltrap Kd 0.5 pM)
  - Fused into Ig hinge
    - Hinge serves as flexible “spacer” between two parts
      - e.g., Ligand binding domain-EPKSCDKTHTCPPCP-Fc

Key features of Fc fusion proteins (cont'd.)

- IgG Fc
  - Retains effector functions
    - ADCC
    - CDC
    - Half-life extension
    - Protein A binding
  - Amenable to molecular engineering in Fc
- Amenable to manufacture using a mAb platform process
  - Acid stabile

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Structural variation: Fc fusion proteins as commercial products

i. ii. iii. iv. v.

i. Enbrel® (etanercept)
ii. Orencia® (abatacept)
iii. Eylea®/Zaltrap® (aflibercept)
iv. Nplate® (romisplostim)
v. Alprolix® [coagulation factor IX (recombinant), Fc fusion protein]

Fc fusion proteins as commercial products (FDA-approved 1998-2014)

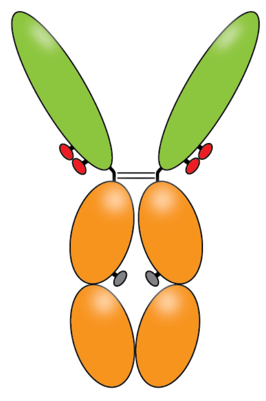
Product	Company	Year Approved	Cell Type	Type	Target
Enbrel	Amgen	1998	CHO	TNFR Fc fusion	TNF
Amiveve	Biogen Idec	2003	CHO	Fc fusion	CD2
Orencia	BMS	2005	CHO	CTLA4 Fc fusion	CD80, CD86
NPlate	Amgen	2008	*E. coli*	TPO mimetic peptide Fc fusion	TPOR
Arcalyst	Regeneron	2008	CHO	IL1R Fc fusion	IL1
Eylea	Regeneron	2011	CHO	VEGFR Fc fusion	VEGF
Nulogix	BMS	2011	CHO	CTLA4 Fc fusion	CD80, CD86
Zaltrap	Regeneron	2012	CHO	VEGFR Fc fusion	VEGF
Alprolix	BiogenIdec	2014	HEK	FIX Fc fusion protein	Blood clotting enzyme
Eloctate	Biogen Idec	2014	HEK	FVIII Fc fusion protein	Blood clotting enzyme
Trulicity	Eli Lilly	2014	CHO	Dulaglutide Fc fusion	Type 2 diabetes

Instability upon purification

Shukla A.A., *et al.* Protein aggregation kinetics during Protein A chromatography: Case study for an Fc fusion protein, *J. Chromatogr. A* **1171**, 22-28 (2007)

Fc fusion protein

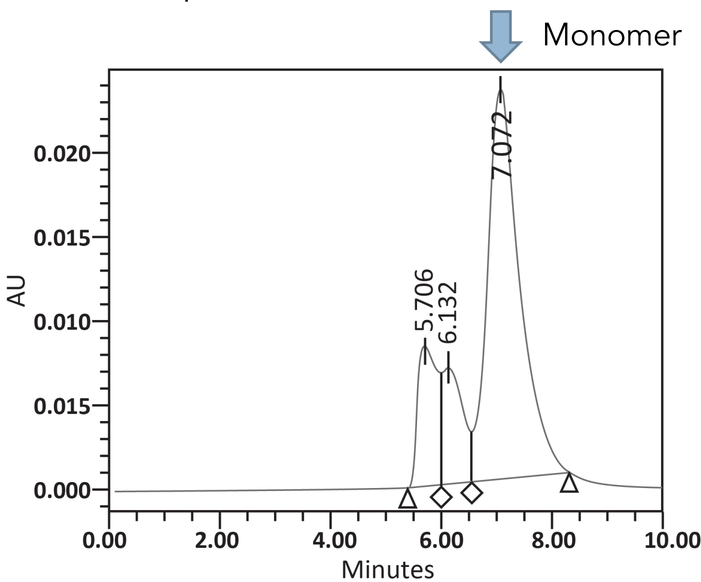
- ECD-IgG1 Fc
  - ECD receptor tyrosine kinase/Hu IgG1 Fc
  - pI 5.2-7.8
  - MW 152 kDa
  - Expressed in CHO
  - 6 N-linked glycosylation sites, sialylated
- Production
  - Capture on Protein A
    - Mab Select
    - Load density: 15 mg/mL
    - Equilibration: 25mM Tris, 100mM NaCl, pH 7.4
    - Wash: 50mM NaCitrate pH 5.5
    - Elution: 50mM Citric acid pH 3.6
    - Product pool neutralized with 1M Tris base



Shukla A.A., *et al. J. Chromatogr. A* **1171**, 22-28 (2007)

Acidic elution from Protein A causes product aggregation

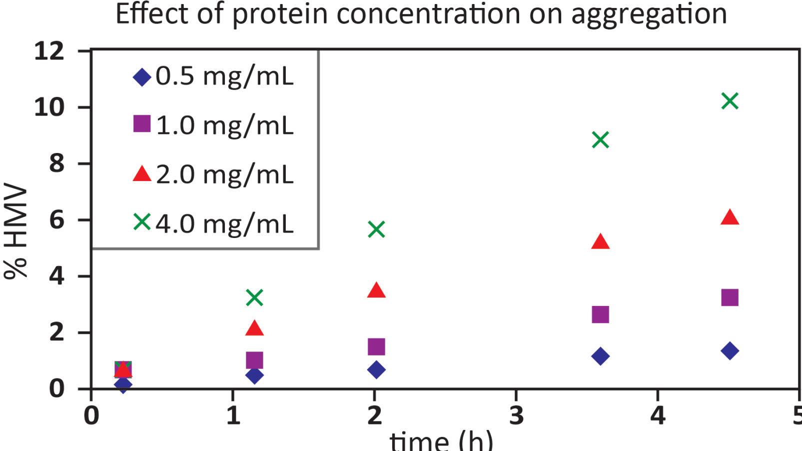
Analytical SEC of neutralized Protein A pool shows high rate of aggregation under low pH conditions



Shukla A.A., *et al. J. Chromatogr. A* **1171**, 22-28 (2007)

Aggregation increases with time of acidic exposure and protein concentration

% HMW forms (0-12) vs. time of acidic exposure (0-5 h) analyzed using analytical SEC



Shukla A.A., *et al. J. Chromatogr. A* **1171**, 22-28 (2007)

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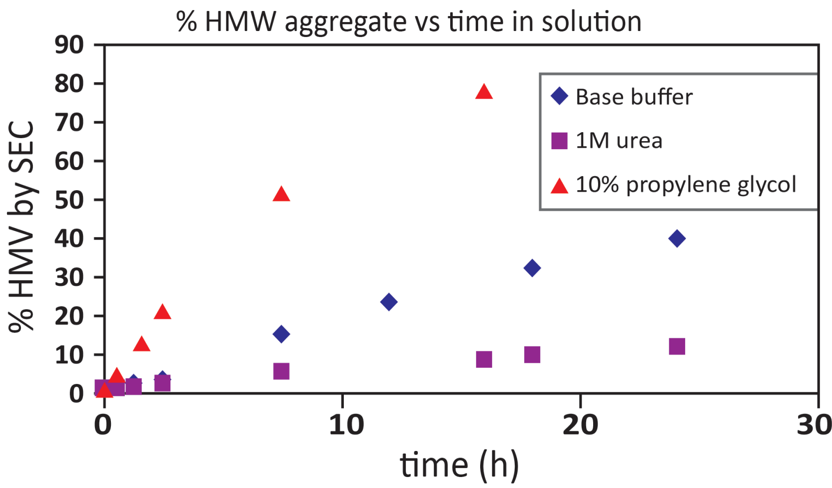
Adding 1M urea to acidic elution buffer stabilizes Fc fusion protein

% HMW forms are increased or decreased by addition of 10% propylene glycol or 1M urea, respectively



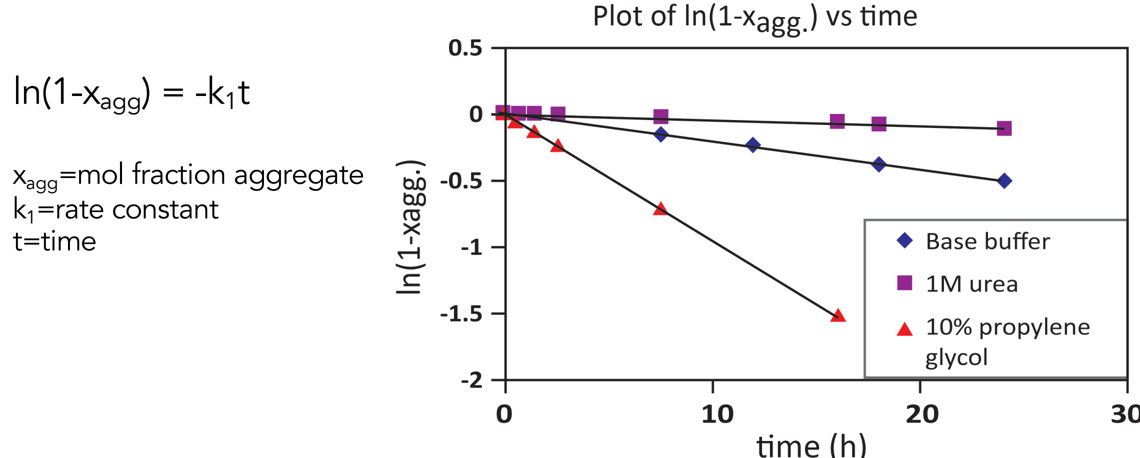
Shukla A.A., *et al. J. Chromatogr. A* **1171**, 22-28 (2007)

Reaction mechanism of aggregation follows first order kinetics

Semi-log plot of (1-mole fraction of aggregated species) vs. time is straight line and k_1 values can be compared

$\ln(1-x_{agg}) = -k_1 t$

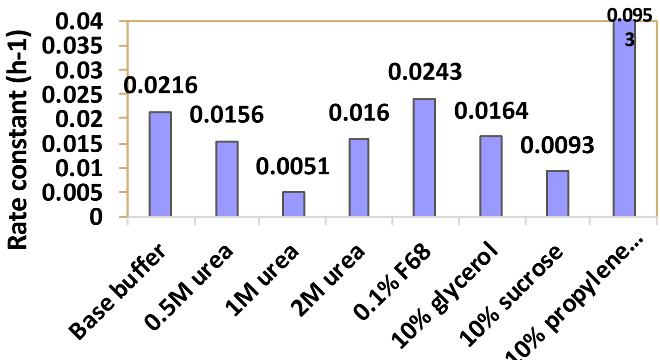
x_{agg}=mol fraction aggregate
k_1=rate constant
t=time



Shukla A.A., *et al. J. Chromatogr. A* **1171**, 22-28 (2007)

1M urea and 10% sucrose have a stabilizing effect

The Fc fusion protein was eluted from Protein A in acidic buffer + additives. Aggregation rate constants were compared.



Shukla A.A., *et al. J. Chromatogr. A* **1171**, 22-28 (2007)

A screening method to predict aggregation problems early

Kohli N., *et al.* A novel screening method to assess developability for antibody-like molecules, *mAbs* **7:4**, 752-758 (2015)

Assessing manufacturability: When is a mAb "developable"?

- Criteria for developability
  - High expression
  - Bioactivity
  - Solubility
    - Good solubility over wide pH and salt range
  - Stability
    - Stable at high concentration with long shelf life
- When do we want to know this?
  - As early as possible during product discovery

Kohli N., *et al.* *mAbs* **7:4**, 752-758 (2015)

SMAC

- Novel high-throughput HPLC based screening method
  - Standup monolayer adsorption chromatography (SMAC)
- Zenix
  - Sepax Technologies (Newark, DE)
  - Silica particles (3 µm) with proprietary surface technology
    - Hydrophobic standup monolayer with terminal hydrophilic groups
  - Pore sizes 100, 150 and 300Å
  - HPLC media commonly used for SEC analysis
- Assess developability factors early in discovery process (μg of mAb)
- Retention times on Zenix HPLC column inversely related to colloidal stability

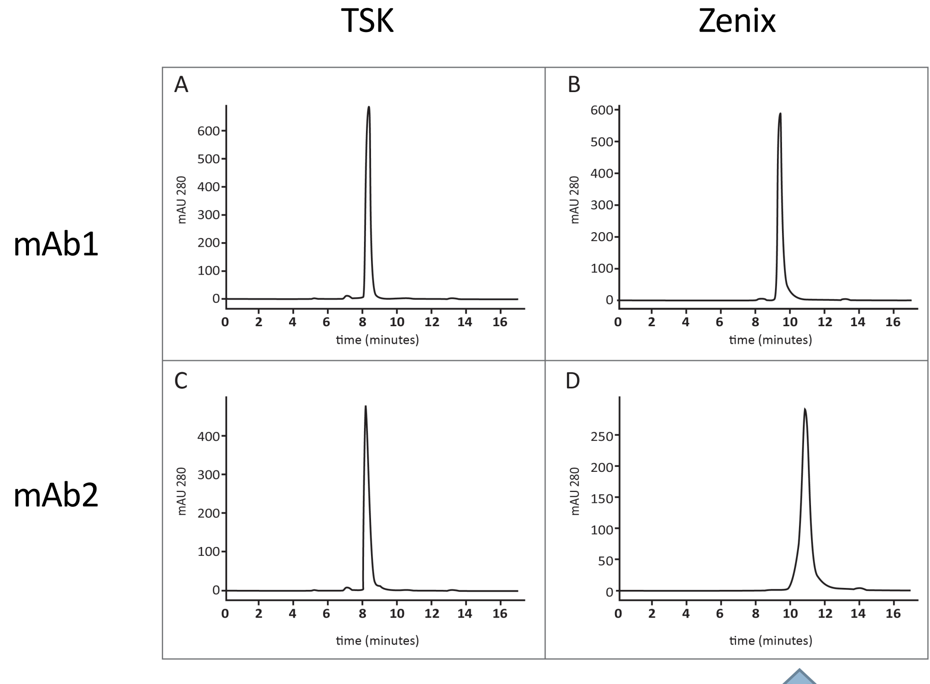
Kohli N., *et al. mAbs* **7:4**, 752-758 (2015)

SMAC Method

- Zenix HPLC
  - Column 4.6 mm ID x 30 cm
  - Mobile phase 150 mM NaPhosphate pH 7.0
  - 50 ug sample injected
  - Agilent 1100 HPLC with diode array detector

Kohli N., *et al. mAbs* **7:4**, 752-758 (2015)

SMAC predicts poor developability of mAb2



Kohli N., *et al. mAbs* **7:4**, 752-758 (2015)

mAbs with colloidal instability are more prone to non-specific interactions with Zenix column

Protein Name	Retention Time on TSKgel (min)	Retention Time on Zenix (min)	Rate of aggregation at 37°C per month
mAb1	8.3	9.4	<2%
mAb2	8.2	10.9	<2%
mAb3	8.6	9.8	<2%
mAb4	9.5	>20 min	Visible precipitation overtime
mAb5	8.1	10.8	>4%
mAb6	8.5	9.5	<2%
mAb7	8.5	11.6	>4%
mAb8	8.6	9.4	<2%
mAb9	8.8	>20	Visible precipitation overtime
mAb10	9.9	>20	Visible precipitation overtime
mAb11	8	>20	Visible precipitation overtime
mAb12	9.8	>20	Visible precipitation overtime
mAb13	8.6	>20	>5%
mAb14	8.8	9.2	<2%
mAb15	8.9	9.4	<2%

TSKgel is 3μm silica with a hydrophilic diol-type bonded phase
Aggregation rate @ 37°C = colloidal stability

mAbs with colloidal instability have longer retention times

Kohli N., *et al.* *mAbs* **7:4**, 752-758 (2015)

mAbs have multiple modes of interaction with the column

1X PBS is optimal mobile phase condition

More electrostatic ← Baseline ↓

Protein	Retention Time in 0.5X PBS (min)	Retention Time in 1X PBS (min)	Retention Time in 2X PBS (min)
mAb1	9.2	9.1	9.4
mAb2	12.3	10.5	10.8
mAb3	15	10.7	10.4
mAb4	>20	>20	>20
mAb5	15	9.6	12
➡ mAb6	>20	10.1	10
mAb7	10.7	10.6	11.3
mAb8	9.6	9.4	9.6
mAb9	>20	>20	>20
mAb10	>20	>20	>20
mAb11	>20	>20	>20
mAb12	>20	>20	>20
mAb13	>20	>20	>20
mAb14	11	9.5	9.6
mAb15	9.6	9.7	9.7

pH 7.4, 10mM NaPhosphate + 75mM/150mM/300mM NaCl = 0.5X/1X/2X PBS

Hydrophobic interactions predominate at 1X and 2X PBS

Kohli N., *et al. mAbs* **7:4**, 752-758 (2015)

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Summary

- Many proteins of potential therapeutic interest can be made "druggable" by conversion into Fc fusion proteins
- Fc fusion proteins share many characteristics with mAbs
  - Capture on Protein A with acidic elution
- Fc fusion proteins can be prone to aggregate at low pH
  - The reaction mechanism for aggregation follows first order kinetics
  - Aggregation increases with time of acidic exposure and protein concentration
  - 1M urea and 10% sucrose have a stabilizing effect
- SMAC is a rapid, high-throughput screening method that can predict early the developability of an Fc fusion protein or mAb

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