Physicochemical and Immunological Comparison of CRM197 from Different Manufacturers and Expression Systems

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Department of Pharmaceutical Chemistry
University of Kansas, Lawrence, KS

8th International Symposium on Higher Order Structure of Protein Therapeutics
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www.mvsc.ku.edu

Macromolecular and Vaccine Stabilization Center (MVSC) is located in

Department of Pharmaceutical Chemistry in School of Pharmacy at KU:

- Characterization, stabilization and formulation
- Vaccines (live and recombinant purified antigens, adjuvants)
- Biopharmaceuticals (proteins, peptides)

Our Team

Distinguished Professor, PI

Dr. David Volkin

Director, co-PI

Dr. Sangeeta Joshi

Scientific Assistant Directors

Dr. Ozan Kumru

Dr. John Hickey

Post Docs, Research Associates

Dr. Ying Wan

Dr. Prashant Kumar

Dr. Vineet Gupta

Dr. Neal Whitaker

Dr. Kawaljit Kaur

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Dr. Swathi Pullagurla

Dr. David Holland

Dr. Lorena Napolitano

Administrative Staff

Emily Thomas-Dykes

Melinda Fish

Research Assistants

Sara Birdjandi

Dara Ogun

Graduate Students

Sanjeev Agarwal

Nishant Sawant

Kaushal Jerajani

Chris Bird

Sakshi Bajoria

➢**Our group** currently has ~25 members (graduate students, post-doctoral fellows, senior scientists, office/lab staff).

- **Our research grants and fee-for service contracts** include complete analytical characterization and formulation development studies as well as per sample testing.

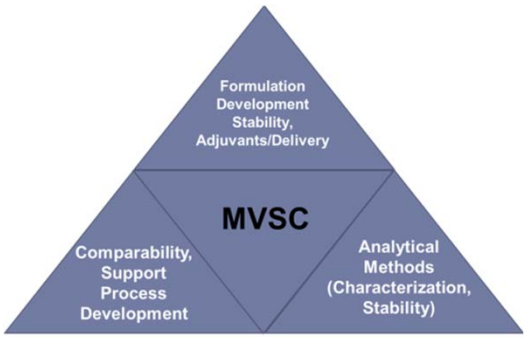
- **Our focus is on the science of CMC development**.

  - ***Early-stage*** Characterize and compare candidates with structural assays, develop stability-indicating assays and identify degradation pathways

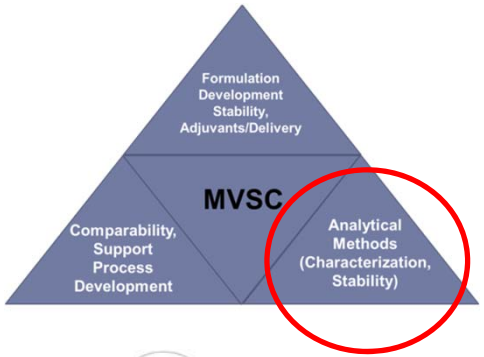
  - ***Translational-stage*** Characterize, stabilize and formulate biological candidates for first-in-human clinical trials (Phase 1).

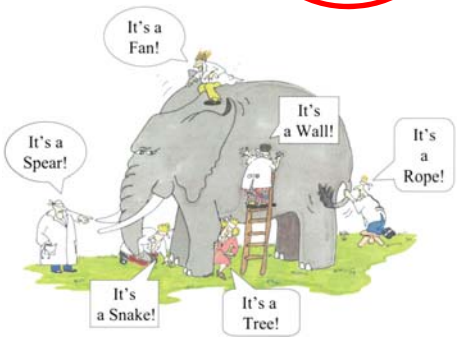
  - ***Late-stage*** Develop final formulations and perform analytical comparability assessments to support Phase 3 trials.

- **Our research goals** include developing analytical tools, elucidating degradation mechanisms, improving stability and correlating physicochemical changes with loss of potency



Analytical Characterization of Vaccines and Biopharmaceuticals





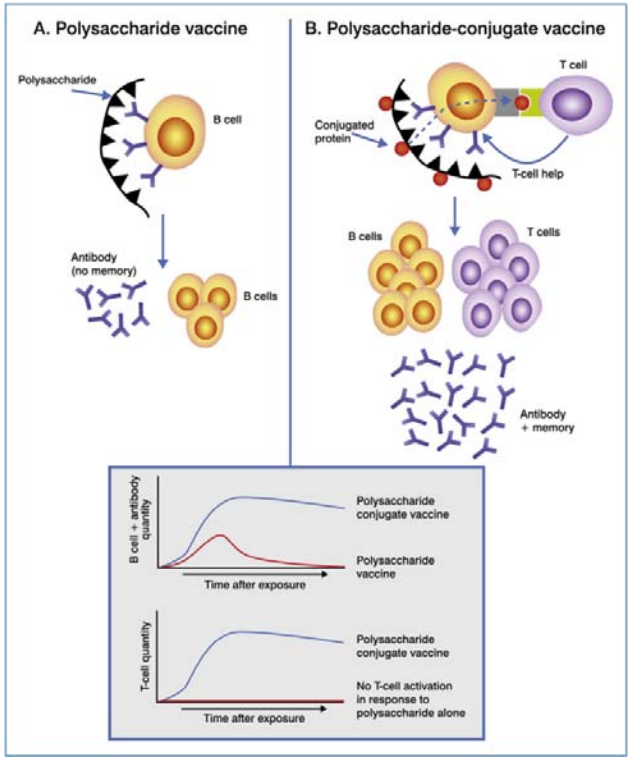
The blind men and the elephant. Poem by John Godfrey Saxe (Cartoon originally copyrighted by the authors; G. Renee Guzlas, artists http://www.nature.com/ki/journal/v62/n5/fig_tab/4493262f1.html

- Requires an overall assessment of results from a family of assays
- Physical, chemical, biological, immunological…
- Key role in vaccine and biopharmaceutical development
  e.g., process/formulation, testing, comparability, stability

Physical & Chemical Attributes

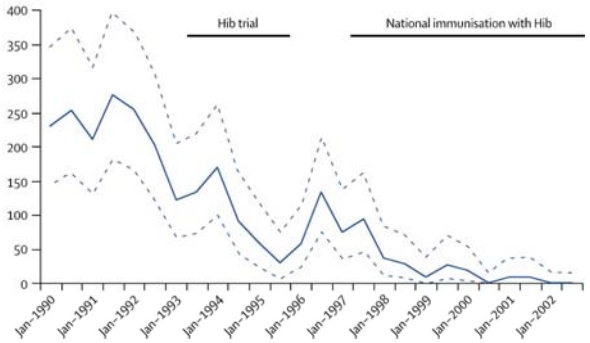
- Appearance
- Purity
- Primary Structure
- Secondary Structure
- Tertiary Structure
- Overall Conformational Stability
- Characterize Size (Oligomeric State)
- Measure Aggregation/Particles
- Charge Heterogeneity

Carrier Proteins In Polysaccharide-Conjugated Vaccines



Vaccine	Polysaccharide Source (Organism)	Manufacturer	Carrier Protein
Menveo®	*Neisseria meningitidis*	Novartis	CRM_{197}
Prevnar®	*Streptococcus pneumoniae*	Pfizer	CRM_{197}
Prevnar-13®	*Streptococcus pneumoniae*	Pfizer	CRM_{197}
Hiberix®	*Haemophilous influenzae*	GlaxoSmithKline	Tetanus Toxoid
ActHIB ®	*Haemophilous influenzae*	Sanofi Pasteur	Tetanus Toxoid
Menhibrix®	*Neisseria meningitidis* *Haemophilous influenzae*	GlaxoSmithKline	Tetanus Toxoid
Menactra®	*Neisseria meningitidis*	Sanofi Pasteur	Diphtheria Toxoid
PedvaxHIB®	*Haemophilous influenzae*	Merck	OMPC

Incidence of Hib meningitis per 100,000 children < 1 yr

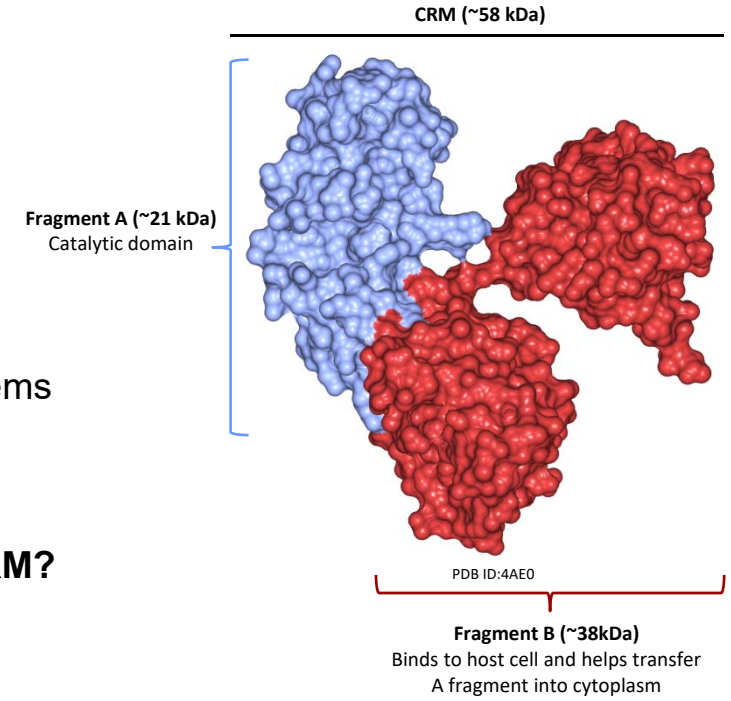


Tetanus Toxoid (TT)	Diphtheria Toxoid (DT)	Cross-Reactive Material 197 (CRM_{197})	*N. meningitidis* Outer Membrane Protein (OMP)	Non-Typeable *H. influenzae* Derived Protein D (PD)
				
• Derived from *Clostridium tetani* • Inactivated with formalin • Purified with ammonium sulfate and filter sterilized prior to conjugation process	• Derived from *Corynebacterium diphtheriae* • Detoxified with formaldehyde • Purified by ammonium sulfate fractionation and diafiltration	• Enzymatically inactive, nontoxic mutant of diphtheria toxin • Requires no formaldehyde detoxification • Obtained at near 100% purity	• Outer membrane protein complex derived from *N. meningitidis* serogroup B strain 11 • Purified by detergent extraction, ultracentrifugation, diafiltration, and sterile filtration	• Antigenically conserved surface lipoprotein found in all *H. influenzae* • Used in a nonacylated, antigenically active form

(2011) Knuf, M. et al, Vaccine 29:4881-4890
(2005) Adegbola, R.A. et al, Lancet 366:144-150
(2016) Hickey, J.M. et al, J Chrom B, 1032:23-38
(2011) Strugnell, R. et al, Underst. Mod. Vaccines Perspect. Vaccinol, 1:61-88

Natural & Recombinant CRM197 ("CRM")

- Produced in *Corynebacterium diptheriae* (low yield) and requires BSL-2 manufacturing facility
- Natural CRM is commercially available but costly and potential issues obtaining sufficient material for preclinical/clinical studies
- CRM can be produced in recombinant expression systems (*E. coli* and *P. fluorescens*)
- **How does recombinant CRM compare to natural CRM?**



(2012) Malito, E. et al, Proc. Natl. Acad. Sci, 109:5229-5234

Recombinant CRM

Natural CRM

FinaBio (Fina Biosolutions LLC)

- Commercial product
- Expression System: ***Escherichia coli***
- Procurement: **Collaboration**

Biological E. Limited (Celebrating Life Every Day)

- Commercial product
- Expression System: ***Escherichia coli***
- Procurement: **Collaboration**

Reagent Proteins (A division of PFEnex)

- Commercial product
- Expression System: ***Pseudomonas fluorescens***
- Procurement: **Purchased**

VaxForm LLC

- Non-commercial product (2006, stored frozen)
- Expression System: ***Corynebacterium diptheriae***
- Procurement: **Donated**

List Biological Laboratories, Inc.

- Commercial product
- Expression System: ***Corynebacterium diptheriae***
- Reference C7 "natural" CRM
- Procurement: **Purchased**

KU The University of Kansas — Analytical Comparison

- Physicochemical Properties
- Physical Stability as a function of stress (pH vs. Temp.)
- Relative Solubility Profiles
- *In vitro* Immunological Assays

(2018) Hickey, J.M. et al, J. Pharma. Sci., 107:1806-1819

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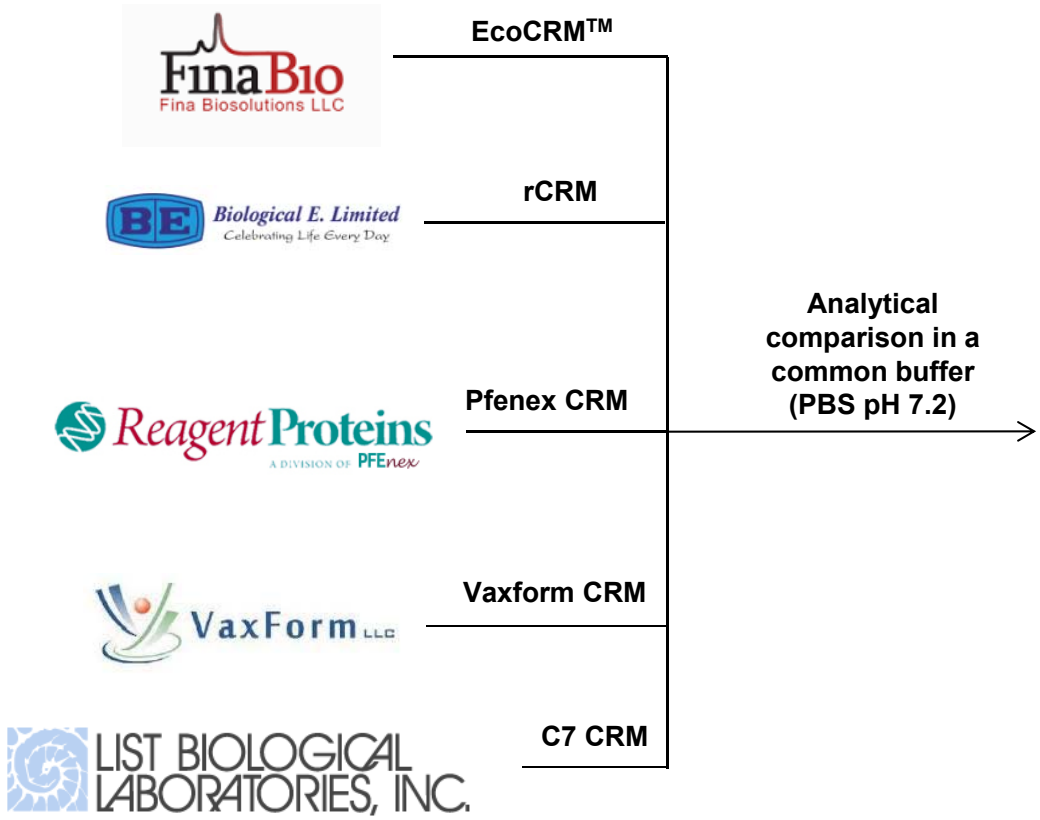
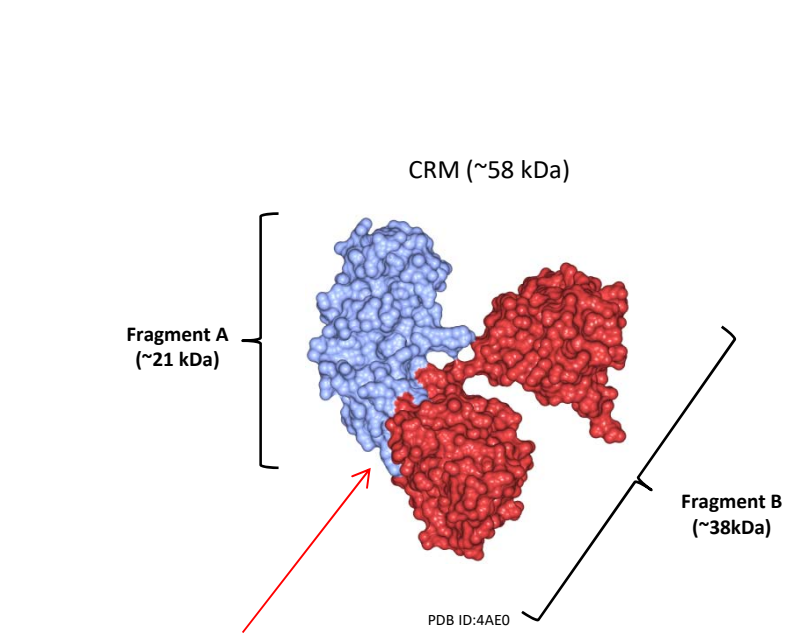
Overview of comparing the physicochemical properties of five CRM samples



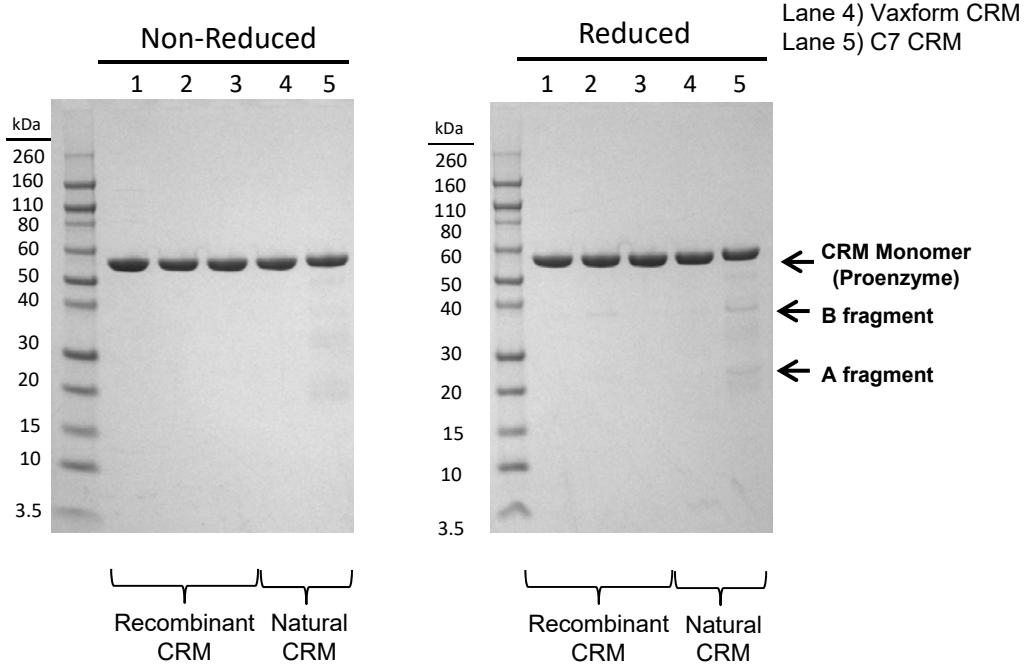
Structural Attribute	Analytical Techniques for CRM Comparison
Appearance, Protein concentration, Purity	UV-Visible Spectroscopy (+/- Light Scattering correction, +/- Centrifugation)
	SDS-PAGE (Reduced vs. Non-reduced)
Primary Structure	Intact Mass (Reduced vs. Non-reduced)
	Peptide Mapping (Non-reduced)
Additional Assays for Primary Structure/Protein Heterogeneity (Purity)	Capillary Isoelectric Focusing (cIEF)
	Anion Exchange Chromatography (AEX)
Secondary Structure	Far UV Circular Dichroism
	Fourier Transform Infrared Spectroscopy
Tertiary Structure	Intrinsic Fluorescence Spectroscopy
	Extrinsic Fluorescence Spectroscopy
Overall conformational stability vs. temperature	Intrinsic Fluorescence Spectroscopy
	Static Light Scattering
	Extrinsic Fluorescence Spectroscopy
	Differential Scanning Calorimetry (DSC)
Characterization of Size and Presence of Aggregates	Sedimentation Velocity Analytical Ultracentrifugation
	Size Exclusion Chromatography (+/-MALS)
	Archimedes (RMS)
	Micro Flow Imaging (MFI)

Comparison of Purity and Presence of Covalent-Linked Aggregates



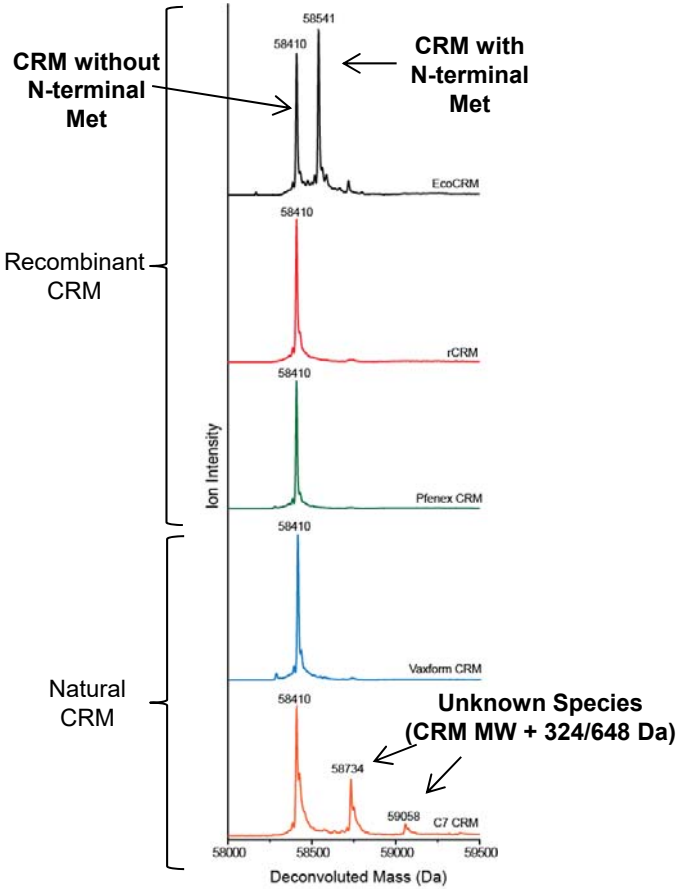
Proenzyme is cleaved by trypsin-like protease in *C. diptheriae* and fragments A and B are associated through one disulfide bond

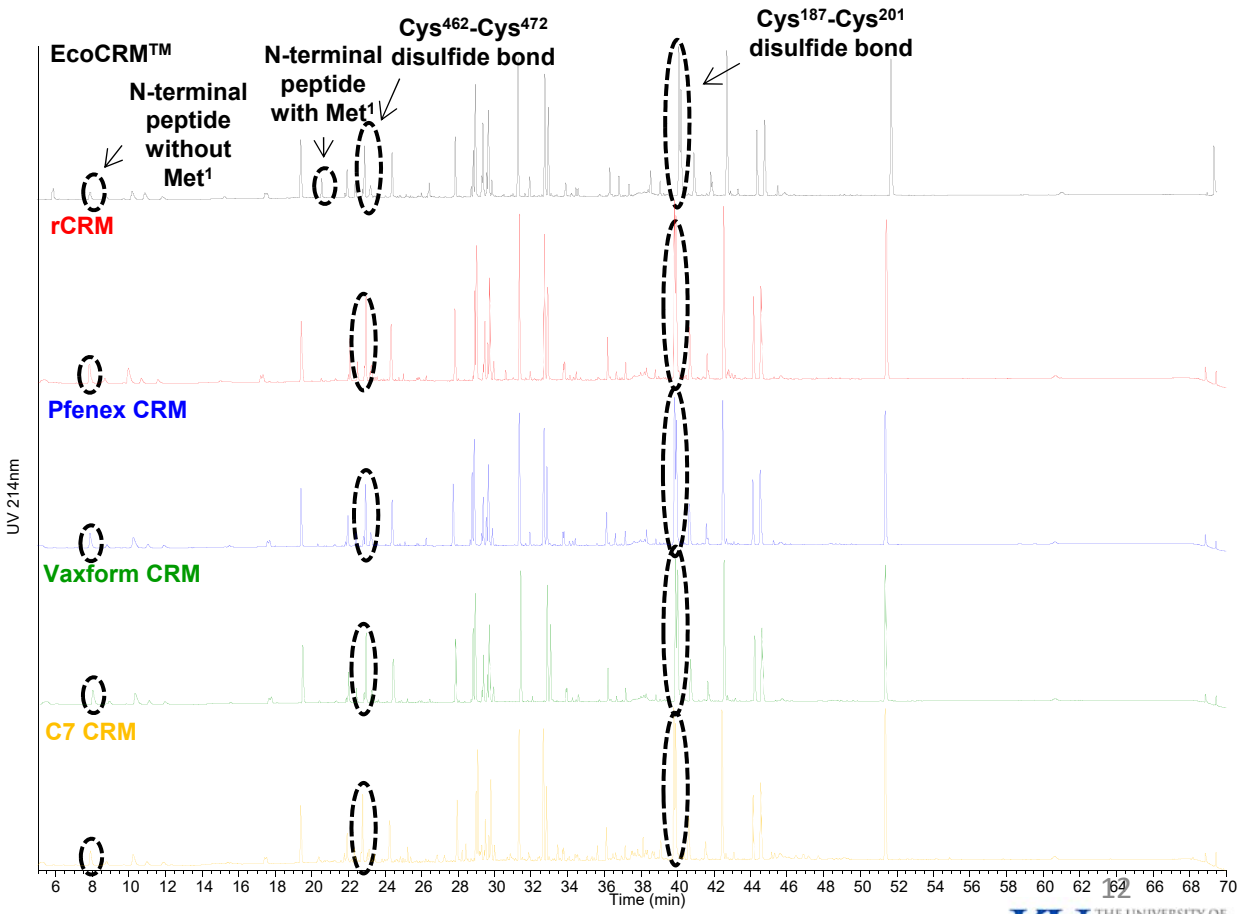
Lane 1) EcoCRM™
Lane 2) rCRM
Lane 3) Pfenex CRM
Lane 4) Vaxform CRM
Lane 5) C7 CRM



(2012) Malito, E. et al, Proc. Natl. Acad. Sci, 109:5229-5234

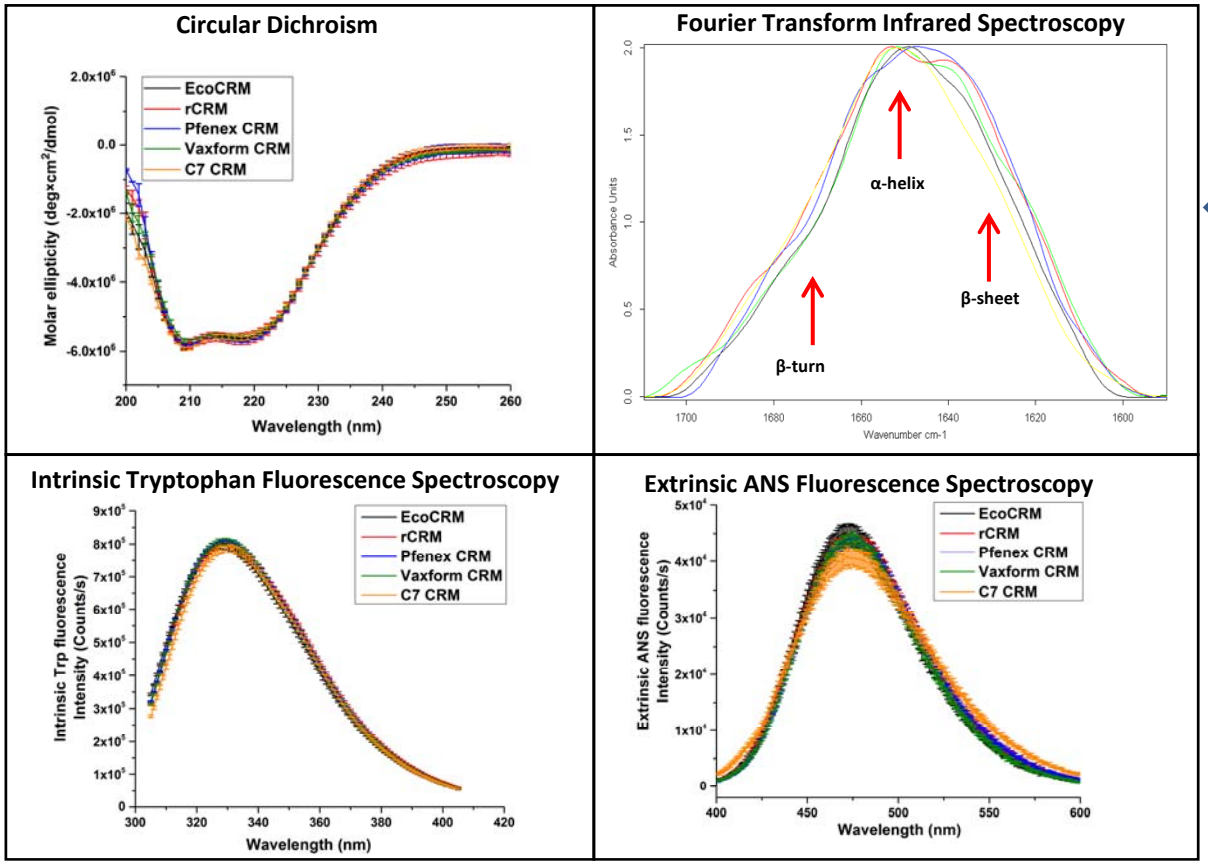
Primary Structure (Intact mass analysis & peptide mapping)





Higher Order Structure Comparison at 10˚C

Secondary Structure



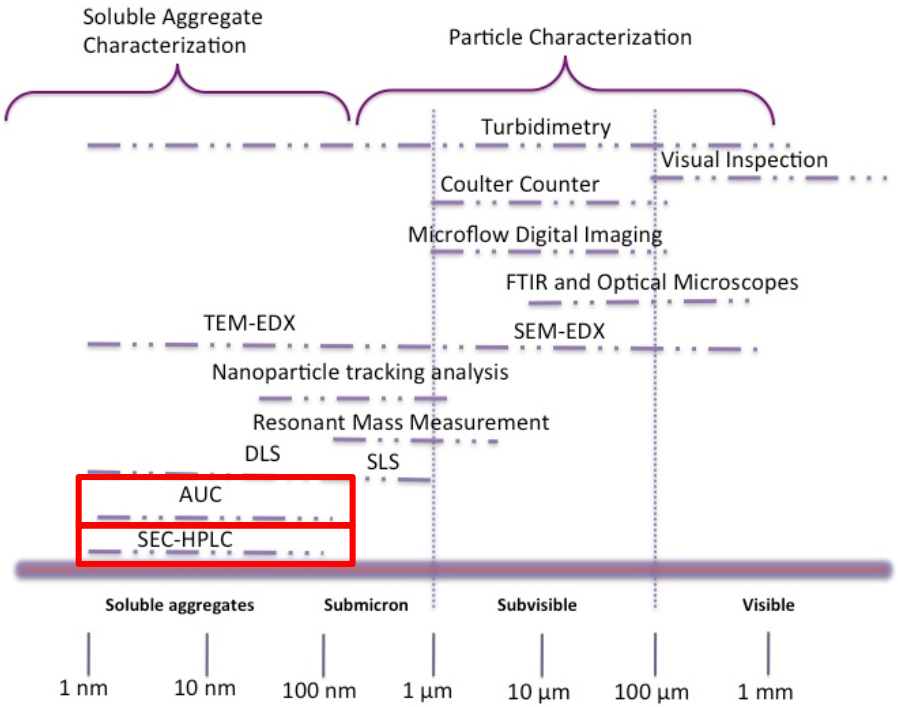
Secondary Structure

Tertiary Structure

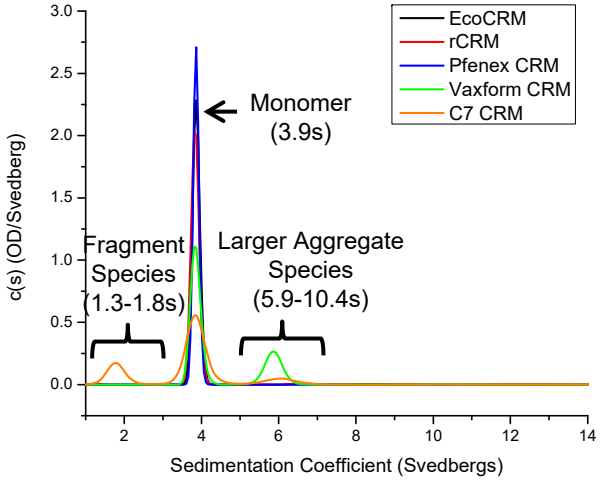
Tertiary Structure/ Exposure of Apolar Surfaces

n = 3

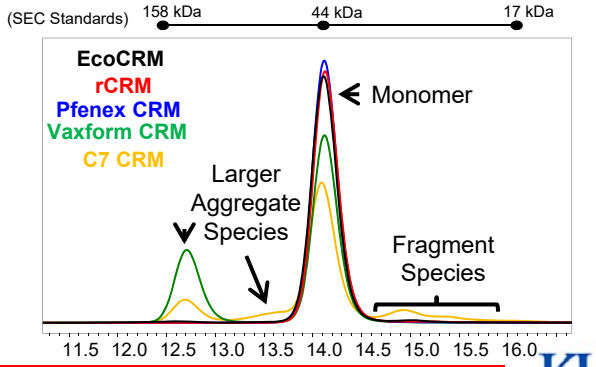
Size Analysis and Quantitation of Soluble Aggregates



Sedimentation Velocity Analytical Ultracentrifugation (SV-AUC)

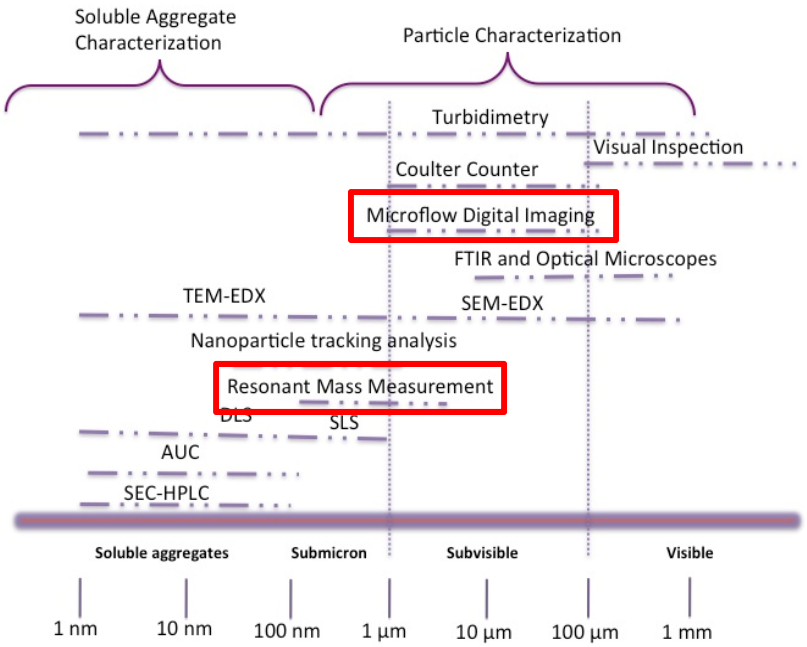


Size Exclusion Chromatography (SE-HPLC)

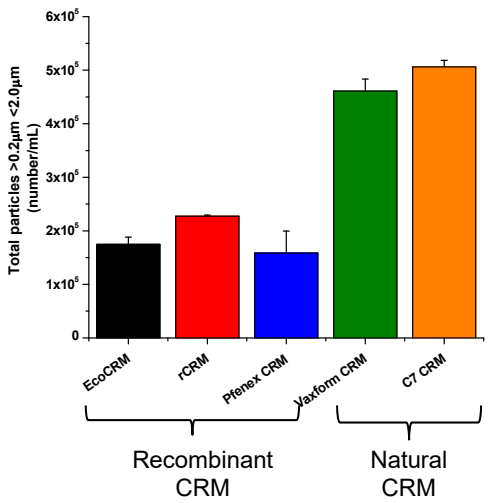


n = 3

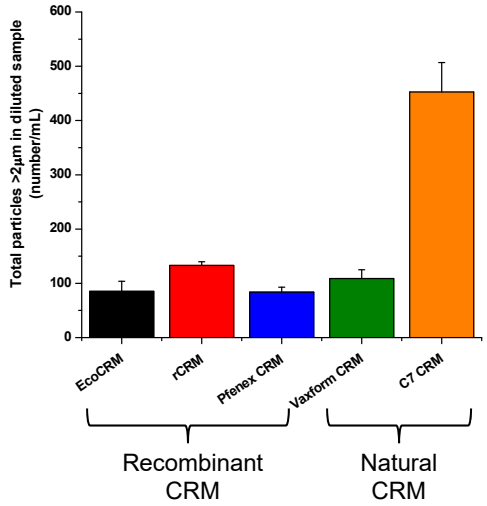
Sub-Micron and Sub-Visible Particle Analysis



Sub-Micron Particle Quantification by Resonance Mass Measurement



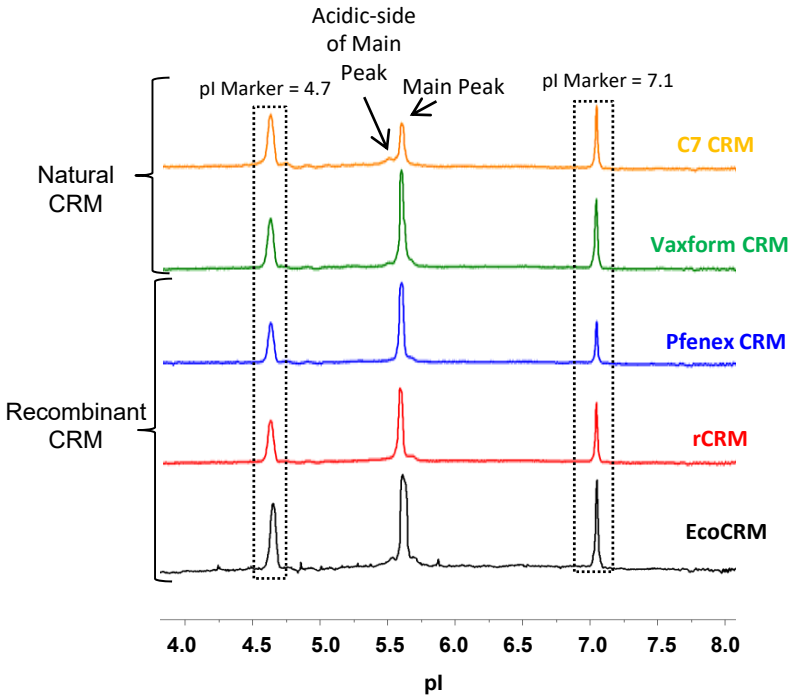
Sub-Visible Particle Quantification by MFI



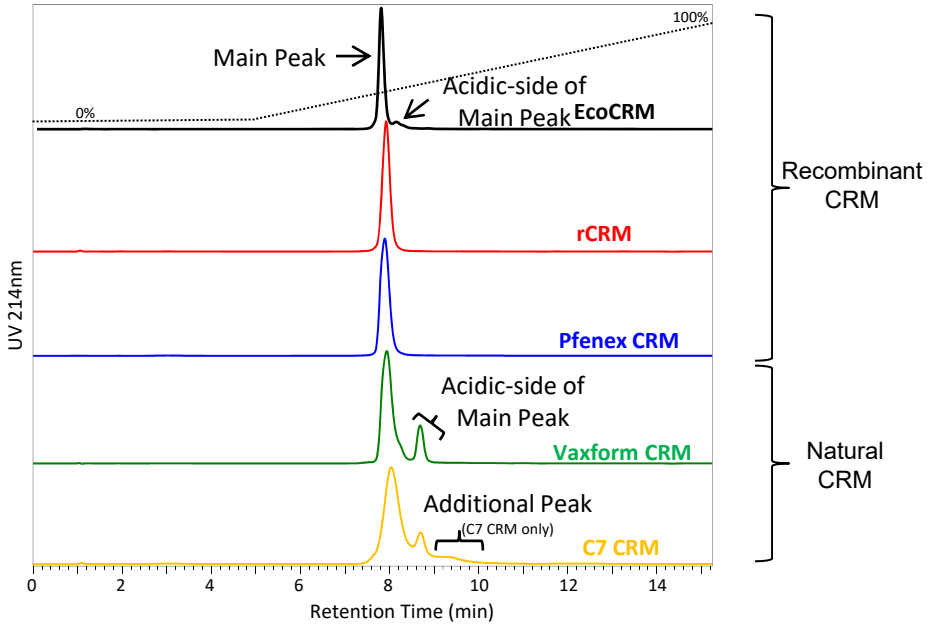
n = 3

Comparison of Protein Charge Heterogeneity

Capillary Isoelectric Focusing (cIEF)



Anion-Exchange Chromatography



Recombinant CRM

Natural CRM

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- Commercial product
- Expression System: ***Escherichia coli***
- Procurement: **Collaboration**

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- Relative Solubility Profiles
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(2018) Hickey, J.M. et al, J. Pharma. Sci., 107:1806-1819

Overview of comparing the physical stability of each CRM molecule as a function of stress (pH & temperature)

EcoCRM™

rCRM

Pfenex CRM

Vaxform CRM

C7 CRM

Structural Attribute	Analytical Techniques
Secondary Structure	Far UV Circular Dichroism
Tertiary Structure and Aggregation Behavior	Intrinsic Tryptophan Fluorescence Spectroscopy
	Static Light Scattering
Overall Conformational Stability	Differential Scanning Calorimetry (DSC)

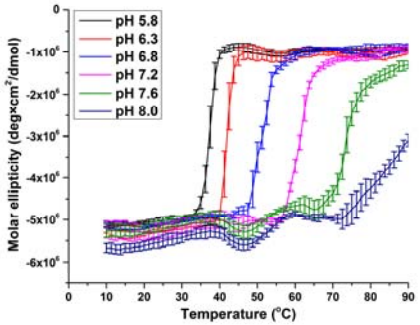
PBS

- **pH 5.8**
- **pH 6.3**
- **pH 6.8**
- **pH 7.2**
- **pH 7.6**
- **pH 8.0**

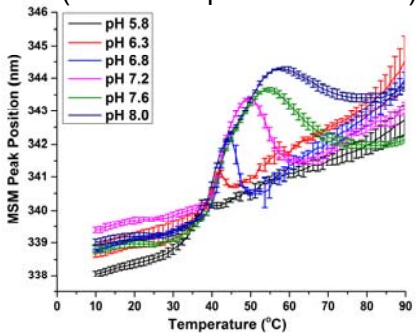
10-90˚C

Construction of Empirical Phase Diagrams
Data Visualization: Large Biophysical Stability Data Sets

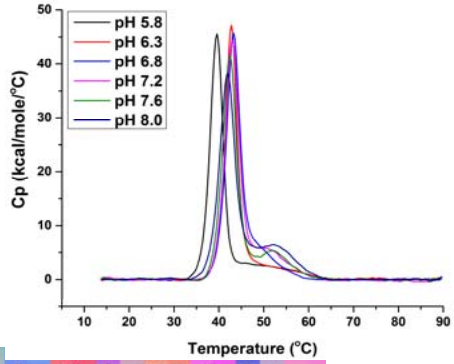
Secondary Structure
(Circular Dichroism)



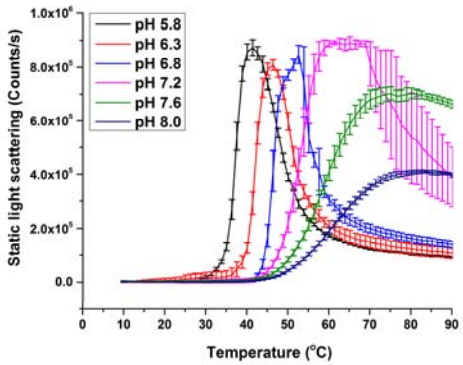
Tertiary Structure
(Intrinsic Trp Fluorescence)

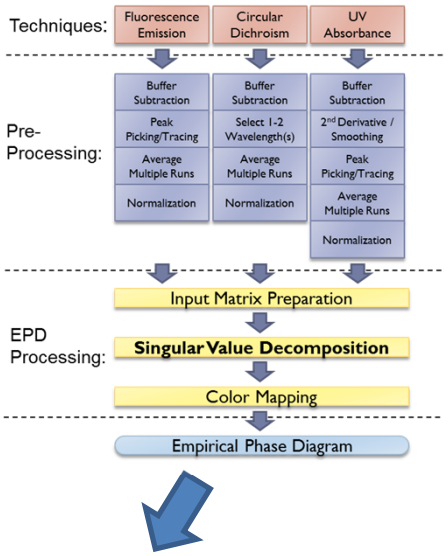


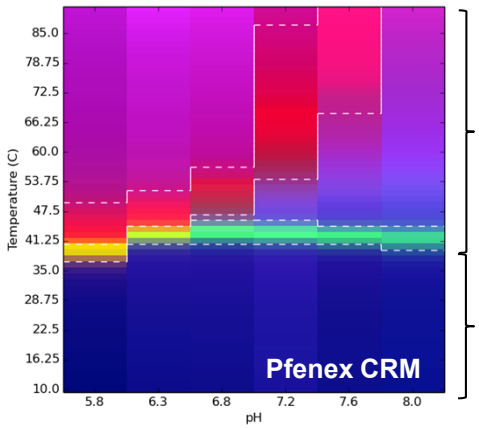
Overall Conformational Stability
(Differential Scanning Calorimetry)



Aggregation Propensity
(Static Light Scatter)







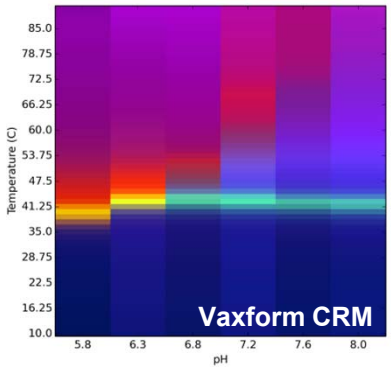
Various states of altered structure and aggregation formation

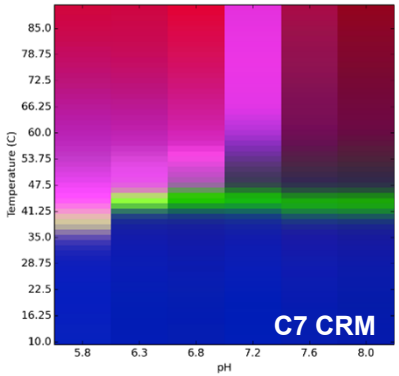
Native-like structure

Review: Maddux et al, *J Pharm Sci* 100: 4171 (2011)

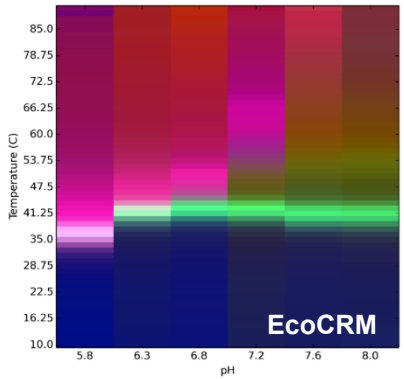
Empirical phase diagram of CRM molecules in PBS

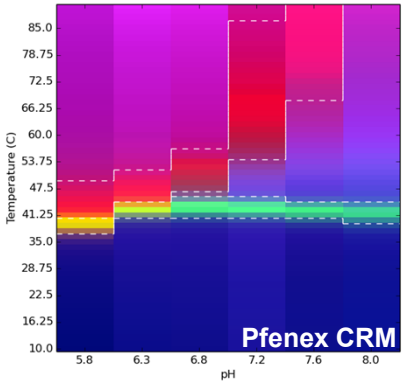
Natural CRM

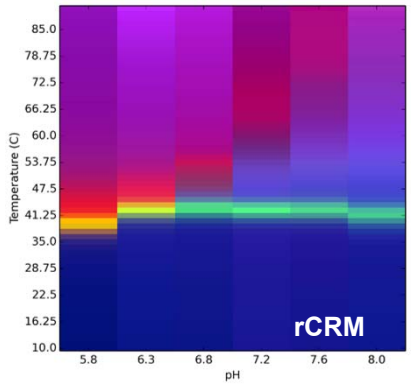




Recombinant CRM







Various states of altered structure and aggregation formation

Native-like structure

Source	CRM	Relative area of native-like structure
Recombinant	EcoCRM	~35%
	rCRM	~37%
	Pfenex CRM	~37%
Natural	Vaxform CRM	~37%
	C7 CRM	~36%

Overall comparison of natural and recombinant CRM

Recombinant CRM

FinaBio Fina Biosolutions LLC	EcoCRM™
Biological E. Limited Celebrating Life Every Day	rCRM
Reagent Proteins A DIVISION OF PFEnex	Pfenex CRM

Natural CRM

VaxForm LLC	Vaxform CRM
LIST BIOLOGICAL LABORATORIES, INC.	C7 CRM

- **Physicochemical Properties**
  - Overall similar structural integrity and conformational stability but measurable differences were observed
- **Physical Stability as a function of stress (pH vs. Temp.)**
  - Similar conformational stability/aggregation propensity as a function of pH (5.8-8.0) and temperature (10-90˚C).
- **Relative Solubility Profiles**
  - Similar solubility profile in PBS pH 7.2
- ***In vitro* Immunological Assays**
  - Similar *in vitro* immunological reactivity

Acknowledgements

- Prof David Volkin
- Dr. Sangeeta Joshi
- Dr. Kawaljit Kaur
- Dr. Vishal Toprani

Collaborators

Dr. Robert Sitrin

Dr. Andrew Lees

Dr. Akshay Goel
Dr. Ravipratapnarayan Mishra

Funding

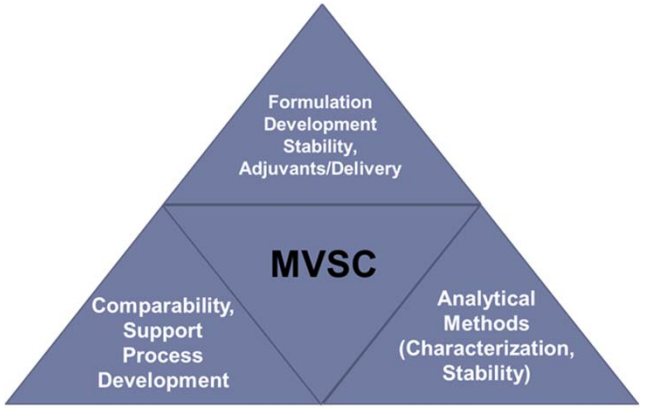
Provided Material

Dr. Garry Morefield

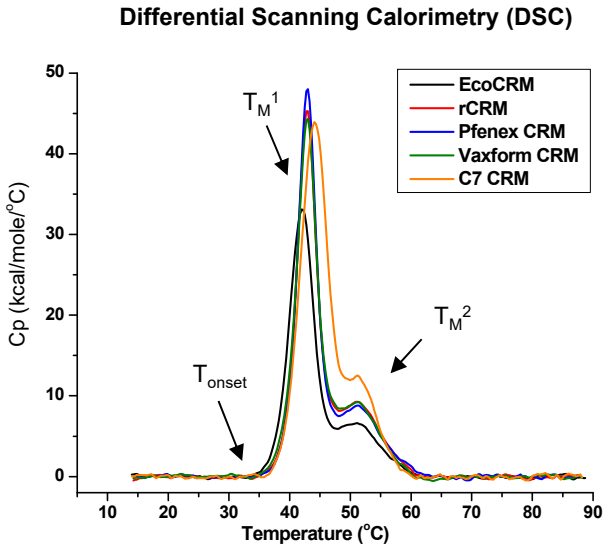
Macromolecule and Vaccine Stabilization Center

Thank you for your attention!

Questions?



Overall Conformational Stability as a Function of Temperature



Source	CRM	T_{onset} (˚C) Mean ± 3SD	T_M1 (˚C) Mean ± 3SD	T_M2 (˚C) Mean ± 3SD
Recombinant	rCRM	35.0 ± 0.2	42.8 ± 0.1	51.1 ± 0.1
	EcoCRM	32.7 ± 0.3	42.0 ± 0.1	51.3 ± 0.2
	Pfenex CRM	34.8 ± 0.2	42.8 ± 0.1	51.1 ± 0.2
Natural	Vaxform CRM	33.5 ± 0.2	42.8 ± 0.1	51.1 ± 0.3
	C7 CRM	35.2 ± 0.6	44.0 ± 0.1	51.7 ± 0.1

n = 3

Recombinant CRM

Natural CRM

FinaBio (Fina Biosolutions LLC)

- Commercial product
- Expression System: ***Escherichia coli***
- Procurement: **Collaboration**

Biological E. Limited (Celebrating Life Every Day)

- Commercial product
- Expression System: ***Escherichia coli***
- Procurement: **Collaboration**

Reagent Proteins (A division of PFEnex)

- Commercial product
- Expression System: ***Pseudomonas fluorescens***
- Procurement: **Purchased**

VaxForm LLC

- Non-commercial product (2006, stored frozen)
- Expression System: ***Corynebacterium diptheriae***
- Procurement: **Donated**

List Biological Laboratories, Inc.

- Commercial product
- Expression System: ***Corynebacterium diptheriae***
- Reference C7 "natural" CRM
- Procurement: **Purchased**

KU The University of Kansas — Analytical Comparison

- Physicochemical Properties
- Physical Stability as a function of stress (pH vs. Temp.)
- Relative Solubility Profiles
- *In vitro* Immunological Assays

(2018) Hickey, J.M. et al, J. Pharma. Sci., 107:1806-1819

Measuring binding affinity between CRM and mAbs

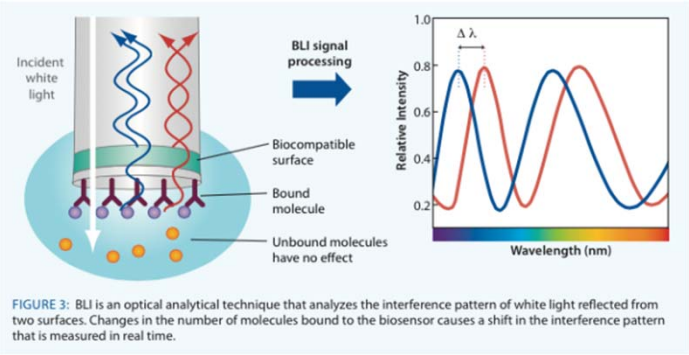
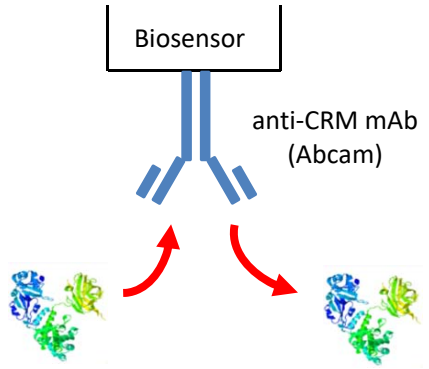


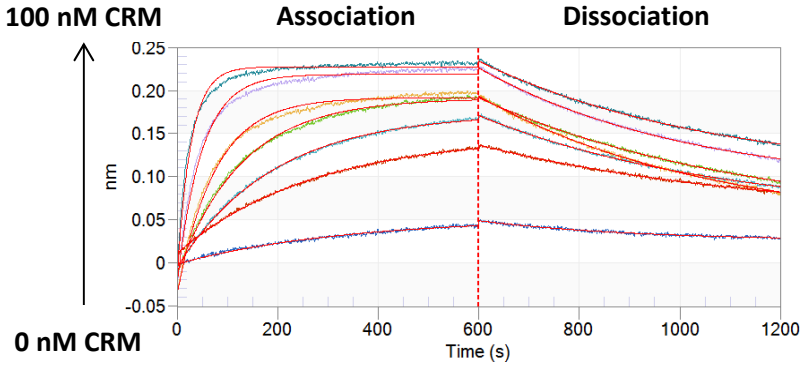
FIGURE 3: BLI is an optical analytical technique that analyzes the interference pattern of white light reflected from two surfaces. Changes in the number of molecules bound to the biosensor causes a shift in the interference pattern that is measured in real time.

Pall Life Sciences 2013

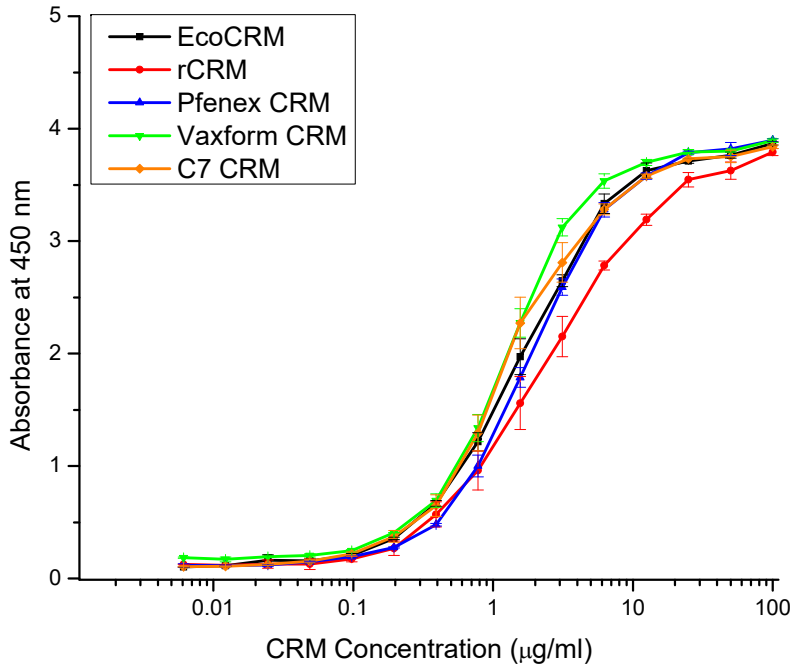


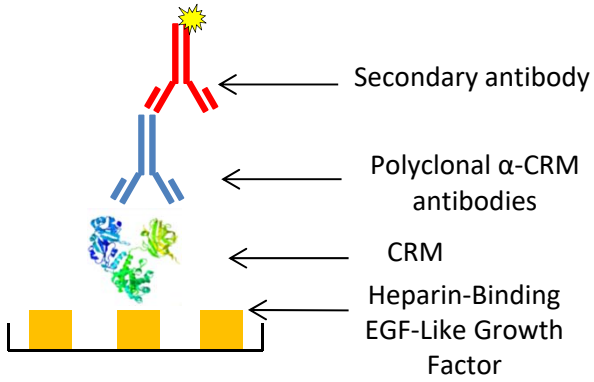
Source	CRM	K_{on} (M^{-1} s^{-1}) Mean ± 3SD	K_{off} (s^{-1}) Mean ± 3SD	K_D Mean ± 3SD
Recombinant	EcoCRM	$4 \pm 1 \times 10^{5}$	$1 \pm 1 \times 10^{-3}$	3 ± 5 nM
	rCRM	$5 \pm 1 \times 10^{5}$	$1 \pm 1 \times 10^{-3}$	3 ± 6 nM
	Pfenex CRM	$5 \pm 2 \times 10^{5}$	$1 \pm 1 \times 10^{-3}$	3 ± 5 nM
Natural	Vaxform CRM	$5 \pm 1 \times 10^{5}$	$2 \pm 1 \times 10^{-3}$	3 ± 3 nM
	C7 CRM	$4 \pm 1 \times 10^{5}$	$2 \pm 1 \times 10^{-3}$	5 ± 6 nM

n = 3



ELISA using polyclonal anti-CRM





Source	CRM	LogEC$_{50}$ Mean ± 3SD (μg/ml CRM)	99% Confidence Interval (μg/ml CRM)	R^2
Recombinant	rCRM	2.3 ± 0.9	1.5 to 3.1	0.97
	EcoCRM	1.6 ± 0.9	0.9 to 2.3	0.99
	Pfenex CRM	1.8 ± 0.3	1.4 to 2.2	0.99
Natural	Vaxform CRM	1.3 ± 0.3	1.1 to 1.5	0.99
	C7 CRM	1.3 ± 0.3	1.0 to 1.6	0.98

n = 3

Recombinant CRM

Natural CRM

FinaBio (Fina Biosolutions LLC)

- Commercial product
- Expression System: ***Escherichia coli***
- Procurement: **Collaboration**

Biological E. Limited (Celebrating Life Every Day)

- Commercial product
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- Non-commercial product (2006, stored frozen)
- Expression System: ***Corynebacterium diptheriae***
- Procurement: **Donated**

List Biological Laboratories, Inc.

- Commercial product
- Expression System: ***Corynebacterium diptheriae***
- Reference C7 "natural" CRM
- Procurement: **Purchased**

KU The University of Kansas — Analytical Comparison

- Physicochemical Properties
- Physical Stability as a function of stress (pH vs. Temp.)
- **Relative Solubility Profiles**
- *In vitro* Immunological Assays

PEG relative solubility of CRM molecules (Overview of PEG assay)

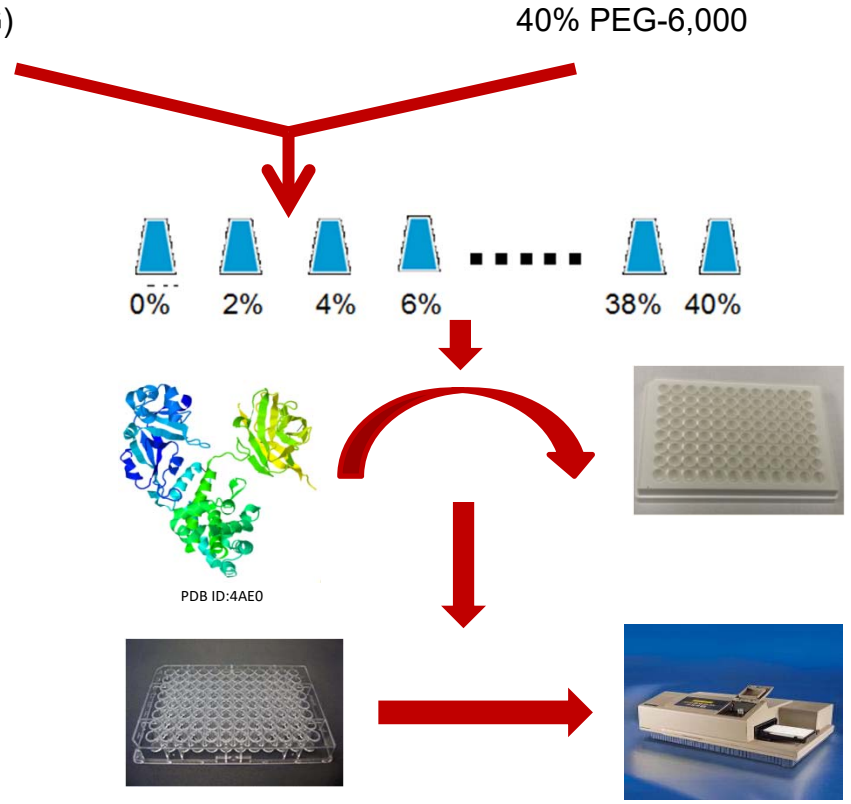
1) Combine buffer and 40% PEG to desired %PEG solutions, and mix thoroughly.

2) Add to 96 well 0.2µm filter plate

3) Add protein, incubate at room temperature overnight.

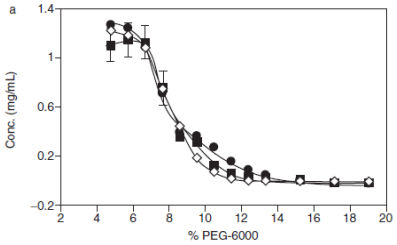
4) Centrifuge plate @3500 rpm for 15 min

5) Transfer equal volumes to a clean UV plate and measure A_{280} on plate reader



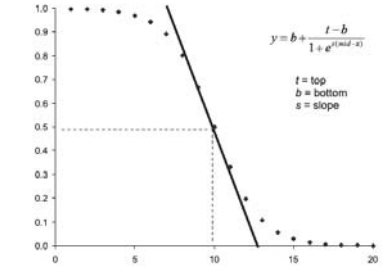
Data analysis from PEG Assay of CRM molecules

Three types of information can be obtained:



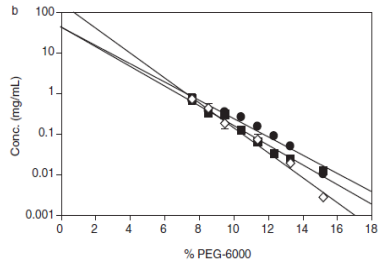
1) Overlaying of curves :

- To compare solution conditions for a single molecule
- To compare different molecules under similar solution conditions



2) % PEG_{midpt} : is the weight% PEG in solution required to decrease the protein concentration by 50%.

- Quicker way of relative solubility assessments, and for high throughput apparent solubility screening of different molecules.



3) Apparent solubility (thermodynamic activity): calculated based on assumptions (not shown) and resulting in the equation.

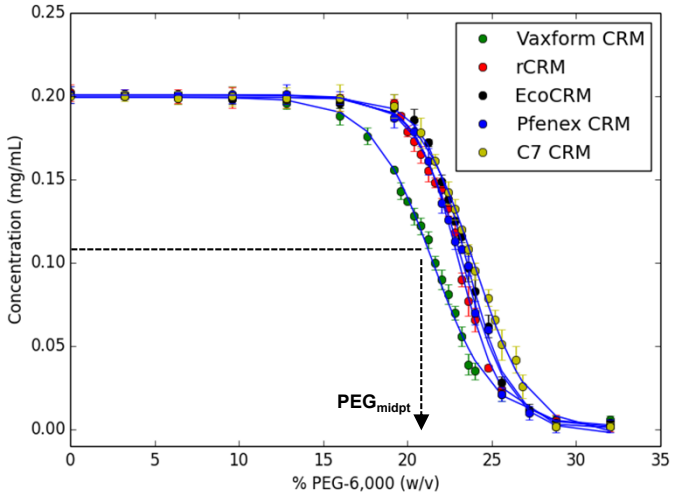
log Sp =loga_0-A_{12}[PEG] where:

Sp is the concentration of soluble protein in a solution containing PEG-precipitated protein in equilibrium with soluble protein
a_0 is the activity of the protein in a saturated PEG-free environment
A_{12} is virial coefficient

(1979) Middaugh CR, et al, JBC, 254(2):367-370
(2011) Gibson TJ, et al *J Pharm Sci, 100*(3):1009-1021

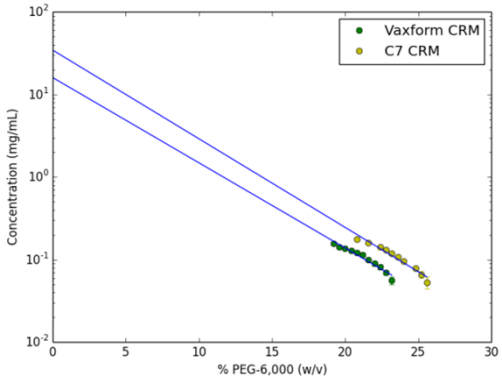
Relative PEG solubility of CRM molecules in PBS pH 7.2

PEG_{midpt} :PEG concentration at which 50% of the protein has precipitated out of solution

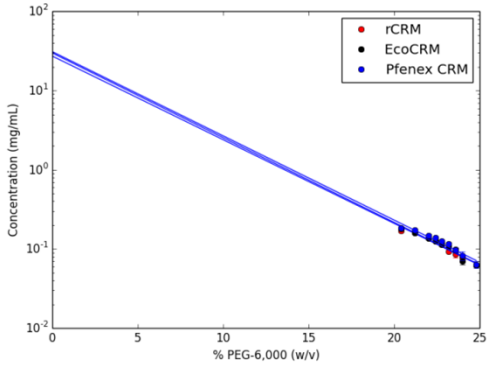


Apparent Solubility

Natural CRM



Recombinant CRM



Source	CRM	$\%PEG_{midpt}$ (w/v) (Mean ± 3SD)	Apparent Solubility (mg/ml) (Mean ± 3SD)
Recombinant	rCRM	23.0 ± 0.2	27 ± 9
	EcoCRM™	23.3 ± 0.2	30 ± 12
	Pfenex CRM	23.5 ± 0.1	31 ± 11
Natural	Vaxform CRM	21.5 ± 0.2	16 ± 10
	C7 CRM	24.0 ± 0.2	34 ± 12

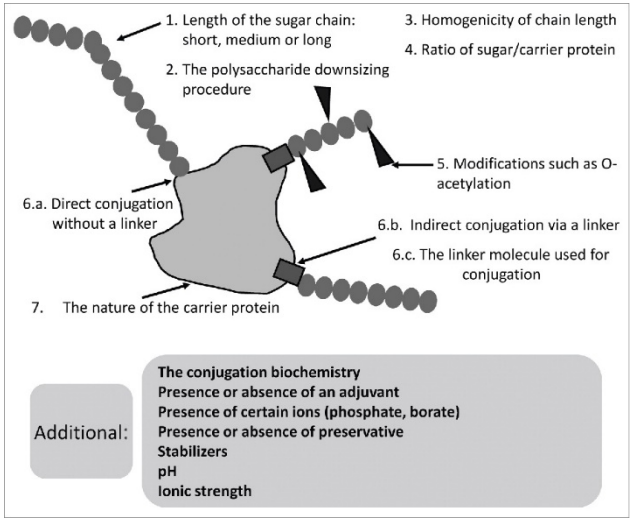
n = 3

Pharmaceutical Quality/CMC of CRM

- Can be used to assess comparability, lot-to-lot variability, process changes, etc.

Structural Attribute	Analytical Techniques
Protein Concentration, Purity, and Proenzyme "nicking"	UV-Visible Spectroscopy
	SDS-PAGE (Reduced vs. Non-reduced)
Primary Structure	Intact Mass
	Peptide Mapping
Additional Assays for Primary Structure/Protein Heterogeneity (Purity)	Capillary Isoelectric Focusing (cIEF)
	Anion Exchange Chromatography (AEX)
Secondary Structure	Far UV Circular Dichroism
Tertiary Structure	Intrinsic Fluorescence Spectroscopy
Overall Conformational Stability vs. Temperature	Differential Scanning Calorimetry (DSC)
Characterization of Size and Presence of Aggregates	Size Exclusion Chromatography (+/-MALS)
	Archimedes (RMS)
	Micro Flow Imaging (MFI)

Factors that could influence immunogenicity:



- Critical quality attributes?

- Polysaccharide conjugation and immunogenicity of recombinant vs. natural CRM?

(2016) Bröker, M. et al, Hum. Vaccin. & Immunother., 12:1808-1824