A Case Study of Early Phase Purification Process Development for an Anti-Cancer Minibody

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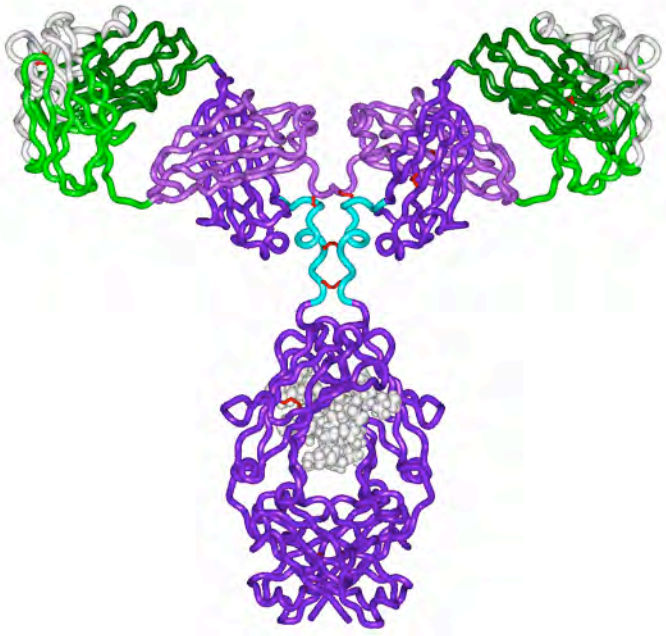
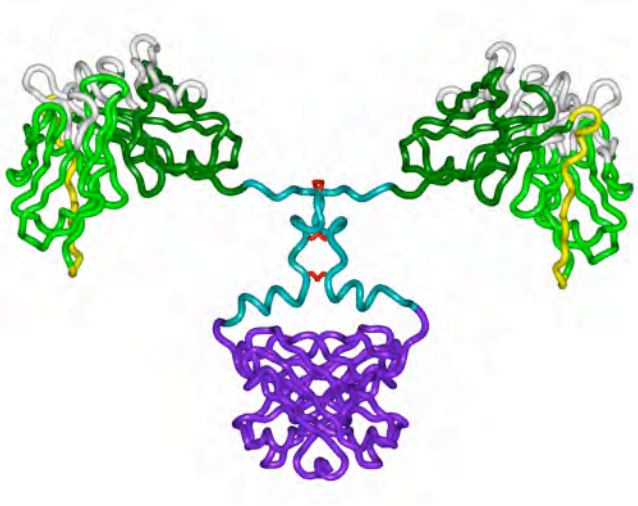
Purification of minibodies

The structural similarity of minibodies to antibodies invites the expectation that their purification might be similarly well defined and simple to develop.



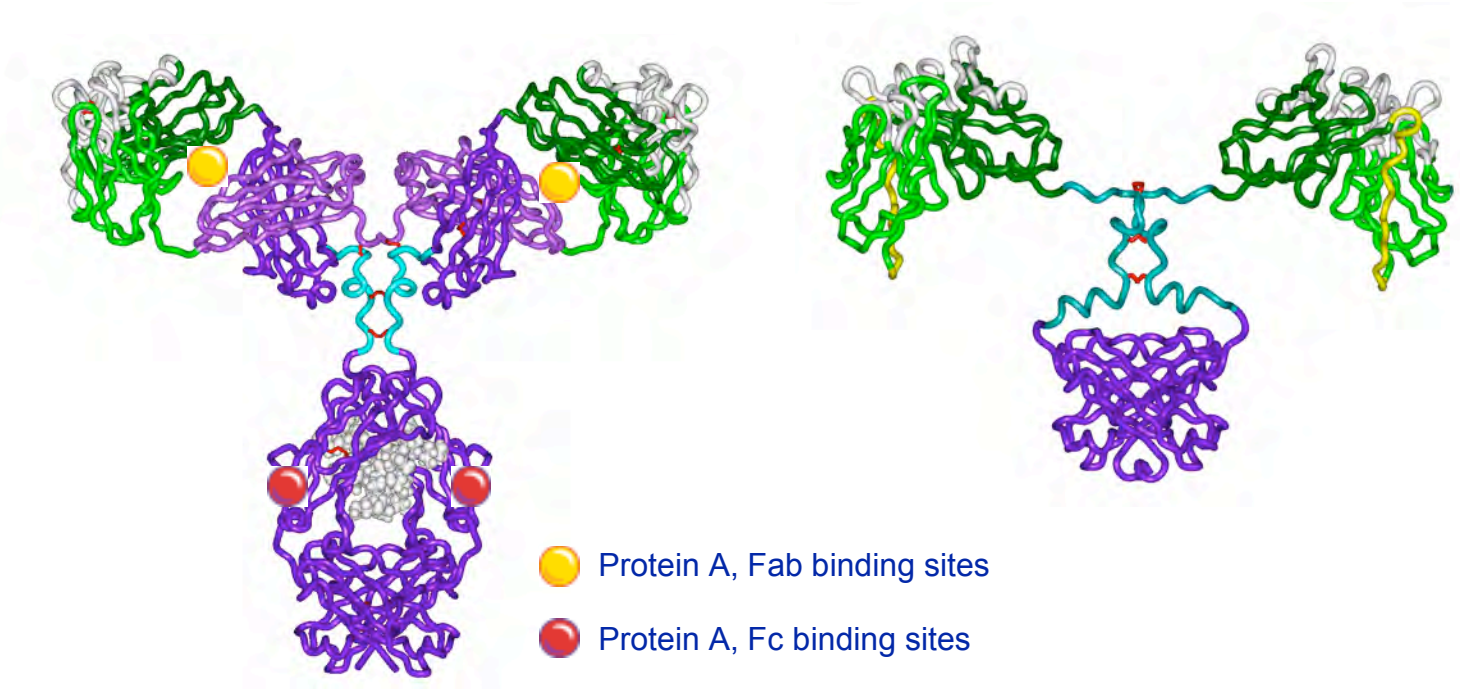


VL: light green
VH: dark green
CDRs: white
CH3: purple

GS18 linker: yellow
Hinge and GS10 linker: cyan
Disulfide bonds: red

Purification of minibodies

But appearances can be deceiving. The principal tool for purification of IgG – the one that enables a simple platform approach to process development – is not applicable to minibodies.



Purification of minibodies

Minibody behavior differs from IgG with non-affinity methods as well.

Cation Exchange

CIM™ SO_3 Monolith

A: 20 mM MES, pH 6.0

B: A + 1 M NaCl

Dilute sample 1:9 with A

Equilibrate column with A

Load sample

Wash A

Elute: 30 CVLG to 50% B

Clean with B

MiniB eluted as a trailing shoulder on the BSA peak. IgGs elute much later.

Anion Exchange

CIM QA Monolith

A: 20 mM Tris, pH 8.0

B: A + 1 M NaCl

Dilute sample 1:9 with A

Equilibrate column with A

Load sample

Wash A

Elute: 30 CVLG to 50% B

Clean with B

MiniB eluted as a leading shoulder on the BSA peak. IgGs elute much earlier.

Hydroxyapatite

CHT™ Type I, 40 µm

A: 10 mM $NaPO_4$, pH 7.0

B: 500 mM $NaPO_4$, pH 7.0

Dilute sample 1:1 with A

Equilibrate column with A

Load sample

Wash A

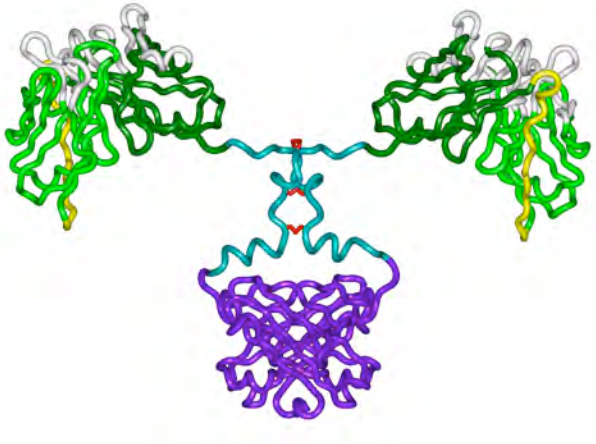
Elute: 30 CVLG to 50% B

Clean with B

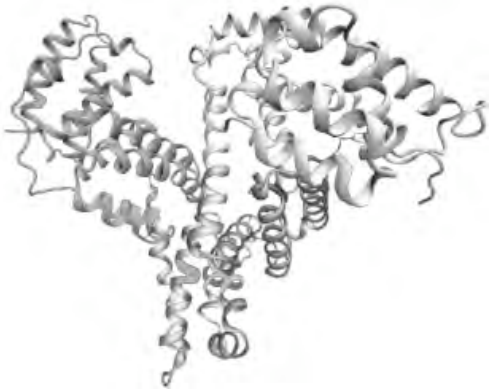
MiniB co-eluted with BSA. IgGs elute much later, often at purity greater than 90%.

BSA removal from the minibody

Shared features of minibodies and BSA.



pI ~5.1, ~80 KDa



pI ~5.2, ~70 KDa

Albumin removal is further complicated by the fact that it spontaneously forms homo-polymers, and stable complexes with a variety of fatty acids, metals, and other small molecules, plus disulfide bonded hybrids with a variety of proteins, all of which increase its chemical heterogeneity. Thus albumin elutes across a much broader zone than well-defined proteins, and is more likely to co-elute with product over a wide range of separation methods and conditions.

Ribbon model of albumin modified from S. Fujiwara and T. Amisaki, 2008, Biophys. J., 94(1): 95-103

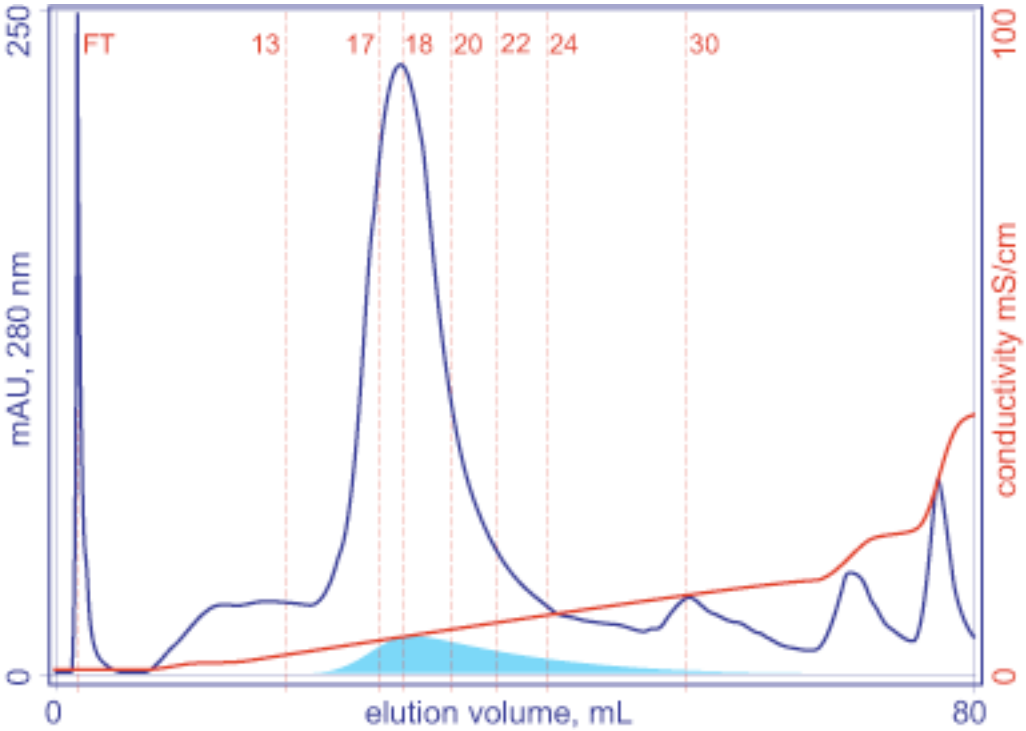
BSA removal from the minibody

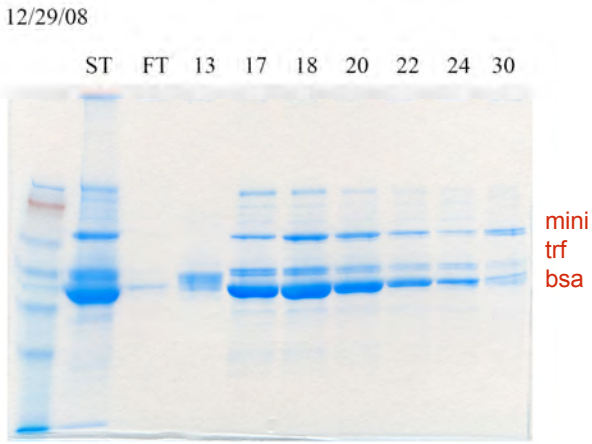
Luck is what happens when preparation meets opportunity.

–Lucius Annaeus Seneca

BSA removal from the minibody

Hydroxyapatite, phosphate gradient elution



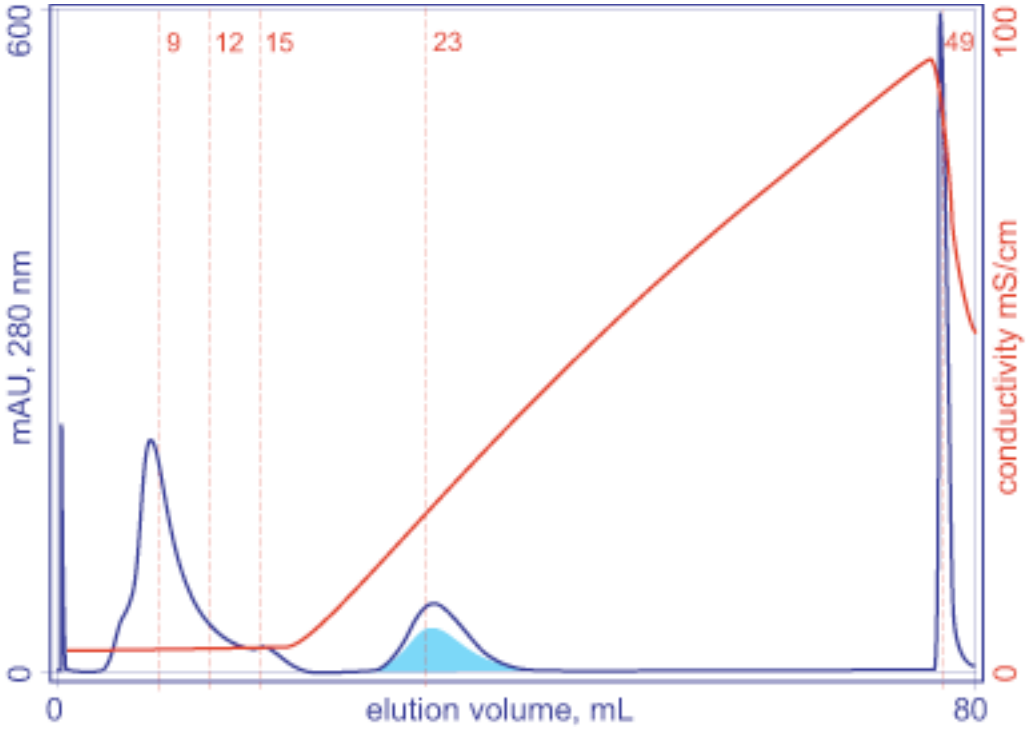


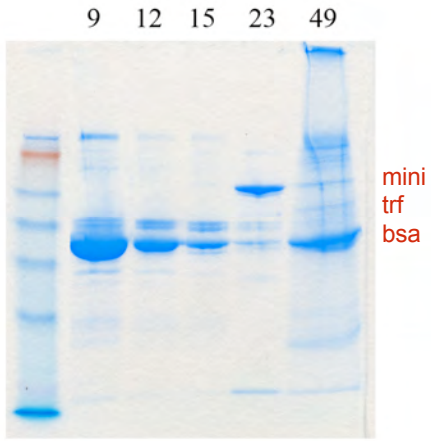
CHT™ type I, 40 µm, 300 cm/hr.
Equilibrate: 20 mM KPO_4, pH 6.5
Load: Mini cell culture supernatant
Wash: 20 mM KPO_4, pH 6.5
Elute: 20 CVLG to 250 KPO_4, pH 6.5
Step to 300 mM KPO_4, pH 6.5
Step to 500 mM KPO_4, pH 6.5
Cyan peak represents minibody

Virtually no separation from BSA or transferrin.

BSA removal from the minibody

Hydroxyapatite, NaCl gradient



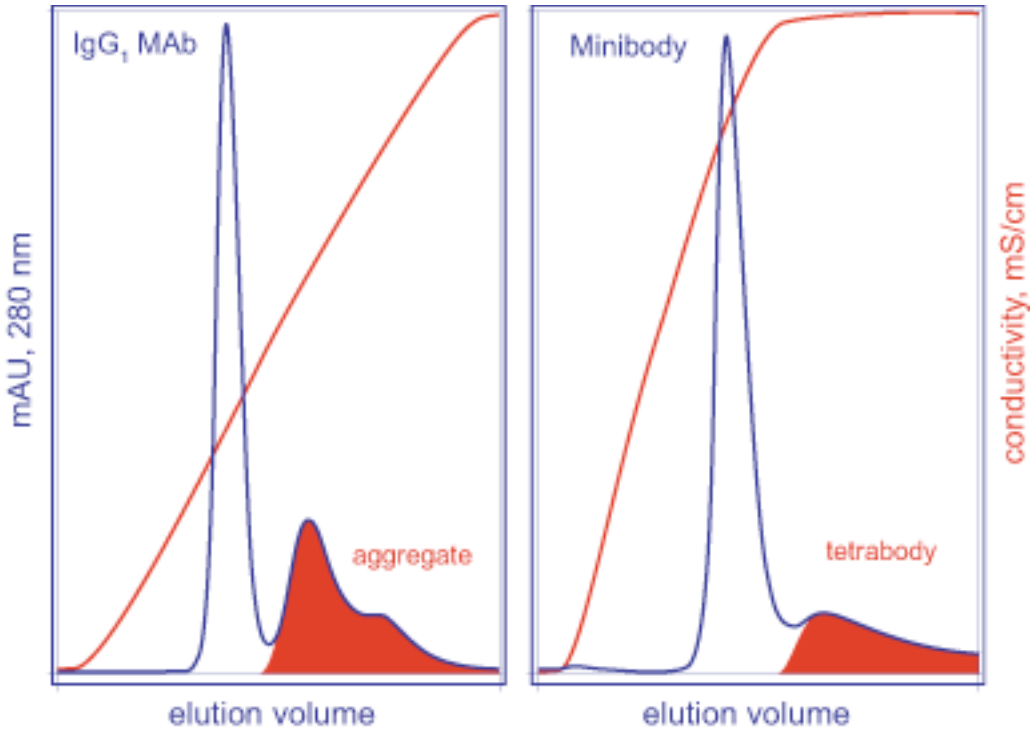


CHT type I, 40 µm, 300 cm/hr.
Equilibrate: 20 mM $NaPO_4$, pH 6.5
Inj: mini cell culture supernatant
Wash: 20 mM $NaPO_4$, pH 6.5
Elute: 30 CVLG to 20 mM $NaPO_4$, 1.0 M NaCl, pH 6.5
Cyan peak represents minibody

Near-total elimination of BSA and transferrin under initial screening conditions! Trf elutes mostly in the wash. BSA elutes mostly in the cleaning step.

Hydroxyapatite with NaCl gradients

Removal of high molecular weight species, IgG vs minibody



*IgG profile modified from Gagnon, P., Beam, K., 2009, Antibody Aggregate Removal with Hydroxyapatite, Current Pharmaceutical Biotechnology, **10**(4) 440-446, with permission.*

IgG
CHT type I, 40 µm, 300 cm/hr.
Equilibrate: 10 mM $NaPO_4$, pH 7.0
Inj: protein A-purified Mab
Wash: 10 mM $NaPO_4$, pH 7.0
Elute: 30 CVLG to 10 mM $NaPO_4$, 1.0 M NaCl, pH 7.0

Minibody
CHT type I, 40 µm, 300 cm/hr.
Equilibrate: 10 mM $NaPO_4$, pH 7.0
Inj: MMC/AX-purified minibody
Wash: 10 mM $NaPO_4$, pH 7.0
Elute: 10 CVLG to 10 mM $NaPO_4$, 1.0 M NaCl, pH 7.0
Tetrabody is believed to form by non-covalent association of “Fab” regions.

Hydroxyapatite with NaCl gradients

Why did minibody behavior mimic IgG in NaCl gradients but not in phosphate gradients?

The results suggest, as with IgG, that minibodies participate in weak calcium affinity interactions with hydroxyapatite. A low concentration of phosphate (10 mM) largely eliminates these interactions, leaving the minibody bound principally by phosphoryl cation exchange, which can then be eluted with the NaCl gradient.

BSA, which participates in strong calcium affinity interactions with hydroxyapatite, requires ~50 mM phosphate to elute. At 10 mM phosphate its retention is thereby protected from NaCl and it remains bound, along with DNA, endotoxin, and viruses.

Hydroxyapatite with NaCl gradients

Purification of IgG

Contaminant	Method	Clearance
DNA	Picogreen	> 3 logs
Endotoxin	LAL (chromo)	> 4 logs
aMULV	Infectivity	> 4 logs
xMULV	Infectivity	> 3 logs
MVM	Infectivity	2 logs

Extensive characterization with many monoclonal IgGs shows that all of these contaminants elute in the 500 mM phosphate strip —not during the NaCl gradient. Thus similar results can be expected with minibodies.

Gagnon, P., 2009, Monoclonal Antibody Purification with Hydroxyapatite, New Biotechnology, ***25*** *287-293*

Capture with hydroxyapatite?

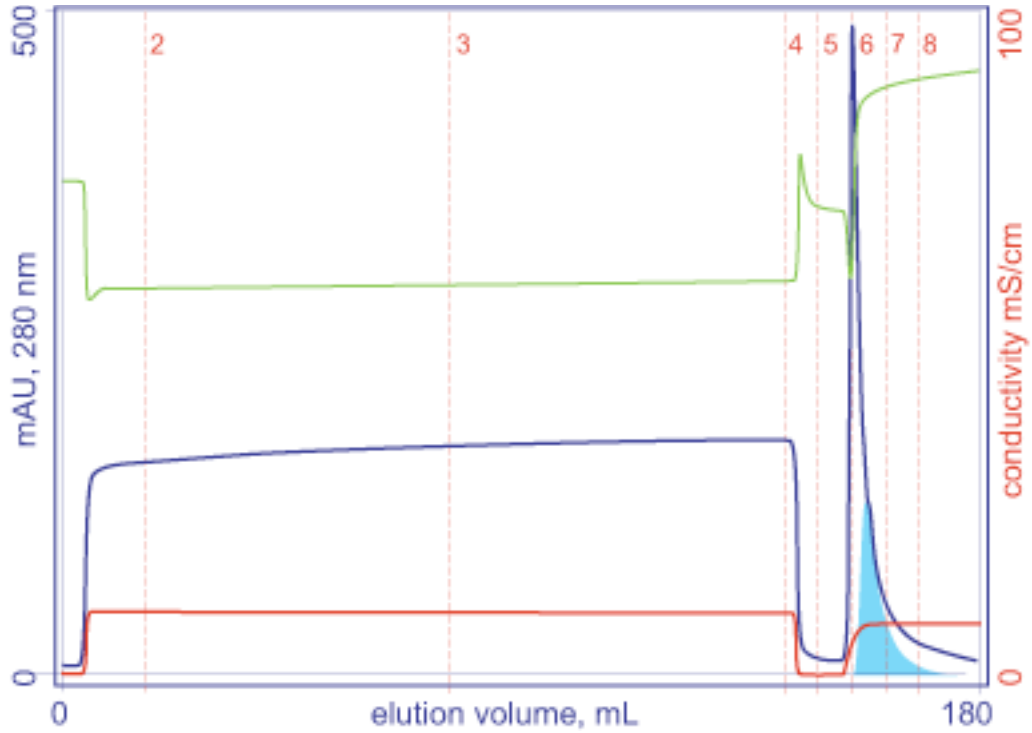
Despite excellent purification, minibody breakthrough binding capacity was only ~12 mL of supe (600 µg minibody) per mL of hydroxyapatite, mostly due to competition from stronger-binding contaminants – chiefly albumin. Not a candidate for capture.

Capture with other methods?

Cation exchange: ~15 mL supe (750 µg) per mL of resin, mostly due to competition from contaminants – chiefly albumin. Purity of the eluted minibody was less than 20%.

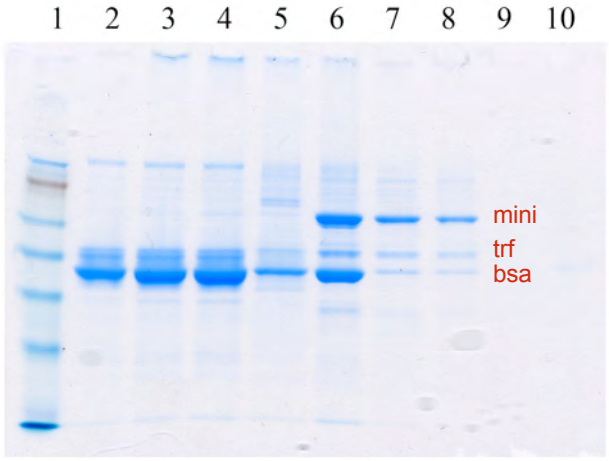
Anion exchange: < 2 mL supe (100 µg) per mL of resin, due to weak minibody binding and competition from albumin. Purity <10%.

Minibody capture with Capto MMC



5-6 times greater capacity than cation exchange and more than twice the purity!

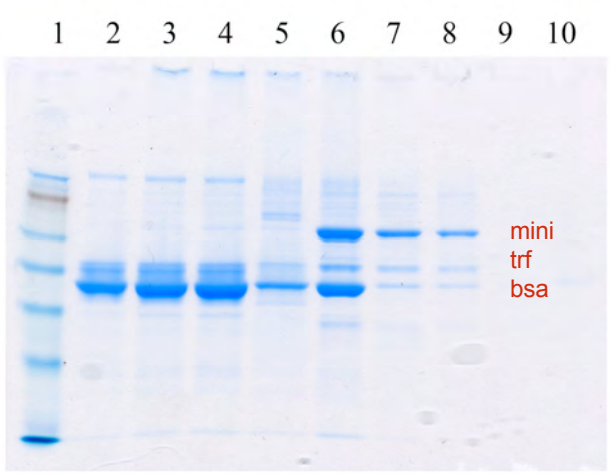
BSA was enriched on the leading side of the peak, minibody on the trailing side.



Capto™ MMC, 1 mL HiTrap™
200 cm/hr (1 mL/min)
Sample prep: dilute mini supe 1:1 with 50 mM MES, pH 6.0
Equilibrate: 20 mM Hepes, pH 7.0
Inj: 140 mL sample
Wash: 20 mM Hepes, pH 7.0
Elute: Step to 20 mM Tris, 60 mM NaCl, pH 8.0

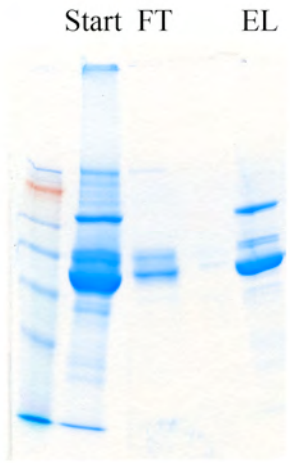
Load-dependent binding selectivity

70 mL supernatant per mL MMC



Minibody/BSA ratio (lane 6) ~1/1

2.5 mL supe



Mini/BSA ~1/5

These results indicate that the minibody outcompetes BSA for binding substrate and displaces it (to a degree) from the column as loading proceeds. This suggests that loading specifications should be based primarily on minibody content of the individual feedstream, but cautions that capacity will be influenced by the ratio of product to BSA.

Load-dependent elution selectivity

Using a 20 mL load (on a 1 mL MMC column), we developed wash conditions to selectively remove most of the leading albumin shoulder, with the goal of improving purity and reproducibility of the MMC step.

But, when the load was increased to 70 mL, more than half of the minibody eluted in the wash.

Managing load-dependent capture

Load-dependent performance is undesirable because it predicts poor reproducibility of product recovery and purity as a function of variations among cell culture production methods, potentially even among different production lots within a single production method.

It is also undesirable because it increases the burden on purification process development:

- *It renders preliminary process modeling with 1 mL columns inaccurate – but larger scale modeling requires proportionately greater sample volumes, which may not be available during early development stages.*
- *Variability at the capture step must be absorbed by subsequent purification steps, which means that more work will be required to screen, identify, and optimize those steps.*

Managing load-dependent capture

Choose the life [chromatography method] that is most useful, and habit will make it the most agreeable.

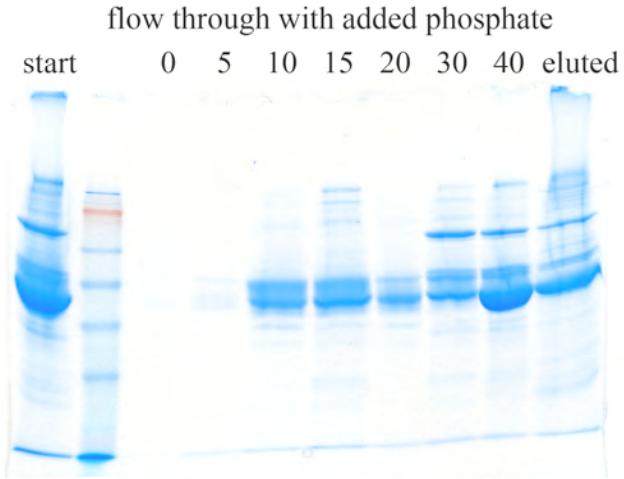
–Sir Francis Bacon

Managing load-dependent capture

Accommodations at the HA step

We evaluated the effect of phosphate concentration on binding selectivity.

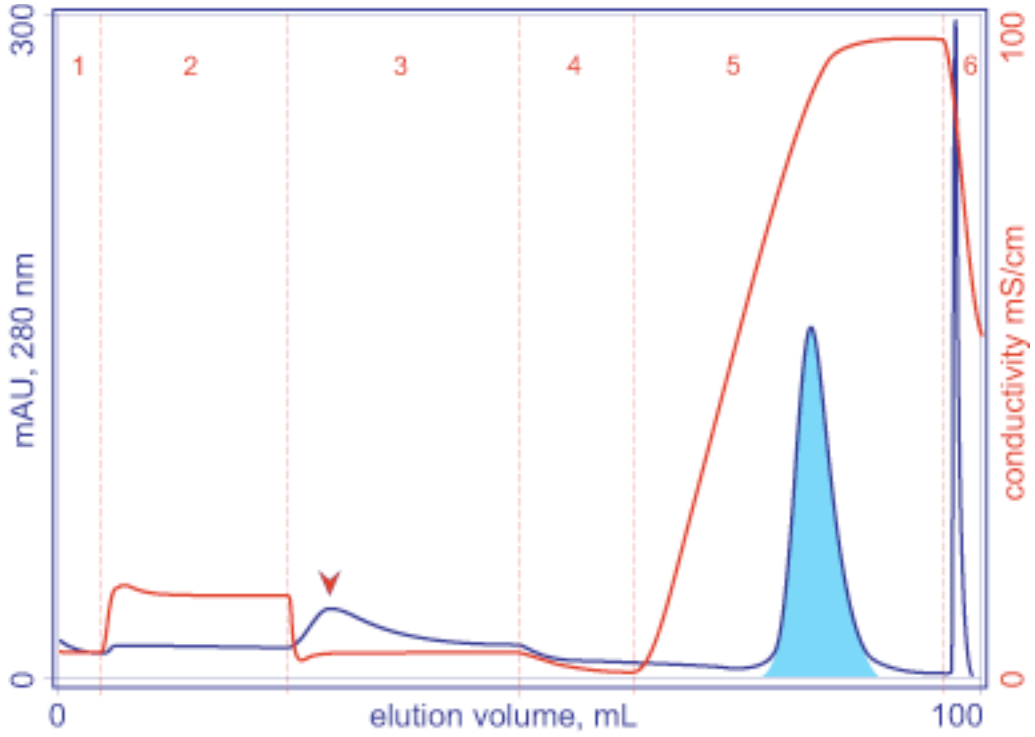
Results suggested that highest capacity would be achieved at 5 mM but the column could be washed at 25 mM phosphate to remove contaminants bound by moderate calcium affinity. Best elution selectivity was achieved with a chloride gradient at 10 mM phosphate.*



**Capacity would be higher in the absence of phosphate, but 5 mM is required to maintain the stability of hydroxyapatite.*

Managing load-dependent capture

Accommodations at the HA step



CHT type I, 40 µm, 300 cm/hr.
Sample prep: dilute AX FT 1:1 with 10 mM $NaPO_4$, pH 7.0 (final 5 mM PO4)
Equilibrate: 30 mM $NaPO_4$, pH 7.0
Inject
Wash1: 30 mM $NaPO_4$, pH 7.0
Wash2: 10 mM $NaPO_4$, pH 7.0
Elute: 10 CVLG to 10 mM $NaPO_4$, 1.0 M NaCl, pH 7.0
Clean: 500 mM $NaPO_4$, pH 7.0
Dashed lines mark buffer changes.
The red arrow marks the peak that eluted when 30 mM phosphate resumed after sample loading. Some minibody was lost in this peak, so the equilibration and first wash were subsequently reduced to 25 mM.

Managing load-dependent capture

We chose anion exchange as a third chromatography step because of its established reputation in the regulatory community for removal of DNA, endotoxin, and virus.

We evaluated both flow-through and bind-elute mode, but chose bind-elute mode because it removed more contaminants and we felt that this added extra insurance against process variation due to load-associated variability at the capture step.

We chose a high capacity porous particle-based anion exchanger because the high load of acidic contaminants (chiefly BSA) would overwhelm the comparatively low protein-binding capacity of membrane-based anion exchangers.

Process sequence and continuity

Anion exchange as a last step was impractical because the minibody eluted from hydroxyapatite at about 800 mM NaCl, which would have required insertion of a diafiltration step.

Instead, we modified MMC elution conditions for high pH and low conductivity so that the sample could be loaded onto the anion exchanger with minimal dilution.

Following MMC with anion exchange also minimized the contaminant load going into the hydroxyapatite step.

Diafiltration was unnecessary because hydroxyapatite was able to tolerate the NaCl concentration from elution of the anion exchanger, as long as the sample was equilibrated to no higher than 5 mM phosphate.

The current process

Capture

Capto MMC

Dilute filtered supernatant 1:1 with 50 mM MES, pH 6

EQ: 50 mM MES, pH 6

Load

Wash: 50 mM MES, pH 6

Elute: Step to 20 mM Tris, 75 mM NaCl, pH 8.5

Clean: 2 M guanidine, pH 5

Sanitize: 1 M NaOH

Intermediate

UNOsphere™ Q

Dilute MMC eluate 1:2 with 20 mM Tris, pH 8.5

EQ: 20 mM Tris, pH 8.5

Load

Wash: 20 mM Tris, pH 8.5

Elute: 10 CVLG* to 20 mM Tris, 225 mM NaCl, pH 8.5

Clean: 1 M NaCl, pH 8.5

Sanitize: 1 M NaOH

Polishing

CHT Type I, 40 µm

To AX eluate, add $NaPO_4$ to final concentration of 5 mM

EQ: 25 mM $NaPO_4$, pH 7

Load

Wash1: 25 mM $NaPO_4$, pH 7

Wash2: 10 mM $NaPO_4$, pH 7

Elute: 10 CVLG* to 10 mM $NaPO_4$, 1 M NaCl, pH 7

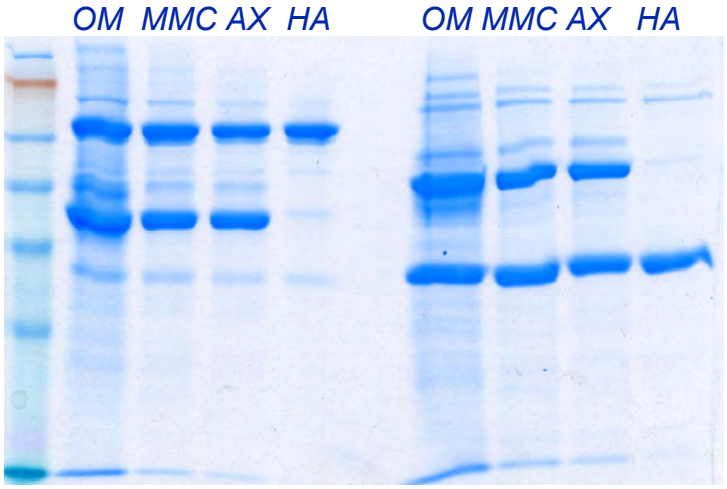
Clean: 500 mM $NaPO_4$, pH 7

Sanitize: 1 M NaOH

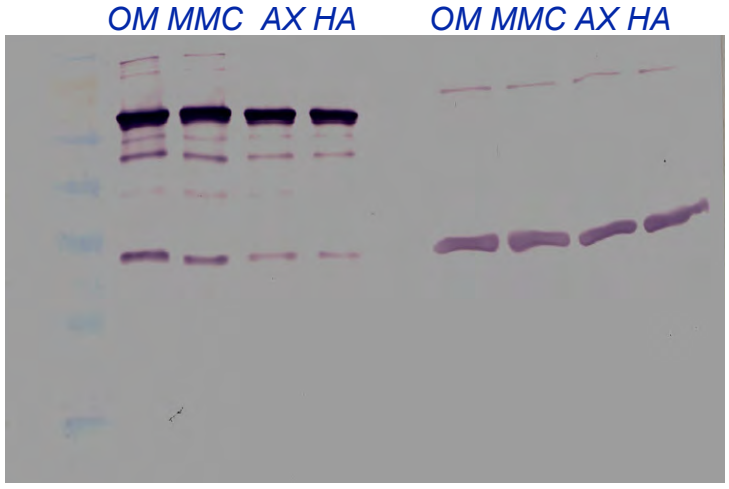
Note that the minibody elutes in phosphate buffered saline at pH 7.0

The current process

PAGE - coomassie



PAGE - Western anti-huFc



Note that the minibody was roughly 10 times more concentrated in this feedstream, and that MMC gave roughly the same purification performance as with the more dilute feedstream.

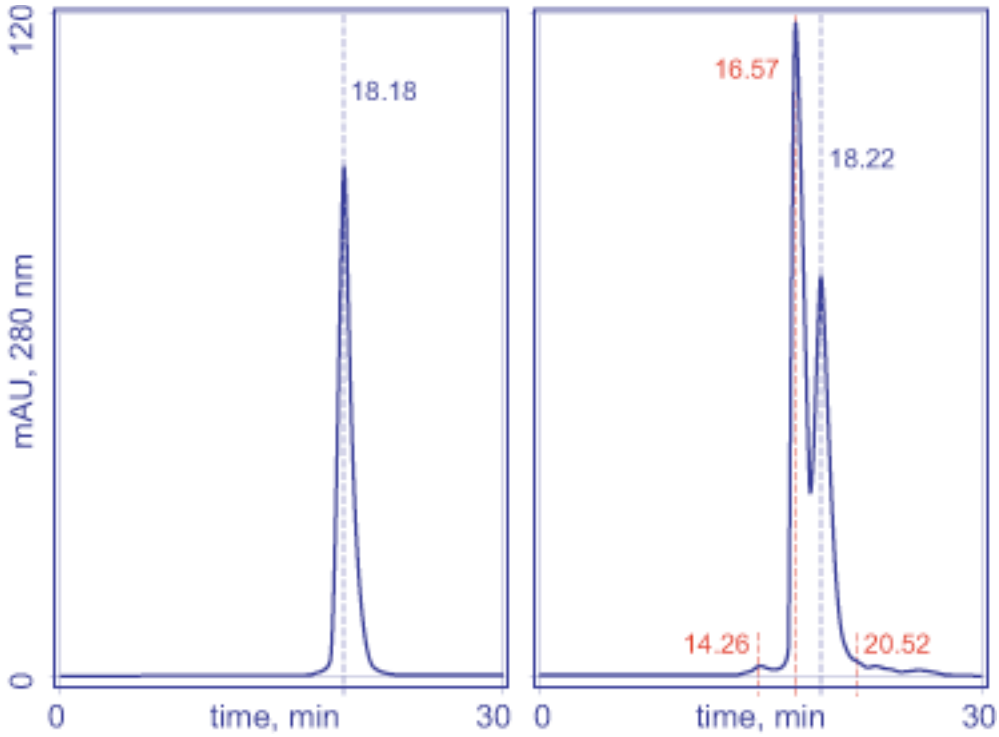
MMC reduces the BSA burden and, in combination with anion exchange, delivers reproducible sample composition to hydroxyapatite.

*Multiple minibody bands in the non-reduced samples were attributed to disulfide scrambling during electrophoresis sample preparation. See Liu et al, 2007, Biotechnol. Lett., **29** 1611-1622. Note also the reduction-resistant high-molecular weight bands on the reduced samples.*

The current process

Analytical size exclusion documenting HA fractionation of minibody and tetrabody

Peak 1 (main) *Peak 2*



Superdex™ 75, HR 10/30
Peak 2 was populated dominantly by tetrabody (retention time 16.57 min.)
The small peak at 14.26 minutes may indicate larger aggregates. With IgG, fragments usually elute on the leading side of IgG so the small peaks eluting from SEC after 20 minutes are likely not minibody-derived.
The relatively large proportion of minibody in peak 2 invites concern about product loss, but peak 2 contains only about 15% the UV absorbance of the main peak (slide 9), and minibody only about 40% of that, so actual product loss is probably less than 6%.

The current process

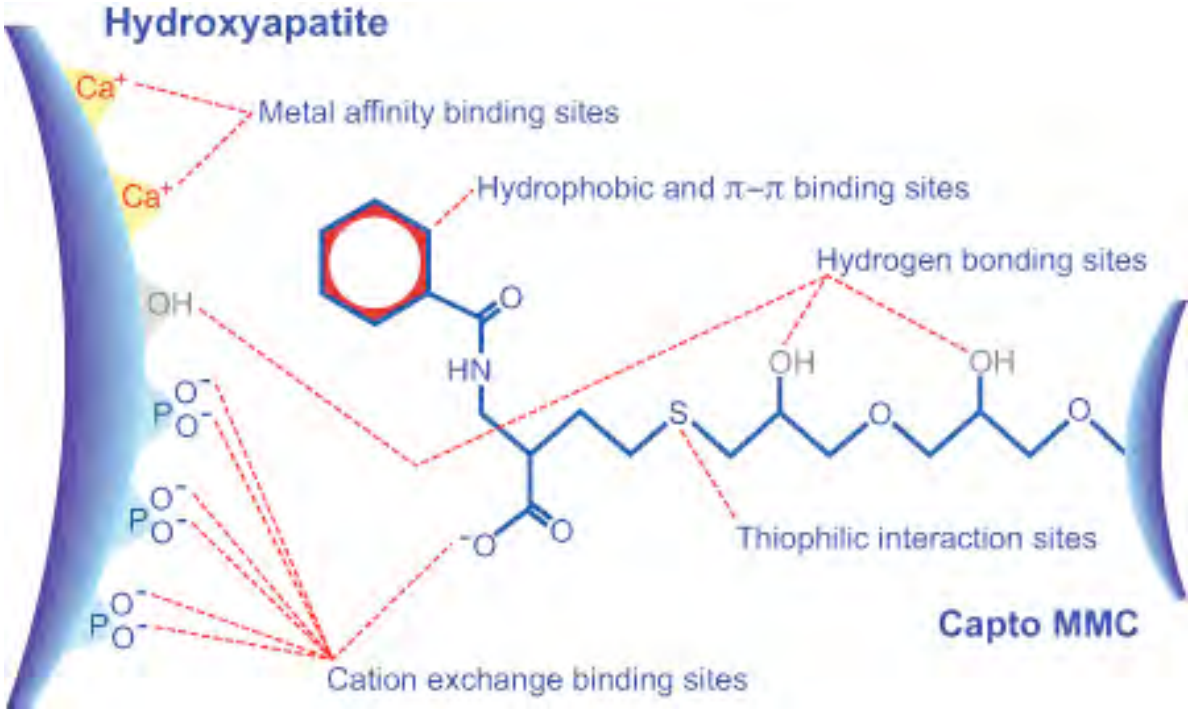
Recovery

Stage	µg/mL	mL	mg	recovery
OM	462	19.7	9.1	100%
MMC elution	362	22	8.0	88%
MMC strip	189	5.5	1.0	11%
Q elution	249	21	5.2	57%
Q strip	117	7.4	0.9	10%
HA elution 1	361	11	4.0	44%
HA elution 2	120	11	1.3	14%*
HA strip	76	3.8	0.3	3%**

**Dominantly Tetrabody.* ***Higher aggregates.*

Conclusions

This presentation, along with a rapidly growing number of others, marks the ascent of multimodal (mixed mode) methods in the field of process chromatography. Method development is more complicated than single-mode methods, but worth the investment.



Conclusions

As shown by MMC, mixed modes can provide an effective capture alternative in the absence of a convenient bioaffinity method.

An important advantage of MMC is that it avoids the problem of bioaffinity ligand leakage. This makes it unnecessary to develop purification methods to remove leachate, or develop analytical methods to measure leachate; and it suspends concerns about potential adjuvancy or immunogenicity of bioaffinity leachates.

MMC also makes an ideal precursor to hydroxyapatite because it removes the majority of contaminants that bind more strongly than the minibody (chiefly BSA and DNA), thereby increasing capacity for the product. It also removes cell culture components that might interact directly with hydroxyapatite (chelating agents and metal ions).

Conclusions

Hydroxyapatite demonstrates that mixed modes can offer unique selectivities, especially for removal of contaminants that are highly similar to the product, such as aggregates, fragments – and in this case, albumin.

Hydroxyapatite's well-documented capabilities for removal of DNA, endotoxin, and virus add extra assurance, in combination with anion exchange, that specifications for reducing these contaminants will be achieved with ease.

Economical regulatory-compliant purification of this minibody might not be possible without mixed modes like MMC and hydroxyapatite.

Acknowledgements

Thanks to Mark Sherman at City of Hope for ribbon models of IgG and minibody. Thanks also to BIA Separations, Bio-Rad Laboratories, and GE Healthcare for providing chromatography media to develop this purification process. Some parts of this research were supported by NCI grant CA43904.

Copies of this presentation can be downloaded at www.validated.com

Disclaimer

If you ask me anything I don't know, I'm not going to answer.

–Yogi Berra