Development of Selective Ligand Adsorbents for use in Downstream Processing of Biotherapeutics

Dev Baines
Director, Research & Development
ProMetic BioSciences Ltd

SEPARATION SCIENCE & TECHNOLOGY GROUP
Mixed Mode Chromatography – A Multimodal Separation Technique for Biopharmaceutical Purification
Wednesday 27th June 2007
PBL is an innovative company bringing new technologies and solutions that enable the production of higher quality, safer and less expensive therapeutic products.

- Materials and technology for the purification of biological products.
- Removal of impurities, contaminants and pathogens.
- Development of purification processes.
PBL - Technology

A Bio-recognition based - approach

- Affinity technology improves yields & purity with fewer process steps, with lower costs.

- Works with all protein products (plasma, rDNA, monoclonal antibodies etc.).

- Purification of target protein or specific removal of undesired compounds/pathogens.

CAPTURE → WASH → RECOVER
Need for DSP performance improvements

- Cost of goods pressure (yield improvements/cost reductions)
- Processes developed for production of early-phase clinical material not always appropriate for large-scale manufacturing
- Product safety (increased purity/contaminant removal)
- Limited biomass availability (yield improvements)
- Improvements in protein expression levels (binding capacity)
- New biological products in development (established platforms may not be applicable to future biological products)
- Follow-on Biologics (process improvements/cost reductions)
- Novel Proteins
## Biotherapeutics in development based on novel scaffolds

Pharma consolidates its grip on post-antibody landscape

<table>
<thead>
<tr>
<th>Company</th>
<th>Molecule</th>
<th>Scaffold, clinical status</th>
</tr>
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<tbody>
<tr>
<td>Ablynx</td>
<td>Nanobodies</td>
<td>Llama heavy chain, phase 1</td>
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<tr>
<td>Adnexus Therapeutics</td>
<td>Adnectins</td>
<td>Fibrinonectin domains, phase 1</td>
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<tr>
<td>Affibody</td>
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<td>Protein binding domain of Protein A, preclinical</td>
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<tr>
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<td>Peptide aptamers</td>
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<td>Avidia</td>
<td>Aimers</td>
<td>A-domain derived cell surface receptors, phase 1</td>
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<td>Transbodies</td>
<td>Transferrin, phase 1</td>
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<tr>
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<td>unnamed</td>
<td>Trimerized tetnectin domains, preclinical</td>
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<tr>
<td>Domantis</td>
<td>Domain antibodies</td>
<td>Heavy and light chain antibodies, preclinical</td>
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<tr>
<td>Evogenics Therapeutics</td>
<td>Evibodies</td>
<td>Derived from V-like domains of T-cell receptors, preclinical</td>
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<td>ESBTech</td>
<td>scFV fragments</td>
<td>Stable single chain antibody fragments, preclinical</td>
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<tr>
<td>Genmab</td>
<td>Unibodies</td>
<td>Monovalent IgG4 mAbs fragments, preclinical</td>
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<tr>
<td>Micromet</td>
<td>BiTEs</td>
<td>Bispecific, T-cell activating single chain antibody fragments, preclinical</td>
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<td>Molecular Partners</td>
<td>DARPins</td>
<td>Designed ankyrin repeat proteins, preclinical</td>
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<tr>
<td>Pieris</td>
<td>Anticalins</td>
<td>Derived from lipocalins, preclinical</td>
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<tr>
<td>Scil Proteins</td>
<td>Affilins</td>
<td>Derived from human lens protein gamma crystalline, preclinical</td>
</tr>
<tr>
<td>Trubion Pharmaceuticals</td>
<td>SMIPs</td>
<td>Custom-designed small modular immunophramaceuticals</td>
</tr>
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</table>
Advantages of Target Selective chromatography
Advantages of Target Selective chromatography

AC is not a new technique!
It is a method in which biospecific and reversible interactions are used for the selective purification of biologically active material from crude samples
(Wilcheck, 2004, Protein Science, 13, 3006, My life with Affinity)

- First reported example: Starkenstein, 1910, Biochem. Z., 24, 210
  - used starch to purify α-amylase

- Modern AC is attributed to Cuatrecasas, Wilchek and Anfinsen, 1968, PNAS, 61, 636, Selective Enzyme Purification by Affinity Chromatography
  – general principles and potential application of affinity chromatography illustrated by purification of staphylococcal nuclease, α–chymotrypsin and carboxypeptidase A

3’-(4-aminophenylphosphoryl)-deoxthymidine-5’-phosphate
Advantages of Target Selective Chromatography

AC is not a new technique!
But, known ligands/inhibitors that bind to the target molecule are usually immobilised to the chromatographic support

- Designer Ligands ‘biomimetics’ – Lowe et al. 1986, J. Chromatogr., 376, 111
  Lowe et al. 1992 Tibtech., 10, 442

- Screening Chemical Combinatorial Libraries – 1995 (Novo Nordisk A/S/ProMetic/Cambridge University – protein targets included immunoglobulins, insulins, FVIII and human growth hormone – WO 97/10887 – based on the concept that the selectivity of the ligands can be increased by increasing the spatial geometry and orientation of the ligand structures
Advantages of Target Selective Chromatography

- Mimetic Ligands exploit complimentary binding groups and the spatial orientation at specific binding site on the target protein
- Mixed Mode Ligands exploit gross physical properties of the target protein through enhanced hydrophobic and ionic interactions
- Both are synthetic ligands
## Synthetic vs biological ligands

<table>
<thead>
<tr>
<th>Criterion</th>
<th>Synthetic/biomimetic ligand</th>
<th>Biological/specific ligands</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cost</td>
<td>Inexpensive</td>
<td>Usually expensive, e.g. monoclonal antibodies/protein A</td>
</tr>
<tr>
<td>Availability</td>
<td>Organic synthesis by adsorbent manufacturer</td>
<td>Biological origin, e.g. ascites, fermentation etc.</td>
</tr>
<tr>
<td>Synthesis</td>
<td>Facile</td>
<td>Often complex and purification needed</td>
</tr>
<tr>
<td>Specificity</td>
<td>Moderate to high</td>
<td>Usually high</td>
</tr>
<tr>
<td>Capacity</td>
<td>High (up to 40 mg protein/mL adsorbent). &gt;10% ligand utilisation</td>
<td>Low. Typically &lt;1% ligand utilisation</td>
</tr>
<tr>
<td>Scale-up</td>
<td>Large scale use: columns at &gt;100 litre scale</td>
<td>Limited application</td>
</tr>
<tr>
<td>Sterilisation</td>
<td>High</td>
<td>Mostly low or not sterilisable</td>
</tr>
</tbody>
</table>
Advantages of Target Selective chromatography

C.I. Reactive Blue 2 \[\xrightarrow{\text{Optimisation of ligand structure and coupling chemistry}}\] Mimetic Blue SA
Approaches to ligand discovery

- Optimisation of existing ligands (analogue synthesis)
- Rational design (computer modeling of new ligand structures)
- Combinatorial libraries (systematic screening of ligand arrays)
Ligand modeling and in-silico design

- Use structural info when available
- Model and refine
- Ligand structure data base
- Search virtual and real spaces
- Design for bioprocess or proteomics
- Design for medical devices
- Design for therapeutics
Ligand library synthesis and screening

- Library design
- Combinatorial synthesis
- Library verification
- Library screening
- Ligand selection
- Development & Scale-up
Chemical Combinatorial Libraries™: triazine ligands on agarose support matrix

- Trivalent
- Base stable
- Aqueous-phase chemistry
- Easy to monitor ligand level
- Well characterised

PuraBead® 6XL (6HF)

- Agarose, 6%, cross-linked
- Near mono-dispersed
- Green, continuous process
Purabead 6HF Flow Properties

![Pressure Drop vs Flow Rate](chart.png)
Ligand synthesis

Step One: Add first amine

Dichlorotriazine agarose

Step Two: Add second amine

Monochlorotriazine intermediate

Final di-substituted product
Library Synthesis

Reactor vessels in the robot

96 well, fritted block

Intermediates

ProMetic Biosciences
Library Synthesis

Addition of Second Amines to rows A-H

96 well fritted block

Left blank for Standard curves
96-well library format
### Library Screening

The image contains a table and a diagram related to library screening. The techniques available are listed as follows:

- ELISA
- SDS PAGE (E-PAGE)
- Western Blot (E-PAGE)
- Total Protein Activity Assays

#### Table:

<table>
<thead>
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</table>

- Grey square = 64 x Samples
- Yellow square = 2 x Controls
- Blue square = LOAD
- Orange square = Calibrants

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**Techniques Available:**

- ELISA
- SDS PAGE (E-PAGE)
- Western Blot (E-PAGE)
- Total Protein Activity Assays
Virtual Library: All possible ligands that can be made from available amine intermediates

General library: Ligands selected for synthesis from a virtual library

Sub-library: Focused library developed on basis of leads from general library screen

Virtual library space

General library

Leads from general library screen

Sub-library
Chemical Combinatorial Libraries®

Screening Strategy

- General Library
- Sub Library 1
- Sub Library 2
- Sub Library 3

Diversity
SAR SAR SAR

Screen Screen Screen

Analysis
Analysis
Analysis

Virtual Library
Ligand/adsorbent development

- Ligand chemistry optimisation
- Attachment chemistry – spacer arm
- Ligand concentration (density)
- Matrix & cross-linking
- Performance optimisation (including optimisation of chromatographic parameters)
- Suitability for manufacture
- Safety studies
Typical ligand discovery programme

2 – 6 months

Project specifications

Milestone 1

Ligand discovery

Adsorbent development

Chromatography development

Milestone 2

Techonology transfer process validation and scale-up

Milestone 3

Purity (selectivity)
Capacity
Recovery
Binding and elution
Alkali resistance
Adsorbent scale-up
Stability
Ligand loss
Safety (Toxicity)
DMF

✓ Purity (selectivity)
✓ Capacity
✓ Recovery
✓ Binding and elution
✓ Alkali resistance
✓ Adsorbent scale-up
✓ Stability
✓ Ligand loss
✓ Safety (Toxicity)
✓ DMF

✓ Purity (selectivity)
✓ Capacity
✓ Recovery
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✓ Adsorbent scale-up
✓ Stability
✓ Ligand loss (leakage)
✓ Safety (Toxicity)
✓ DMF

Prometic Biosciences
Discovery of an affinity ligand for the purification of MI3 insulin precursor
### Substituent effects

<table>
<thead>
<tr>
<th>Ring Pos.</th>
<th>Substituents</th>
<th>MI3 Binding</th>
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<tbody>
<tr>
<td>R₁</td>
<td>OH</td>
<td>bad</td>
</tr>
<tr>
<td>R₂</td>
<td>OH NO₂</td>
<td>bad</td>
</tr>
<tr>
<td>R₃</td>
<td>OH</td>
<td>good</td>
</tr>
<tr>
<td>R₄</td>
<td>OH</td>
<td>good</td>
</tr>
<tr>
<td>R₅</td>
<td>OH</td>
<td>good</td>
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</table>
## Conditions for MI3 purification

<table>
<thead>
<tr>
<th><strong>Equilibration</strong></th>
<th>0.2 M Na-acetate pH 5.5</th>
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</thead>
<tbody>
<tr>
<td><strong>Application</strong></td>
<td>Broth adjusted to pH 5.5</td>
</tr>
<tr>
<td><strong>Wash</strong></td>
<td>0.1 M Na-acetate 5.5</td>
</tr>
<tr>
<td><strong>Elution</strong></td>
<td>0.1 M acetic acid</td>
</tr>
<tr>
<td><strong>C.I.P.</strong></td>
<td>0.5 M NaOH</td>
</tr>
</tbody>
</table>
Breakthrough curve for MI3 on 2/2 agarose
Purification of MI3 insulin precursor from fermentor broth
Purification of MI3 Insulin precursor from fermentor broth: SDS PAGE

1. Mw standards
2.
3. Application
4. Flow through
5. Wash
6. Wash
7.
8. Pool
9.
10. C.I.P
11.
12. MI3 Standard
Purification of MI3 insulin precursor from fermentor broth: visual appearance
Protein A interaction with Fc domain
Rational design of synthetic affinity ligands for IgG purification

Protein A

Phe 132                   Tyr 133

Protein A Mimic
MAbsorbents® were developed to mimic the Phe-132, Tyr-133 dipeptide binding site in the hydrophobic core of Protein A.

MAbsorbent® A2P was developed by screening triazinyl scaffold based chemical ligand libraries.

MAbsorbent® A2P-HF has been developed with improved cross-linking chemistry giving a very rigid but hydrophilic base matrix that resists shrinking and swelling and permits high operational flow rates.
MAbsorbent® A2P

- Binds IgG at the Fc region of the molecule
- Binds human and murine IgG
- Binds all human IgG subclasses
- Binds IgG at neutral pH and is salt tolerant
- Mild elution of IgG (acidic pH or ethylene glycol)
- Can be sanitised with 1 M NaOH
- Can be autoclaved
Identification of an affinity ligand for purification of a tPA-Urokinase fusion protein check

Ligand #23/B2 Analogue 1

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<tr>
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<th>Fraction</th>
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<tbody>
<tr>
<td>1</td>
<td>Load (cell culture fluid)</td>
</tr>
<tr>
<td>2</td>
<td>MW Marker</td>
</tr>
<tr>
<td>3</td>
<td>Flow through (1)</td>
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<tr>
<td>4</td>
<td>Flow through (2)</td>
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<td>5</td>
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<td>6</td>
<td>Elution (2)</td>
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<tr>
<td>7</td>
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<td>8</td>
<td>MW Marker</td>
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<tr>
<td>9</td>
<td>Lysine Affinity elution P007/01</td>
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<tr>
<td>10</td>
<td>Lysine Affinity elution P008/01</td>
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</table>
Identification of an affinity ligand for purification of a tPA-Urokinase fusion protein

<table>
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</table>

Lane | Fraction
---|--------
1 | Load
2 | B1 Flow through
3 | B1 Elution
4 | C1 Flow through
5 | C1 Elution
6 | D1 Flow through
7 | D1 Elution
8 | B7 Flow through
9 | B7 Elution
10 | B8 Flow through
11 | B8 Elution
12 | F8 Flow through
13 | F8 Elution
14 | MW Marker
15 | Amediplase standard
Identification of an affinity ligand for purification of a tPA-Urokinase fusion protein

» Column dimensions: 10mL column - 4.5cm bed height, 1.6cm diameter
» Adsorbent: Ligand #23/B2 Analogue 1 attached to Purabead™ 6HF; ligand density 17 μmol/g.
» Flow rate: 300cm/hr
» Equilibration / wash buffer: 25mM sodium phosphate, pH 6.5, 100mM NaCl
» Loading with concentrated cell culture supernatant, pH 6.5
» Elution buffer: 25mM sodium phosphate, pH 6.5, 0.5M NaCl, 40% v/v ethylene glycol
» Sanitization: 0.5M NaOH

- Capacity: 11.4 mg/ml
- Purity: 99%
- Recovery: 92%
- Loading flow rate: 300 cm/h
- Alkali stable (0.5 M NaOH)
Advantages of Target Selective chromatography
Basic plasma protein fractionation scheme using the Cohn process (1946)

Plasma

Ethanol precipitation

Cryosupernatant

Supernatant I

Supernatant II+III

Supernatant IV

Supernatant V

Cryoprecipitate (Factor VIII)

Precipitate I (Fibrinogen)

Precipitate II+III (IgG, IgA, IgM)

Precipitate IV (Alpha1-antitrypsin)

Precipitate V (Albumin)
Affinity Purification of Fibrinogen

<table>
<thead>
<tr>
<th>Lane</th>
<th>Loading</th>
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<tbody>
<tr>
<td>1</td>
<td>MW Marker</td>
</tr>
<tr>
<td>2</td>
<td>Fibrinogen Standard</td>
</tr>
<tr>
<td>3</td>
<td>Plasma (HSA reduced)</td>
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<tr>
<td>4</td>
<td>Flow through</td>
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<tr>
<td>5</td>
<td>Fibrinogen Elution</td>
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</tbody>
</table>
Capture Purification – Plasminogen

4 mL column
Linear flow rate: 50cm/hour
Equilibration buffer: 50mM Sodium Phosphate pH 7.5 (5CV)
Load: 100 mL filtered human plasma
Post load wash buffer: 50mM Sodium Phosphate pH 7.5 (10CV)
Elution buffer: pH 7.5 (5CV)

Lane 1: MW Marker
Lane 2
Lane 3: Human plasma load
Lane 4: Flow through fraction
Lane 5: Elution fraction.
Lane 6:
Lane 7: MW Marker
ProMetic-ARC New Fractionation Process

**Backbone**

**Plasma**

**Downstream processes and viral inactivation**

- vWF/Factor VIII
- Plasminogen
- Fibrinogen
- Immunoglobulin G
- Albumin
- Alpha₁-proteinase inhibitor
Mimetic™ ligands developed

Target Biomolecule

- Albumin
- IgG
- Insulin
- Factor VII
- Factor VIII
- Alpha-1-Antitrypsin
- Fibrinogen
- Plasminogen
- tPA
- tPA-Urokinase
- Alkaline Phosphatase
- Endotoxin
- Prions
Benefits of ProMetic Approach

- Screening performed with actual feed-stock.
- Screening performed with synthetic (Mimetic Ligands™) firmly attached to a high-flow chromatographic matrix.
- Adsorbents used for screening can be scaled-up and used in manufacture with little or no modifications.
- Early availability of adsorbent samples for in-house evaluations by sponsor/end-user.
- Bulk quantities of “cGMP” grade adsorbent produced as an integral part of the development programme.
- Synthetic ligands – inexpensive; no materials of animal origin.
- Ligand screening, adsorbent development and adsorbent manufacture performed by same company.
- Short development times; modular approach.
- Proven technology.
Manufacturing Validation & Scale-up

- ISO 9001:2000
- “cGMP” standard manufacture
- Class 100,000, 10,000 & 100 areas
- Drug Master files/regulatory support files
- Single batch sizes to 250 litres

(Applies to entire UK operations)
more of what you want
less of what you don’t