Aptamers: Powerful and Innovative Ligands in Affinity Chromatography

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Outline

1. Aptamers as affinity ligands: definition, selection and general properties
2. Selection of an Aptamer designed for affinity chromatography application
3. Examples of aptamo-purification
4. The path to industriability
5. Conclusions
Definition and Generation

- Aptamer from « Aptus » (from the Latin *aptus* - fit, and Greek *meros* - region)

- Oligonucleotides (RNA or single-strand DNA) selected to bind a target [biological macromolecules (e.g. proteins) or little organic molecules]

- Specific 3D structures which allow aptamers to display high affinity and specificity for their targets
  - First time reported in 1990: two publications\(^1,2\) in Nature and Science
  - First selection against non nucleic acid binding protein in 1992: Thrombin\(^3\)

- *In vitro* Selection process inspired from natural selection: The SELEX
  - This process enables the isolation, from a highly diverse combinatorial library of oligonucleotides, of the best binder(s) under a given set of conditions

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The SELEX: Systematic Evolution of Ligand by Exponential enrichment

- The combinatorial Library

- Single stranded DNA chemically synthesized
- Central randomized region of 20-40nt
  - Huge diversity: $10^{15}$ to $10^{16}$ of different molecules
  - 2000 pmols of a 40nt library $\cong$ 47 µg ssDNA $\Rightarrow$ 1.2x $10^{15}$ unique sequences
- Flanked by fixed region (for PCR amplification and cloning)
The SELEX: Systematic Evolution of Ligand by Exponential enrichment

- Iterative process of selection/counter-selection/amplification of the more fitting species followed by an identification step.
Number of cycles needed for an efficient evolution of the library: 6 to 16
  - Stopped when the enrichment or the affinity of the pool is deemed sufficient enough
  - One SELEX leads to many specific candidates

Sequencing performed on the evolved Aptamer pool allows
  - Identification of repeated consensus sequences
  - Characterization of the secondary structures and identification of the minimal core-sequence needed for binding

Minimal size of the core-sequence: 10 to 60 nt
  - 15 nt anti-thrombin aptamer: GGTTGGTGTGGTTGG
    (Kd: 200nM)
Aptamers: global view

General properties
- Binds the target: Kd pM to µM
- Possibly, Inhibits the biological activity of the target

4000+ Aptamer sequences - 370 sequences of artificial Ribozymes referenced in 500 publications and patents (DataBase: Aptamer.freebase or RiboaptDB)

2000+ publications on Aptamers and/or SELEX

Some examples of target/Kd

<table>
<thead>
<tr>
<th>Target</th>
<th>Kd</th>
</tr>
</thead>
<tbody>
<tr>
<td>VEGF</td>
<td>2 nM</td>
</tr>
<tr>
<td>Adenine</td>
<td>8 µM</td>
</tr>
<tr>
<td>C5</td>
<td>2-5 nM</td>
</tr>
<tr>
<td>B12 (vitamin)</td>
<td>500 nM</td>
</tr>
<tr>
<td>HIV-RT</td>
<td>25 pM</td>
</tr>
<tr>
<td>PrPsc</td>
<td>40 nM</td>
</tr>
<tr>
<td>PrPc</td>
<td>20 nM</td>
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</table>
Aptamers: main features

- Generation of aptamers against non immunogenic, or difficult to handle targets (Prion, toxin, small molecules)

- Quick *in vitro* process: Generation in ~ 8 weeks against virtually any target
  - Less for optimized /automatized process

- Their chemical nature (5’P-3’OH) allows for precise derivatization (biotin, fluorescent reporter, spacer arm, etc.)

- Owing to their small size: anti-protein aptamers can access/contact relatively small binding pockets on their targets
  - Powerful inhibitors

- High specificity:
  - An Aptamer exhibits greater than 10,000-fold binding affinity for theophylline over caffeine

- Can be selected under a wide range of conditions
  - Select species that bind under non physiological conditions (pH, ionic strength, solvent, temperature)
  - Binding under precise conditions to meet a specific need (process, type of sample)
Affinity ligand Ideal attributes?

- Dedicated grafting function to allow oriented immobilization
- Capable to exclusively recognize the target protein
- Possibility to easily release the captured proteins by a chosen condition suitable for the product
- Reusable and stable under quite harsh conditions
- Neither leakage nor molecular hydrolysis
- Molecular mass as small as possible
- Non-toxic
- Easy synthesis and affordable cost
Aptamer as ligand for affinity chromatography: Ideal ligand?

- **High specificity and selectivity**
  - Generally considered to be superior to antibody-derived ligands

- **High physical and chemical stability: reusable and resistant to harsh sanitization**
  - High physical and chemical stability of DNA chemistry
  - Modified nucleotides provide nuclease resistance

- **Behavior which may perfectly fit specific process requirements**
  - Behavior chosen and modulated during the SELEX process
  - Elution under optimal conditions for the product or considering the process constraints (modality of selection)
  - Improvement selectivity through counter-selection
    - Particular interest for transgenic proteins: counter-selection against undesirable endogenous proteins
Aptamer as ligand for affinity chromatography: Ideal ligand?

- **Manufacturing cost: large scale production by chemical synthesis**
  - High reproducibility and moderate cost: 2 to 5 K€ per gram of Aptamer with spacer and terminally-modified nucleotide (MW = 1/10 to 1/50 of Mab)
  - No potential biological contamination

- **Availability of highly sensitive assays when considering aptamers as leachables**: Molecular biology methods

- **Lack of immunogenicity (in case of leakage)**
  - Lack of immunogenicity demonstrated during pre-clinical tests for therapeutic Aptamers when 1,000-fold higher doses were administered to monkeys (by Eyetec for anti-VEGF165).
Selection of an Aptamer designed for affinity chromatography applications
Selection of an Aptamer designed for affinity chromatography applications  
Case of human Recombinant FVIIa

<table>
<thead>
<tr>
<th>SELEX strategy</th>
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<tbody>
<tr>
<td><strong>Selection buffer</strong></td>
</tr>
<tr>
<td><strong>Target</strong></td>
</tr>
<tr>
<td><strong>Counter-selections</strong></td>
</tr>
<tr>
<td></td>
</tr>
<tr>
<td><strong>Wash / Elution</strong></td>
</tr>
</tbody>
</table>

*Significant Proportion of interesting species in round 7 confirmed in round 10*

⇒ Cloning and sequencing
Selection of an Aptamer designed for affinity chromatography applications
Case of human Recombinant FVIIa

Bioinformatics and SPR analysis (Biacore) for confirmation of Monoclonal Aptamer (Mapt) sequences

Bioinformatics alignment for family identification

Affinity confirmation against immobilized target: R FVIIa
Selection of an Aptamer designed for affinity chromatography applications
Case of human Recombinant FVIIa

SPR analysis for best binder determination in a aptamer family followed by core sequence identification

Mapt 2  Mapt 2.2
Selection of an Aptamer designed for affinity chromatography application
Case of human Recombinant FVIIa

Selectivity assessment and analyses of their binding properties

Selectivity assessment

Plasma derived human FVII

Plasma derived rabbit FVII

Resistance under different wash conditions evaluation with immobilized aptamer
Examples of Aptamo-purification
Aptamo-purification as a polishing step
Example of Recombinant FVIIa

From **highly purified** material
Purified recombinant FVIIa from slightly stressed raw material containing 10% of product-related impurities (other impurities less than 10ppm)

**Material:** Purified hRFVIIa

**Equilibration buffer:**
- 50 mM Tris-HCl (pH 7.4)
- 150 mM NaCl
- 10 mM CaCl$_2$

**Washing Buffer:** None

**Elution:** 50 mM EDTA pH 8

⇒ Capacity to eliminate very closely related impurities
Highly purified product by aptamo-purification

Example of Factor H protein

From **pre-purified** material
Plasmatic FH obtained after 4 chromatographic steps, including a pseudo affinity chromatography

From **crude** material
Recombinant FH in supernatant

Highly purified FH product obtained in one single step (with 1M NaCl wash) from pre-purified or crude material
Highly purified product by aptamo-purification

Example of coagulation Factor IX

Material: Intermediate product containing 94 UI/mL of FIX

Equilibration buffer:

- 50 mM Tris-HCl (pH 7.4)
- 150 mM NaCl
- 2 mM CaCl$_2$
- 1 mM MgCl$_2$

Elution: 200 mM EDTA (pH 8)

SDS PAGE gel - Blue staining
The path to Industriability: Grafting and resistance demonstration
On the path to Industriability: High grafting yield

A specific concern for this technology has been addressed through the use of a proprietary immobilization approach (efficient chemical grafting of an NH2-derivatized oligonucleotide on a classical support)

<table>
<thead>
<tr>
<th>Quantity targeted in µg (for 1mL gel)</th>
<th>100</th>
<th>3500</th>
<th>6000</th>
</tr>
</thead>
<tbody>
<tr>
<td>Quantity grafted in µg</td>
<td>≈100</td>
<td>3498</td>
<td>5556</td>
</tr>
<tr>
<td>Grafting yield (%)</td>
<td>100.0</td>
<td>100.0</td>
<td>92.6</td>
</tr>
</tbody>
</table>

| Immobilization (mg) | 1.0  | 3.5  | 5.7  |
| Dynamic Capacity* (mg) | 1.6  | 5.2  | 8.0  |

*Capacity for a 50 KDa protein at 60 cm/H

⇒ New grafting approach which provides a very high yield of immobilization
⇒ High efficiency (functionality/grafting)

\[ y = 1.3783x + 0.2362 \]
\[ R^2 = 0.9989 \]
For most of affinity ligands, the development of a specific assay especially in the presence of the target remains a challenge

- Sensitivity required less than pg/mL
- Overcomes the probable complex formation with the target and/or the probable matrix effect by the concentrated target itself in the final product

For aptamers different methods developed in the genomic and molecular biology fields can be adapted for this purpose

- Highly sensitive methods available from molecular biology for DNA assay including a specific DNA extraction and/or target denaturation
As a model: chemically immobilized anti-FVII aptamers with inverted 3’T
⇒ 2 anti-FVII aptamers with 2 different spacers (hydrophobic C6 and hydrophilic C11)

Parameters monitored:

- Capacity at 80% = 6.5-7.5 g/L (initial values)
- Specific activity of the eluted product: 0.8-1.0 (initial values)

<table>
<thead>
<tr>
<th>Résistance tested</th>
<th>Mapt 2 C11</th>
<th>Mapt 2,2 C6</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Capacity 80% g/L</td>
<td>Specific activity (FVIIam/FVIIAg)</td>
</tr>
<tr>
<td>100 Hours Sodium hydroxide 1 M</td>
<td>7.1</td>
<td>0.9</td>
</tr>
<tr>
<td>100 Hours in cryosupernatant</td>
<td>7.3</td>
<td>0.9</td>
</tr>
<tr>
<td>100 Hours in clarified milk</td>
<td>ND*</td>
<td>ND</td>
</tr>
<tr>
<td>+ 30 add. cycles buffer/ sodium hydroxide 1M</td>
<td>ND</td>
<td>ND</td>
</tr>
</tbody>
</table>

*not determined

⇒ Very high resistance of aptamer resin

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Conclusions
Most promising technology for improving yield and COGs of biotherapeutics including Plasma fractionation

- 10 patents submitted
- One major patent: original process for Aptamer immobilization

The last 3 years, investment made by LFB aimed at demonstrating:

- High selectivity (vs contaminants, related-protein contaminants, homologous proteins)
- Capacity to discriminate proteins with correct PTMs (i.e. complete $\gamma$-carboxylation for FIX)
- High resistance: ultimate sanitization with 1M NaOH, compatibility with biological media (serum, milk)
- High yield of immobilization using a specific chemistry on a classical support
- High efficiency of immobilized aptamers
- Very sensitive assay of residual aptamers in case of leakage

First positive feedback from EMA (Innovation Task Force)
Special Thanks

- Sami Chtourou, Patrick Santambien, Laurent Siret and Nicolas Bihoreau
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- Egisto Boschetti (Jam Conseil) and Frédéric Ducongé (CEA)

Thank you for your attention!