Enzyme Immobilization: Why, What and How

Roger A. Sheldon

- Enzyme Immobilisation
- Entrapment
- Carrier-bound
- Cross-Linking
Why use enzymes?

• Renewable, biodegradable feedstock
• Mild conditions (pH, T & P)
• High rates
• Higher quality product
• High chemo, regio & enantioselectivity
• No special equipment needed
• Environmentally & economically attractive (GREEN)
The Challenge

• Disadvantages of enzymes
  – Low operational stability & shelf life
  – Cumbersome recovery & re-use
  – Product contamination
  – Allergic reactions to proteins

• Non viable biocatalytic applications
  – Enzyme costs too high
  – Not practical
The Solution: Immobilization

- Immobilization is Enabling Technology

High waste / low profit

Low waste / high profit

- Advantages
  - stability, stability, stability ...
  - repeated re-use of biocatalyst (batch)
  - easier downstream processing
  - continuous process technology
Types of Immobilization

• Binding to a carrier
  – e.g. ion exchange resins

• Entrapment
  – e.g. in silica sol-gel

• Cross-Linking
  – e.g. Cross-Linked Enzyme Aggregate (CLEA)

Carrier-bound / entrapped enzymes have inherently low volumetric & catalyst productivities (90->99% non-catalytic mass)

Cross-Linking with Glutaraldehyde

The monomeric structure of glutaraldehyde does not reflect the complexity of glutaraldehyde behavior in solution and its reactivity with proteins!
Glutaraldehyde in Practice

Glutaraldehyde Reactions with Proteins

Cross-Linking with Glutaraldehyde

• Common, inexpensive and effective protein cross-linking agent
• Cross-linking chemistry still not fully understood
• Type of covalent bond formed depends heavily on glutaraldehyde concentration, amine concentration, pH, and temperature
• Reduction of Schiff bases with NaBH₄ or NaCNBH₃ usually not necessary
• Other aldehyde cross-linkers, such as dextranpolyaldehyde generally do need a reduction step
The CLEA Technology
Basic CLEA Properties

Very high enzyme loading

Particle size typically 5-50 µm

Good filterability and centrifugability

Packed bed possible

Mechanically robust

Excellent operational stability
  heat, organic solvents and proteolysis (autolysis)

Tuneable hydrophobicity/hydrophillicity
Advantages of CLEAs

1. Improved properties
   - Better storage and operational stability
   - Hypoallergenic
   - No leaching of enzyme in aqueous media

2. Cost-effective
   - No need for pure enzyme (crude cell lysate sufficient)
   - Easy recovery and recycle (easier DSP)
   - High productivities (kg product/kg enzyme)

3. Broad scope & short time to market
Magnetic CLEAs

- Synthesis of magnetic nanoparticles in silica
- Functionalisation of nanoparticles with aminopropyl groups
- CLEAtion: cross-linking the enzyme and the nanoparticles

Characteristics

- Magnetic decantation
- Magnetic strength can be adjusted
- No change in CLEA activity
- e.g. hydrolases, oxidoreductases, nitrile hydratases
Additional Properties of mCLEAs

Separation of the enzyme catalyst by magnetic decantation

Magnetic strength of the mCLEA can be adjusted for the particular application

No changes in the structure by the introduction of magnetic particles

No changes in enzyme activity of the immobilised enzyme by the introduction of magnetic particles

mCLEA of any enzyme can be manufactured – currently examples with hydrolases and oxidoreductases

Potential application in the pharmaceutical, food and feed industries, and diagnostics
Combi CLEAs

Synthesis of CombiCLEAs

- Two or more enzymes in one CLEA
- Used for cascade reactions
Storage Stability – NHase CLEA

7 months storage no decrease in activity!

- Storage in 0.01M TRIS buffer pH 8, no additions at room temperature
- Instability of free enzyme due to dissociation of multimeric enzyme

van Pelt, Green Chem. 10 (2008) 395-400
Thermostability – Papain CLEA

- Papain (protease from *C. papaya*) incubated at pH 7 and 50 °C.
Recyclability – PaHNL CLEA

- Effect of recycling on the performance of (R)-oxynitrilase CLEA in the hydrocyanation of o-chlorobenzaldehyde
Scope of the CLEA Technology

**Hydrolases**
- Pen. Acylases (2)
- Lipases (19)
- Esterases (3)
- Proteases (9)
- Nitrilases (5)
- Aminoacylase
- Phytase
- Galactosidase
- Carbonic anhydrase

**Oxidoreductases**
- KRED
- FDH
- Glucose oxidase
- Galactose oxidase
- Amino acid oxidase
- Laccase (3)
- Catalase
- Chloroperoxidase
- HRP

**Lyases**
- R- & S- HNLases (5)
- PDC
- DERA
- Nitrile hydratase (9)

**Transferases**
- Transaminases
  - (R) selective (3)
  - (S) selective (5)
Scope of the Technology

Hydrolases

• Pen. Acylases (2)
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• Aminoacylase
• Phytase
• Galactosidase
• Carbonic anhydrase
Hydrolases – Pen. Acylase

\[
\text{Biocatalyst} \quad \text{Conv. (\%)} \quad \text{S/H ratio} \quad \text{Rel. Productivity}
\]

<table>
<thead>
<tr>
<th>Biocatalyst</th>
<th>Conv. (%)</th>
<th>S/H ratio</th>
<th>Rel. Productivity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Free enzyme</td>
<td>88</td>
<td>2.0</td>
<td>100</td>
</tr>
<tr>
<td>T-CLEA</td>
<td>85</td>
<td>1.58</td>
<td>151</td>
</tr>
<tr>
<td>PGA-450</td>
<td>86</td>
<td>1.56</td>
<td>0.8</td>
</tr>
</tbody>
</table>

Conclusion – High productivity and S/H

Hydrolases – Protease

- Alcalase CLEA: \( B. licheniformis \) protease
- Savinase CLEA: \( B. clausii \) protease
- Esperase CLEA: \( B. lentus \) protease
- BS CLEA: \( B. subtilis \) protease
- Papain CLEA: \( C. papaya \) protease
- Protease CLEA Discovery Platform
Hydrolases - Proteases

Antifouling Agent in Paint

• To replace toxic organotin compounds (banned in the EU since 2008)
• Cross-linked enzyme aggregates (CLEAs) of proteases were tested in artificial seawater (ASW) both as it is and as a component of the paint.
• It is found that all CLEAs have tolerance to xylene and have great stability in dried paint.
• The maximum increase in relative activity was found for CLEA *B. licheniformis*.
• CLEA *B. licheniformis* has shown 900% activation during storage in ASW.
• In the paint, non-modified subtilisin lost more than 90% of activity in 28 days.
Hydrolases – Proteases

Amidation in Organic Media

\[
\begin{align*}
\text{CbzHN} & \quad \text{PhNH}_2 \\
\text{H} & \quad \text{Alcalase-CLEA} \\
\text{OR} & \quad \text{MTBE / 50°C} \\
\end{align*}
\]

<table>
<thead>
<tr>
<th>R</th>
<th>Yield (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CH(_3)</td>
<td>93</td>
</tr>
<tr>
<td>PhCH(_2)</td>
<td>94</td>
</tr>
<tr>
<td>H</td>
<td>93</td>
</tr>
</tbody>
</table>

Nuijens, Cusan, Kruijtzer, Rijkers, Liskamp, Quaedflieg, J. Org. Chem. 74 (2009) 5145
Regioselective Esterifications

\[
\begin{align*}
R^1 \text{HN} \rightarrow \text{COOH} & \quad \xrightarrow{\text{Alcalase-CLEA}} \quad R^2 \text{OH} \\
& \quad \text{O} \\
& \quad \text{OH}
\end{align*}
\]

\[n = 1 \text{ or } 2\]

\[R^1 = \text{Cbz, Boc, Fmoc}\]

\[R^2 = \text{allyl, Me}_3\text{SiCH}_2\text{CH}_2^-\]

92-98% Yield

84-89% Isolated yield

\[
\begin{align*}
R^1 \text{HN} \rightarrow \text{COOH} & \quad \xrightarrow{R^2 \text{OH}} \quad R^1 \text{HN} \rightarrow \text{COOR}^2 \\
& \quad \text{O} \\
& \quad \text{OH}
\end{align*}
\]

\[n = 1 \text{ or } 2\]

\[R^1 = \text{CBz, BOC, FMOC}\]

\[R^2 = \text{allyl or } (\text{CH}_3)_3\text{SiCH}_2\text{CH}_2^-\]

95-98% Yield

77-87% Isolated Yield

Nuijens, Cusan, Kruijtzer, Rijkers, Liskamp, Quaedflieg, *Synthesis* (2009) 809

Peptide Synthesis

\[
\begin{align*}
X' & \text{H} \quad \text{O} \\
\quad & \text{N} \\
\quad & \text{C} \quad \text{O} \\
\quad & \text{R} \quad \text{O} \quad \text{R} \\
\quad & \text{N} \\
\quad & \text{C} \quad \text{O} \\
\quad & \text{R} \quad \text{H} \\
\quad & \text{N} \\
\quad & \text{C} \quad \text{O} \\
\quad & \text{R} \quad \text{O} \quad \text{R} \\
\quad & \text{N} \\
\quad & \text{C} \quad \text{O} \\
\quad & \text{R} \quad \text{H} \\
\quad & \text{N} \\
\quad & \text{C} \quad \text{O} \\
\quad & \text{R} \quad \text{O} \quad \text{R} \\
\quad & \text{N} \\
\quad & \text{C} \quad \text{O} \\
\quad & \text{R} \quad \text{H} \\
\quad & \text{N} \\
\quad & \text{C} \quad \text{O} \\
\quad & \text{R} \quad \text{O} \quad \text{R} \\
\quad & \text{N} \\
\quad & \text{C} \quad \text{O} \\
\quad & \text{R} \quad \text{H} \\
\quad & \text{N} \\
\quad & \text{C} \quad \text{O} \\
\quad & \text{R} \quad \text{O} \quad \text{R} \\
\end{align*}
\]

\[\text{alcalase CLEA}\]

\[\text{ROH}\]

\[\text{alcalase CLEA}\]

\[\text{X} = \text{N-protecting group}\]

\[\text{R}^1, \text{R}^2, \text{R}^3 = \text{amino acid side-chains}\]

Resolution of Amino Ester with Alcalase-CLEA

\[
\begin{align*}
\text{COOCH}_3 & \quad \text{alcalase CLEA} \\
\text{Cl} & \\
\text{NHBOc} & \\
\text{H}_2\text{O} / \text{THF} & \\
\end{align*}
\]

\[
\begin{align*}
\text{COOH} & \\
\text{Cl} & \\
\text{NHBOc} & \\
\end{align*}
\]

\[
\begin{align*}
\text{COOCH}_3 & \\
\text{Cl} & \\
\text{N} & \\
\text{S} & \\
\end{align*}
\]

Clopidogrel

**Rhodococcus erythropolis** amidase CLEA: Enantioselective Hydrolysis (AstraZeneca)

![Chemical Reaction Diagram]

A. Wells, presented at the SCI Meeting on *Biocatalysis & Biotransformations*, London, October 14, 2010
Hydrolases - Lipases

- *Candida antarctica* lipase B (CaLB)
- *Candida antarctica* lipase A (CaLA)
- *Thermomyces lanuginosus* (Lipolase)
- *Rhizomucor miehei*
- *Candida rugosa*
- *Alcaligenes sp.*
- *Pseudomonas stutzeri*

*Lipase CLEA discovery platform*

*Candida antarctica* Lipase B CLEA

The only commercially available immobilized form of CaLB completely stable to leaching in water
Hydrolases - Lipases

\[
\text{HN-} \text{COOCH}_3 \xrightarrow{\text{Pseudomonas sp. lipase CLEA}} \text{HN-} \text{COOCH}_3 + \text{HN-} \text{COOH}
\]

\((S)\) \quad \text{E value} = > 100

\[
\text{ibuprofen} + \text{C. rugosa lipase} \rightarrow \text{ester}
\]

Free enzyme \quad E = 13
CLEA \quad E = 23


Hydrolases - Lipases

\[
\text{citronellol} \xrightarrow{B. cepacia lipase CLEA} R - \text{citronellol acetate}
\]

**Free Enzyme**  \( E = 19 \)

**CLEA**  \( E = 74 \)


\[
\begin{align*}
\text{Ar} & \quad \text{R} \\
\text{OH} & \quad \text{Ar} \quad \text{R} \\
\text{OAc} & \quad \text{Ar} \quad \text{R} \\
\end{align*}
\]

\[
\begin{align*}
\text{Ar} = \text{Ph, 2-furyl,} \\
\text{R} = \text{Me, CH}_2\text{NHCOPr}
\end{align*}
\]

\[
\text{B. cepacia lipase-CLEA} \\
\text{in PhMe or MTBE} \\
\text{RT, 24-48h}
\]

\[
\text{47-56% conversion} \\
E = 35 - >200
\]

Hydrolases - Lipases

**B. cepacia** lipase-CLEA

- Reaction in PhMe or MTBE
- Reaction at RT, 24-48h
- Products: OH
- Conversion: 47-56%
- Enantiomeric excess: E = 35 - >200

1. Ar = Ph, R = Me
2. Ar = 2-furyl, R = Me
3. Ar = Ph, R = CH₂NHCO₃⁻


**CaLA-CLEA**

- Reaction with OH
- Products: OH
- Conversion: 47-56%
- Enantiomeric excess: E = 35 - >200

n = 1 or 2

Hydrolases - Lipases

\[
\text{CaL B CLEA in a Fixed Bed Reactor} \\
100\% \text{ activity after } >300 \text{ h on stream}
\]
CalB CLEA in Organic Media

\[
\text{Reaction Scheme:} \quad \text{NH}_2 \quad + \quad \text{O} \quad \text{OPr} \quad \xrightarrow{\text{Lipase}} \quad \text{HN} \quad \text{O} \quad \text{OPr}
\]

<table>
<thead>
<tr>
<th></th>
<th>Activity in H\textsubscript{2}O (U/g)</th>
<th>Activity in (i-Pr)\textsubscript{2}O (U/g)</th>
<th>Ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>CaL B CLEA–ST</td>
<td>38000</td>
<td>50</td>
<td>21</td>
</tr>
<tr>
<td>CaL B CLEA-OM</td>
<td>31000</td>
<td>1500</td>
<td>760</td>
</tr>
<tr>
<td>Novozym 435</td>
<td>7300</td>
<td>250</td>
<td>29</td>
</tr>
</tbody>
</table>
CalB CLEA in scCO$_2$

Catalyst | Conversion (%) | E  
---|---|---  
Novozym 435 | 17 | 280  
CaL B CLEA | 48 | 640

CalB CLEA in Ionic Liquid

\[
\begin{align*}
\text{OEt} & + \text{OH} & \xrightarrow{40^\circ \text{C}} & \text{EtOH} \\
\end{align*}
\]

<table>
<thead>
<tr>
<th>Solvent</th>
<th>Lipase</th>
<th>Time (h)</th>
<th>Conv. (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>t-BuOH</td>
<td>Nov 435</td>
<td>6</td>
<td>83</td>
</tr>
<tr>
<td>[bmim][dca]*</td>
<td>Nov 435</td>
<td>24</td>
<td>0</td>
</tr>
<tr>
<td>t-BuOH</td>
<td>CaL B CLEA</td>
<td>3</td>
<td>83</td>
</tr>
<tr>
<td>[bmim][dca]</td>
<td>CaL B CLEA</td>
<td>6</td>
<td>80</td>
</tr>
</tbody>
</table>

*dca = (CN)₂N

Scope of the Technology

Hydrolases
- Pen. Acylases (2)
- Lipases (19)
- Esterases (3)
- Proteases (9)
- Nitrilases (5)
- Aminoacylase
- Phytase
- Galactosidase
- Carbonic anhydrase

Oxidoreductases
- KRED
- FDH
- Glucose oxidase
- Galactose oxidase
- Amino acid oxidase
- Laccase (3)
- Catalase
- Chloroperoxidase
- HRP
Oxidoreductases – Combi CLEA

<table>
<thead>
<tr>
<th>Activity</th>
<th>Free enzyme</th>
<th>CLEA</th>
</tr>
</thead>
<tbody>
<tr>
<td>1\textsuperscript{st} use</td>
<td>100%</td>
<td>100%</td>
</tr>
<tr>
<td>2\textsuperscript{nd} use</td>
<td>-</td>
<td>100%</td>
</tr>
</tbody>
</table>

\textit{combiCLEA 1} = \textit{Glucose oxidase / catalase}

\textit{combiCLEA 2} = \textit{Galactose oxidase / catalase}
Oxidoreductases – Laccase

- TEMPO/NaOCl environmentally unfriendly
- Laccase / TEMPO / O₂:
  - Green Alternative
  - Enzyme costs too high (owing to suicide inactivation)
- Increase operational stability with a laccase CLEA
  - Recycle
- Also with cellulose to carboxycellulose (shampoo)
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- HRP

**Lyases**
- $R$- & $S$- HNLases (5)
- PDC
- DERA
- Nitrile hydratase (9)
Lyases – Hydroxynitrile Lyases

- Low reaction temp. (5°C)
- Microaqueous environment (0.18% H₂O in DCM)
- Immobilization as a CLEA

"The use of a dichloromethane reaction system with enzyme aggregates and free hydrogen cyanide was crucial in improving cyanohydrin stereoselectivity through minimizing background racemic cyanide addition and enzyme-catalyzed racemization of the product."

Lyases – Combi CLEA

Step Economy a Tri-enzymatic Cascade with a Triple-Decker Combi CLEA

- Buffer: DIPE (10:90)
- pH 5.5 / RT / < 5h
- HnL/ NLase / Pen.acylase Combi-CLEA
- Conv. 96% / ee >99%
(1) Aggregation/purification using ammonium sulfate

(2) Cross linking using glutaraldehyde

Remaining activity in CLEA: >50%
Lyases – Nitrile Hydratase

\[ \text{C} = \text{N} \quad \xrightarrow{10 \text{ mM Tris-}\text{HCl pH8}} \quad \text{C} = \text{NH}_2 \]

\[ 21^\circ \text{C} \]

- Cells 1600 mmol/L
- Cells 2000 mmol/L
- Extract 1600 mmol/L
- Extract 2000 mmol/L
- CLEA 1600 mmol/L
- CLEA 2000 mmol/L

<table>
<thead>
<tr>
<th>Run</th>
<th>Yield</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>90 %</td>
</tr>
<tr>
<td>2</td>
<td>95 %</td>
</tr>
</tbody>
</table>
Fed-Batch Production Acrylamide

Run | Residual Activity
--- | ---
1 | 95 %
2 | 85 %
3 | 65 %
4 | 50 %

21 °C
NHases - Storage and Recycle Stability

- 1000 µL 0.01 M Tris buffer pH 8
- 80 mM hexanenitrile
- 3.9 mg protein in CLEA

Exposure to 2 phase system for 60 minutes

- Storage: 3 days on ice
- Storage: 2 weeks on ice
- Storage: 5 days on ice
- Storage: 20 hours at rt.
- Storage: 1 day on ice
- Storage: 5 days at rt.
- Storage: 7 days at rt.
- Storage: 1 day at rt.
- Storage: 5 days at rt.
- Storage: 10 days at rt.

Produced Hexanamide (mmol/L)

Cycle #
Scope of the Technology

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- PDC
- DERA
- Nitrile hydratase (9)

**Transferases**

**Transaminases**
- (R) selective (3)
- (S) selective (5)
Transferases - Transaminases

- Combi CLEA – TAm and AlaDH

Conversion to MPPA after 24 h
Triple Combi CLEAs

Comparison to FE (TA:LDH:GDH)

Different TAm : LDH : GDH ratios in the triple combi CLEAs
CLEAs in Reactors

Pen Acylase in Filter Slurry Reactor (FSR)

Alcalase® CLEA in fluidized bed

Microchannel Reactors

- Numbering up vs Scaling up
- $10^2 \times$ larger surface/volume ratio
- Efficient mass & heat transfer
- Rapid screening of enzyme scope

- $\gamma$-Lactamase CLEAs in microchannel reactor
  - 100% Activity retention

Conclusions

• Biocatalysis is Green & Sustainable

• Immobilization is a Key Enabling Technlogy

• The CLEA Technology has many Benefits
  — Simple, broadly applicable & cost-effective
  — Improved stability & operational performance
  — High productivity & product quality
  — Applicable to crude cell lysates
  — No leaching of the enzyme in aqueous media
  — Combi-CLEAs for biocatalytic cascades
  — Smart CLEAs (e.g. magnetic CLEAs)
Thank you.......