Site-selective bioconjugation to protein

Literature seminar
2016/01/23
Takashi Ishiyama
Topics

1. Introduction
2. Importance control of bioconjugation
3. Conventional protein modification
4. Site-selective bioconjugation strategy
   - $\pi$-clamp-mediated cysteine bioconjugation
   - Ligand-directed selective bioconjugation
5. Summary
What is Bioconjugation?

Bio(生体)conjugation(共役)

A : Biomolecules (mainly protein)
B : Molecules adding other function

A + B → Forming Covalent Bond → A-B Conjugate

add new function
Use of Bioconjugation

• Creating experimental tool

Antibody-enzyme conjugate
Fluorescently labeled antibody
Biotinylated antibody

Immunoassay
Western blotting
Affinity chromatography
Use of Bioconjugation

- Polyethylene glycol-protein conjugate drugs

![Chemical structures and reaction](image)

<table>
<thead>
<tr>
<th></th>
<th>Native Interferon (\alpha)-2a</th>
<th>PEGASYS</th>
</tr>
</thead>
<tbody>
<tr>
<td>Half-life</td>
<td>3-8 h</td>
<td>65 h</td>
</tr>
<tr>
<td>Dosing interval</td>
<td>thrice-weekly</td>
<td>weekly</td>
</tr>
<tr>
<td>Sites of attachment</td>
<td>Lys 31, 121, 131 or 134</td>
<td></td>
</tr>
</tbody>
</table>

Improvement of patients' QoL

Use of Bioconjugation

• ADC (Antibody-drug conjugate)

(Kadcyla)

Trastuzumab (Herceptin)
Inhibition of HER2 signal

+ eптansine

Inhibition of tubulin polymerization

Stronger therapeutic effect and less side effects
than conventional chemotherapy
Conditions necessary for bioconjugation

- Physiological conditions not to interfere with proper protein folding and function
  - Temperature (<37°C)
  - pH(6-8)
  - Aqueous solvent

- Chemoselectivity (residue-selective reaction)
  - Distinction of different amino acids

- Site-selectivity and control of the number of modifications
  - Distinction of local environment of amino acids
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Importance of control of modification

• PEGylation of proteins

Activity of proteins is reduced due to blocking active site by PEG chain

Importance of control of modification

Activity of PEGylated chymotrypsin

Need for controlled modification to the amino acid away from the active site
Importance of control of modification

• Influence of conjugation site to ADC’s stability and therapeutic activity

![Diagram showing conjugation sites](image)


• Solvent accessibility: FC-S396C > HC-A114C > LC-V205C
• LC-V205C conjugation site is in a positively charged environment
Importance of control of modification

These ADCs’ *in vivo* efficacies and pharmacokinetics properties:

- In vivo activities and durabilities: LC-V205C > HC-A114C > FC-S396C
- No differences in total antibody clearance rates

⇒ Differences in intact ADC levels from linker stability
Importance of control of modification

Difference of stability of antibody-drug bonding

⇒ Site-selective bioconjugation is important to improve the stability and activity of antibody-drug conjugate
Importance of control of modification

- Influence of conjugation number to ADC’s stability and therapeutic activity

**In vitro** cytotoxic activities of ADC

- IC50...E2 : 6.2 ng/mL
- E4 : 2.9 ng/mL
- E8 : 1.0 ng/mL

*in vitro* potency of ADC E2 < E4 < E8

Importance of control of modification

The effect of drug loading on in vivo antitumor activity

Relationship of ADCs’ activity and DAR or dose

Therapeutic effect
E4 0.5 mg/kg ≅ E8 0.5 mg/kg
E2 1.0 mg/kg > E4 0.5 mg/kg > E8 0.25 mg/kg

in vivo activity isn’t proportional to drug number
Importance of control of modification

Relationship of conjugation number and pharmacokinetics properties

in vivo stability: E2 > E4 > E8

ADCs with higher DAR level are more sensitive to stress such as temperature.


⇒ The number of bioconjugation is also important to improve the stability and activity of antibody-drug conjugate
Importance of control of modification

• Modification location and number of the protein are important for its activity and stability

⇒ Site-selective bioconjugation reaction
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Target of protein bioconjugation

• 20 natural amino acids

Ala | Arg | Asn | Asp | Cys | Glu | Gln
---|---|---|---|---|---|---
H2N-CH-C-OH | H2N-CH-C-OH | H2N-CH-C-OH | H2N-CH-C-OH | H2N-CH-C-OH | H2N-CH-C-OH | H2N-CH-C-OH
CH3 | CH2 | CH2 | CH2 | CSH | CH2 | CH2
| | | | | | | NH2
| | | | | | |
Ala | Arg | Asn | Asp | Cys | Glu | Gln
---|---|---|---|---|---|---
H2N-CH-C-OH | H2N-CH-C-OH | H2N-CH-C-OH | H2N-CH-C-OH | H2N-CH-C-OH | H2N-CH-C-OH | H2N-CH-C-OH
H | CH2 | CH2 | CH2 | CH2 | CSH | CH2
| | | | | | | CH2
| | | | | | | NH2
| | | | | | | |
Gly | His | Ile | Leu | Lys | Met | Phe
---|---|---|---|---|---|---
H2N-CH-C-OH | H2N-CH-C-OH | H2N-CH-C-OH | H2N-CH-C-OH | H2N-CH-C-OH | H2N-CH-C-OH | H2N-CH-C-OH
NH2 | CH2 | CH2 | CH2 | CSH | CH2 | CH2
| | | | | | | CH2
| | | | | | | | CH3
Gly | His | Ile | Leu | Lys | Met | Phe
---|---|---|---|---|---|---
H2N-CH-C-OH | H2N-CH-C-OH | H2N-CH-C-OH | H2N-CH-C-OH | H2N-CH-C-OH | H2N-CH-C-OH | H2N-CH-C-OH
| | | | | | | CH2
| | | | | | | OH
| | | | | | | |
Pro | Ser | Thr | Trp | Tyr | Val | C-terminal
---|---|---|---|---|---|---
H2N-CH-C-OH | H2N-CH-C-OH | H2N-CH-C-OH | H2N-CH-C-OH | H2N-CH-C-OH | H2N-CH-C-OH | H2N-CH-C-OH
| | | | | | | CH2
| | | | | | | | CH3
| | | | | | | | OH
| | | | | | | | |
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---|---|---|---|---|---|---
H2N-CH-C-OH | H2N-CH-C-OH | H2N-CH-C-OH | H2N-CH-C-OH | H2N-CH-C-OH | H2N-CH-C-OH | H2N-CH-C-OH
| | | | | | | CH2
| | | | | | | | | CH3
| | | | | | | | | OH
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---|---|---|---|---|---|---
H2N-CH-C-OH | H2N-CH-C-OH | H2N-CH-C-OH | H2N-CH-C-OH | H2N-CH-C-OH | H2N-CH-C-OH | H2N-CH-C-OH
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| | | | | | | | | OH
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---|---|---|---|---|---|---
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| | | | | | | CH2
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| | | | | | | | | OH
| | | | | | | | | |
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---|---|---|---|---|---|---
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| | | | | | | CH2
| | | | | | | | | CH3
| | | | | | | | | OH
| | | | | | | | | |
Pro | Ser | Thr | Trp | Tyr | Val | C-terminal
---|---|---|---|---|---|---
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| | | | | | | CH2
| | | | | | | | | CH3
| | | | | | | | | OH
| | | | | | | | | |
Conventional protein bioconjugation

1. Targeting natural lysine
2. Targeting N-terminal amine
3. Targeting natural cysteine
Targeting natural lysine

• One of the most nucleophilic functional group in a protein
• Abundance on the solvent-exposed outside surfaces of protein

⇒ Most reactive amino acid
Targeting natural lysine

• Random modification of Lys residues

40 of identified modification sites $\Rightarrow$ millions of types of products
Modified lysine residues either are located on the protein surface or have
relatively large structural flexibilities.

But site-selectivity is remarkably low

Wang L., Amphlett G., Blattler W.A., Lambert J. M., Zhang W.
Targeting N-terminal amine

• Only one N-terminal amine
  ⇒ No diversity of conjugation products

• Possible to distinguish between N-terminal α amine and Lysine ε amine

But impossible to introduce multiple modifications
Targeting natural cysteine

- Rarity of cysteine in proteins
  ⇒ Low heterogeneous products
- Thiol also has high nucleophilicity
- Different reactivity of Thiol (soft nucleophilic group)

But Cys in protein form disulfide bonds ⇒ Necessity to reduction of S-S bonds
Targeting natural cysteine

• Reduction-Alkylation strategies

Intermolecular and intramolecular disulfide bonds were distinguished (intermolecular S-S only reduced)
Partial reduction can’t distinguish intermolecular S-S bonds ⇒ various reduction product
⇒ various conjugation product

Site-selective Cys reduction-modification was difficult

Strategy of site-selective protein bioconjugation

• Impossible to distinguish same amino acids
  Lys and Lys, Cys and Cys

⇒ Recognize other than the target amino acids
  ➢ Local environment around the reaction point
  ➢ Ligand recognition site away from reaction point
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π-clamp-mediated cysteine bioconjugation

- Thiol’s Nucleophilic aromatic substitution ($S_{\text{N}Ar}$) reactions

- Glutathione-S-transferase are capable of selectivity arylationing

⇒ Arylation of cysteine can be used to Cys-selective bioconjugation.
π-clamp-mediated cysteine bioconjugation

- Cys-perfluoroarylation reaction

Cys-selective
Room temperature
But organic solvent needed

<table>
<thead>
<tr>
<th>Amino acid</th>
<th>Yield (^{19}F NMR)</th>
</tr>
</thead>
<tbody>
<tr>
<td><img src="image1.png" alt="Amino acid 1" /></td>
<td>&gt; 95%</td>
</tr>
<tr>
<td><img src="image2.png" alt="Amino acid 2" /></td>
<td>&gt; 95%</td>
</tr>
<tr>
<td><img src="image3.png" alt="Amino acid 3" /></td>
<td>No reaction</td>
</tr>
<tr>
<td><img src="image4.png" alt="Amino acid 4" /></td>
<td>No reaction</td>
</tr>
</tbody>
</table>

\(\pi\)-clamp-mediated cysteine bioconjugation

- Enzyme-catalyzed Cys-bioconjugation

Fast
Cys-selective
Regioselective
Aqueous condition
But enzyme needed

$\pi$-clamp-mediated cysteine bioconjugation

- Cysteine bioconjugation using without
  1. Organic solvent
  2. Enzyme (GST)

$\Rightarrow$ Search of substrates reacting in water

$\Rightarrow$ Phe-Cys-Pro-Trp-... reacted in water

$\Rightarrow$ Aromatic amino acids activated the cysteine thiol and interact with the perfluoroaryl group?
\(\pi\)-clamp-mediated cysteine bioconjugation

- \(\pi\)-clamp mediated conjugation

<table>
<thead>
<tr>
<th>peptide</th>
<th>(K_2 (M^{-1}s^{-1}))</th>
<th>yield(%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gly-Cys-Pro-Gly-</td>
<td>N/A</td>
<td>&lt;1</td>
</tr>
<tr>
<td>Gly-Cys-Pro-Phe-</td>
<td>0.09</td>
<td>50</td>
</tr>
<tr>
<td>Phe-Cys-Pro-Phe-</td>
<td>0.73</td>
<td>&gt;99</td>
</tr>
<tr>
<td>Tyr-Cys-Pro-Tyr-</td>
<td>0.08</td>
<td>47</td>
</tr>
<tr>
<td>Trp-Cys-Pro-Trp-</td>
<td>0.20</td>
<td>79</td>
</tr>
</tbody>
</table>

Aromatic amino acids accelerated reaction
Only Phe-Cys-Pro-Phe reacted quantitatively


- Applicable to reaction of protein(antibody)
π-clamp-mediated cysteine bioconjugation

• Mechanism of π-clamp mediated cysteine bioconjugation
  ◆ Activation of thiol of Cysteine (stabilization of thiolate)

Structure of Phe-Cys-Pro-Phe

\[ \text{←-SH (pK}_a=7.69 \pm 0.09) \]

More acidic than thiol of Gly-Cys-Pro-Gly (pK\(_a=8.30 \pm 0.05\))

⇒ Nucleophilicity of thiol increases
π-clamp-mediated cysteine bioconjugation

- Lower activation energy and product free energy

\[ \Delta E = E_{TS} - E_{\text{peptide}} \]
\[ \Delta G = E_{\text{product}} + E_{\text{HF}} - E_{\text{peptide}} - E_{\text{perfluoroaromatics}} \]

π-π interaction stabilizes transition state and product ⇒ reaction acceleration
π-clamp-mediated cysteine bioconjugation

Interaction between reagent and amino acids around target

Cysteine increases site-selectivity of bioconjugation

• Problem
  ◆ Necessity of structure X-Cys-Pro-X to drive bioconjugation reaction

  ⇒ Introduction of X-Cys-Pro-X to target protein by genetic manipulation

• Next step
  ◆ Recognition of native amino acids around target residue
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Ligand-directed selective bioconjugation

- Concept of an affinity labeling probe

Site-selective
Protein-selective
Not react to amino acids in active-site

Ligand-directed selective bioconjugation

• Problem

⇒ Active site was blocked, reactivity ↓
⇒ Reagents not yield covalent protein-ligand adducts

• Ligand-directed Tosyl (LDT) chemistry

Ligand-directed selective bioconjugation

• Design of LDT reagents (spacer structure)

SLF (Ligand for FKBP12)
Ligand-directed selective bioconjugation

• Reactivity of reagent

\[ \text{Ligand-directed reagent} \]

FKBP12

11 μM, pH 8.0, 37 °C, 48 h

<table>
<thead>
<tr>
<th>entry</th>
<th>spacer</th>
<th>probe</th>
<th>yield</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>a</td>
<td>Dc</td>
<td>3%</td>
</tr>
<tr>
<td>2</td>
<td>b</td>
<td>Dc</td>
<td>19%</td>
</tr>
<tr>
<td>3</td>
<td>c</td>
<td>Dc</td>
<td>6%</td>
</tr>
<tr>
<td>4</td>
<td>b</td>
<td>Fl</td>
<td>21%</td>
</tr>
<tr>
<td>5</td>
<td>d</td>
<td>Fl</td>
<td>71%</td>
</tr>
</tbody>
</table>

⇒ Spacer D increases reactivity and site-selectivity

• Site-selectivity of reagent

<table>
<thead>
<tr>
<th>Entry</th>
<th>His90</th>
<th>Tyr85</th>
<th>Glu57</th>
</tr>
</thead>
<tbody>
<tr>
<td>4</td>
<td>43%</td>
<td>4%</td>
<td>53%</td>
</tr>
<tr>
<td>5</td>
<td>4%</td>
<td>0%</td>
<td>96%</td>
</tr>
</tbody>
</table>
Ligand-directed selective bioconjugation

• Consideration of high reactivity and site-selectivity

- Length of spacers b, d is close to the distance between ligand and target amino acids

- Piperazine type spacer may fix probe towards Glu57

<table>
<thead>
<tr>
<th>Labeling site</th>
<th>Distance from ligand</th>
</tr>
</thead>
<tbody>
<tr>
<td>His90</td>
<td>8.51 Å</td>
</tr>
<tr>
<td>Tyr85</td>
<td>6.74 Å</td>
</tr>
<tr>
<td>Glu57</td>
<td>6.88 Å</td>
</tr>
</tbody>
</table>

⇒ Hard spacer with appropriate length is important to reactivity and selectivity
Ligand-directed selective bioconjugation

Binding of ligand and active site accelerate bioconjugation reaction

- Problem
  - Slow reaction rate and low labeling efficiency of ligand-directed tocylate chemistry

⇒ More reactive reagent
Ligand-directed selective bioconjugation

• Ligand-directed acyl imidazole (LDAI) chemistry

  Acyl transfer is faster than $S_N2$ reaction?
  Much more reactive than LDT reagent

• Ligand-directed dibromophenyl benzoate (LDBB) chemistry

  pKa of leaving group : 5.6 (imidazole 14.5)
  $\Rightarrow$ More reactive

  Modification yield 50% (30 min)
  85~90% (3 h)

Matsuo. K. et al., Chem. Sci., 2013, 4, 2573
Takaoka. J. et al., Chem. Sci., 2015, 6, 3217
Ligand-directed selective bioconjugation

• Problem

◆ Modification point is limited to around active site

indicated that the original molecular recognition ability of CAII is fully retained.


⇒ Not applicable to modification of amino acids away from active site

• Next step

◆ Recognition of structure of protein surface other than the active site
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Summary

◆ Importance of modification location and number of the protein

• Resent study

◆ Transferring the microenvironment of the protein surface to the site-selectivity of bioconjugation
  
  ➢ $\pi$-clamp-mediated cysteine bioconjugation
  
  ➢ Ligand-directed selective bioconjugation

• Next step

◆ Development of technique for recognition of protein surfaces