Experiment Protocol 016

Immobilization of ligands (compounds with OH groups) on COOH beads

In affinity purification (screening) of ligand-binding proteins, it is first necessary to optimize the amount of immobilization of ligands on beads. You can change the amount of immobilization of ligands by changing the concentration of ligands. This experiment protocol shows a method to immobilize the ligand concentrations at four steps, i.e. 0, 3, 10, 30 mM when immobilizing ligands on COOH beads.

1. Materials

1.1 Beads and ligands（compounds）

- COOH beads (TAS8848N1140):10 mg (Functional groups：Approx. 200nmol/mg)
- Ligands: Approx. 20 mg

1.2 Reagents

- *N*,*N*'-dimethylformamide (DMF): Dehydrated 30 mL
- Fluoro-*N*,*N*,*N'*,*N'*-Tetramethylformamidinium Hexafluorophosphate (TFFH)：
  M.W. 264.12 4.0 mg
- *N*,*N*-Diisopropylethylamine(*i*-Pr_2NEt) M.W.129.24 8.8 μL
- oxyma pure M.W.142.11 2.1 mg
- 2-amino ethanol M.W. 61.08 126 μL
- N-hydroxysuccinimide (NHS) M.W. 115.09 51.8 mg
- 1-etyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC･HCl) M.W. 191.70 76.8 mg

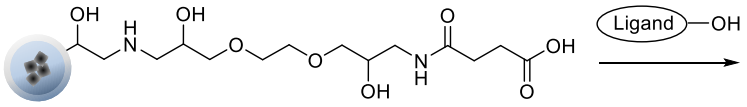
1.3 Apparatus

- Micro centrifuge
- Micro tube mixer (TOMY MT-360, etc.)
- Ultrasonic dispersing device
  We have performed operation checks with an ultrasonic homogenizer:VP-15S with a cup horn(TAITEC), or an ultrasonic dispersing device.

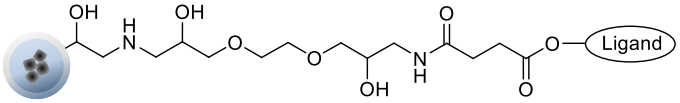
2. Method

2.1 Outline

The following is a schematic view of ligand immobilization. Refer to the next section 2.2 “Procedures” for details.



COOH beads



OH groups ligand immobilized beads

2.2 Procedures

2.2.1 Immobilization of ligands

1) Dissolve the ligand (compound) in DMF to prepare 250 μL of 100 mM ligand solution.
2) Mix *i*-Pr_2NEt (8.8 uL) into DMF (41.2 uL) to prepare 50 uL of 1M *i*-Pr_2NEt. Dilute 1M *i*-Pr_2NEt to prepare 120 uL of 100 mM *i*-Pr_2NEt.
3) Dissolve TFFH (4.0 mg) in DMF (150 uL) to prepare 150 uL of 100 mM TFFH solution.
4) Dissolve oxyma pure (2.1 mg) in DMF (1.5 mL) to prepare 1.5 mL of 10 mM oxyma pure solution.
5) Add 2.5 mg of COOH beads (TAS8848N1140) to each of the four 1.5 mL microtubes. (total 10 mg)
6) Centrifuge at 15,000 rpm for five minutes at room temperature, and discard the supernatant.

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7) Add 500 µL of DMF and disperse the beads with an ultrasonic device.
8) Centrifuge at 15,000 rpm for five minutes at room temperature, and discard the supernatant.
9) Repeat the above 7) - 8) two more times. (Wash the beads three times in total.)
10) Add the solutions prepared in 1) to 4) above as shown in the table below, and disperse the beads with an ultrasonic device.

Immobilization concentration	0	3	10	30	mM
COOH beads	2.5	2.5	2.5	2.5	mg
DMF	500	440	315	147	µL
100 mM *i*-Pr_2NEt or 1M *i*-Pr_2NEt	0	25 (100 mM)	75 (100 mM)	23 (1M)	µL
100 mM TFFH	0	10	30	90	µL
100 mM ligand	0	15	50	150	µL
10 mM oxyma pure	0	10	30	90	µL
Total	500	500	500	500	µL

11) React them overnight (16-20 hours) at room temperature by using a micro tube mixer.
12) Centrifuge at 15,000 rpm for five minutes at room temperature, and discard the supernatant.
13) Add 500 µL of DMF, and disperse the beads.
14) Centrifuge at 15,000 rpm for five minutes at room temperature, and discard the supernatant.
15) Repeat the above 13) - 14) two more times. (Wash the beads three times in total.)

2.2.2 Masking

16) Dissolve NHS (51.8 mg) in DMF (450 uL) to prepare 450 µL of 1M NHS solution.
17) Mix aminoethanol (126 uL) and DMF (1974 uL) to prepare 2.1 mL of 1M aminoethanol solution.
18) Weigh 19.2 mg (100 µmol) of EDC / HCl into four separate 1.5 mL microtubes.
19) Add 400 µL of DMF to the beads washed in above 15) to disperse the beads.
20) Add 100 µL of each 1M NHS solution and mix.
21) Transfer the entire amount of above 20) to the tube of above 18) and mix.

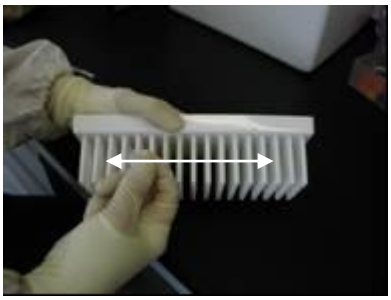
Beads (after reaction)	2.5	mg
DMF	400	µL
1M NHS	100	µL
EDC・HCl	19.2	mg
Total	500	µL

22) React them for two hours at room temperature by using a micro tube mixer.
23) Centrifuge at 15,000 rpm for five minutes at room temperature, and discard the supernatant.
24) Add 500 µL of DMF, and disperse the beads.
25) Centrifuge at 15,000 rpm for five minutes at room temperature, and discard the supernatant.
26) Repeat the above 24) - 25) two more times. (Wash the beads three times in total.)
27) Add 500 µL (4 tubes) of 1M aminoethanol to the beads from which the supernatant has been discarded to disperse the beads.
28) React them for two hours at room temperature by using a micro tube mixer.
29) After 2 hours, centrifuge at 15,000 rpm for five minutes at room temperature, and discard the supernatant.
30) Add 500 µL of ultrapure water to disperse the beads.
31) Centrifuge at 15,000 rpm for five minutes at room temperature, and discard the supernatant.
32) Repeat the above 30) - 31) two more times. (Wash the beads three times in total.)

13) Disperse the beads in 100 μL of ultrapure water, and store them at 4℃. (Concentration of ligand immobilized beads:0.5 mg/20 μL)

3. Supplements

・ Beads are easily dispersed by using an ultrasonic dispersing device. But if you do not have such a device, they are dispersed by using an ultrasonic washer, or by the manual agitation. In the manual dispersion method, the bottom of a micro-tube is glided over an uneven surface (side of plastic test tube rack in this case) creating turbulence through the collisions. (see left side picture below)
Please make sure to use well-constructed tubes with the caps tightly secured in order to prevent leakage/breakage. Use of cap lock is recommended in order to prevent leakage. (see right side picture below).
For more information, please visit FG beads web site and see the movie of the method.
(Please click:
https://fgb.tamagawa-seiki.com/english/faq/please-tell-me-how-to-disperse-fg-beads-ultrasonic-method-and-rattling-method for moving pictures.)



・ Recover beads dispersed in DMF or ultrapure water not by magnetic separation but by centrifugation because the magnetic separation takes a longer time.
・ Use DMF which is hydrated with a molecular sieve, or a low-moisture solvent. If the solvent contains moisture, succinimide may be liberated from beads, and ligands are not properly immobilized on the beads.

4. Notes

・ TFFH and EDC･HCl are easy to absorb moisture, so be careful of moisture contamination.
・ The TFFH solution does not last long, so prepare it just before use if possible.
・ After using TFFH, fill the reagent bottle with an inert gas and store it. Do not use TFFH that has been opened for a long time, as the activity of the reagent will decrease due to the moisture in the air.
・ These ligand-immobilized beads cannot be stored for a long period of time because the ligand and the bead bond portion are ester-bonded and hydrolysis is likely to occur. Please use as soon as possible after immobilization.
・ If you want to immobilize a ligand with a phenolic OH group on a bead, see Protocol 003, Immobilization of a Ligand on a Epoxy bead (a compound with a phenolic OH or amino group).