Experiment Protocol 008

Immobilization of ligands (compounds with NH_2 groups) on COOH beads

For screening, you need, first of all, 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. 0mM, 0.1mM, 0.3mM, and 1mM when immobilizing ligands on COOH beads.

1. Materials

1.1 Beads and ligands (compounds)

- COOH beads (TAS8848N1140):10mg (Functional groups:Approx. 200nmol/mg)
- Ligands: Approx. 2mg

1.2 Reagents

- N,N'-dimethylformamide (DMF) 18mL
- N-hydroxysuccinimide (NHS) M.W. 115.09 0.1g
- 1-etyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC・HCl) M.W. 191.70 100mg
- Amino ethanol M.W. 61.08 200μL
- Triethylamine M.W. 101.19
- Methanol (MeOH) 4mL

1.3 Apparatus

- Micro centrifuge
- Micro tube mixer (TOMY MT-360, etc.)
- Ultrasonic dispersing device

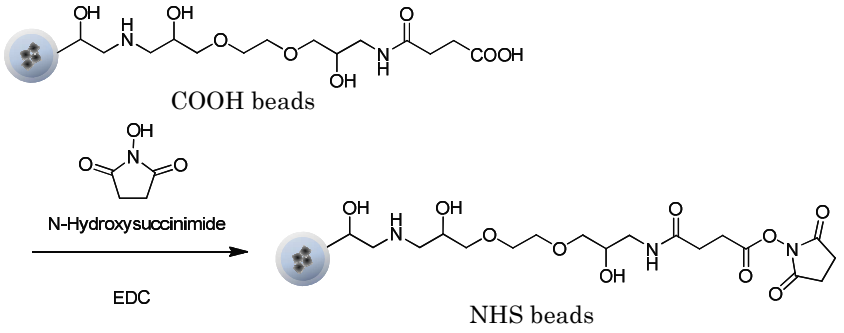
  We have performed operation checks with an ultrasonic homogenizer:VP-15S with a cup horn (TAITEC), or an ultrasonic dispersing device:TA4905 (Tamagawa Seiki).

2. Method

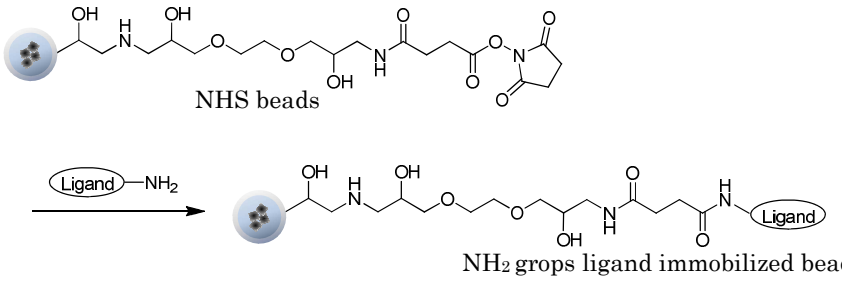
2.1 Outline

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

1) Active esterification



2) Ligand immobilization



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2.2 Procedures

2.2.1 Active esterification

1) Dissolve succinimide in DMF, and prepare 500µL of 1M succinimide solution.
2) Add COOH beads (TAS8848N1140) into a 1.5ml micro-tube. Repeat this one more time. (total 10mg)
3) Centrifuge at 15,000rpm for five minutes at room temperature, and discard the supernatant.
4) Add 500µL of DMF, and disperse the beads.
5) Centrifuge at 15,000rpm for five minutes at room temperature, and discard the supernatant.
6) Repeat the above 4) - 5) two more times. (Wash the beads three times in total.)
7) During the washing, add 38.4mg (200µmol) of EDC・HCl into another 1.5mL micro-tube.
8) After DMF washing, add 800µL of DMF, and disperse the beads.
9) Add 200µL of the prepared 1M succinimide solution, and mix them.
10) Transfer the total volume of the above 9) to the micro-tube of the above 7), and mix them.

COOH beads	(mg)	5
DMF	(µL)	800
1M succinimide	(µL)	200
EDC・HCl	(mg)	38.4
Total	(µL)	1000

11) React them for two hours at room temperature by using a micro tube mixer.
12) Centrifuge at 15,000rpm for five minutes at room temperature, and discard the supernatant.
13) Add 500µL of DMF, and disperse the beads.
14) Centrifuge at 15,000rpm for five minutes at room temperature, and discard the supernatant.
15) Repeat the above 13) - 14) four more times. (Wash the beads five times in total.)
16) Disperse the beads into 100µL DMF each. (Concentration of NHS beads：2.5mg/50µl)

2.2.2 Immobilization of ligands

1) Dissolve ligands (compounds) in DMF, and prepare 1mL of 1mM ligand solution.
2) Dissolve amino ethanol in DMF, and prepare 3mL of 1M amino ethanol solution.
3) Add 2.5mg (50µL) of the NHS beads to each of the four micro-tubes. Centrifuge at 15,000rpm for five minutes at room temperature, and discard the supernatant.
4) Add 200µL of DMF to each of them, and disperse the beads. Centrifuge at 15,000rpm for five minutes at room temperature, and discard the supernatant.
5) Add DMF and the prepared ligand solution, and disperse the beads with an ultrasonic dispersing device.

Concentration	(mM)	0	0.1	0.3	1
NHS beads	(mg)	2.5	2.5	2.5	2.5
DMF	(µL)	500	450	350	0
1mM ligand	(µL)	0	50	150	500
Total	(µL)	500	500	500	500

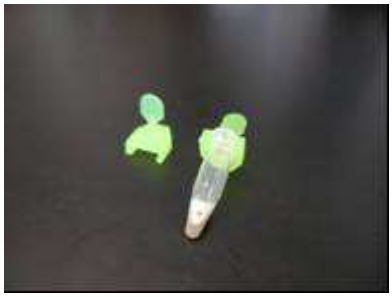
Note 1: If the ligand solution is directly added to the beads, the concentration could be locally raised. Add, therefore, the ligand solution after adding the DMF to the beads.

Note 2: When ligands are hydrochloride, add twice mole tri-ethyl amine to them. In this case, it will be easier to prepare 4 mM triethylamine and 2mM ligand solution, and mix them in equal volume.

6) React them for seventy minutes at room temperature by using a micro tube mixer.
7) Centrifuge at 15,000rpm for five minutes at room temperature, and transfer the supernatant to a fresh micro-tube. (Supernatant A)
8) Add 500µL of amino ethanol to the remaining beads each, and disperse the beads with an ultrasonic dispersing device.
9) React them for two hours at room temperature by using a micro tube mixer.
(Masking of ligand-non-binding carboxyl groups)
10) Centrifuge at 15,000rpm for five minutes at room temperature, and transfer the supernatant to a fresh micro-tube. (Supernatant B)
11) Add 500µL of 50% MeOH, and disperse the beads with an ultrasonic dispersing device.
12) Centrifuge at 15,000rpm for five minutes at room temperature, and discard the supernatant.
13) Repeat the above 11) - 12) two more times. (Wash the beads three times in total.)
14) Disperse the beads in 100µL of 50% MeOH, and store them at 4℃. (Concentration of ligand immobilized beads:0.5mg/20µL)

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 : http://www.magneticnanoparticle.jp/en/htdocs/af-notes.html for moving pictures.)



・ Recover beads dispersed in DMF or 50% MeOH 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.
・ Although we recommend using 50% MeOH for storing ligand immobilized beads in view of the decrease of dispersibility of beads due to immobilization of hydrophobic compounds, you can satisfactorily use ultrapure water, too.
・ You can investigate the following by determining quantity of succinimide in the supernatant A and B by means of HPLC.
(Refer to Experiment Protocol 201 for the method.)

A : The amount of immobilization of ligands
(The amount of succinimide liberated when immobilizing ligands.)

B : The amount of NHS groups on which ligands are not immobilized.
(The amount of succinimide liberated when masking.)
A+B : The amount of NHS groups of beads

・ You can enhance the reactivity by adding tri-ethyl amine even when ligands are not hydrochloride if the volume of the immobilization is small.