Experiment Protocol 005

Immobilization of ligands (carboxylic compounds) on NH_2 beads (1) A method of using HOSu

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 protocol shows a method to immobilize ligands at four various concentrations, i.e. 0 mM, 0.4 mM, 2 mM, and 10 mM when immobilizing ligands on NH_2 beads.

1. Materials

1.1 Beads and Ligands (compounds)

- NH_2 beads (TAS8848N1130):10mg (Functional groups：Approx 200nmol/mg)
- Ligands: approximately 5mg

1.2 Reagents

- N,N'- Dimethylformamide (DMF)　25ml
- N- Hydroxysuccinimide (HOSu)　M.W. 115.09　5mg
- 1- Ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC) M.W. 155.24
  5mg (Peptide Institute 1020, etc.)
- Triethylamine　200µl
- Acetic anhydride　M.W. 102.09　80µl
- Methanol (MeOH)　5ml

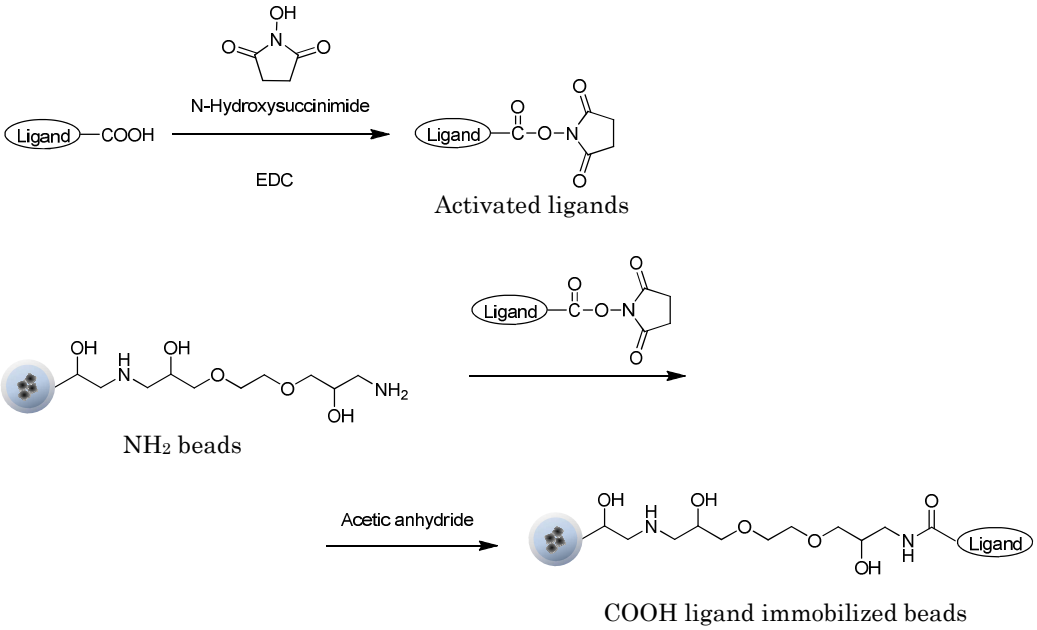
1.3 Apparatus

- 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), and 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.



2.2 Procedures

1) Dissolve ligands (compound) in DMF, and prepare 10µmol/500µL (20mM) solution. (Final 10mM chemical compound)
2) Dissolve succinimide in DMF, and prepare 100µL of 200mM succinimide solution.
3) Dissolve EDC in DMF, and prepare 100µL of 200mM EDC solution.
4) Add 400µL of DMF, 50µL of 200mM succinimide solution, and 50µL of 200mM EDC solution to 500µL of 20mM ligand solution as below, and mix for two hours at room temperature by using a micro tube mixer. (Mix them in equal mol.)

20mM ligand (compound)	(µL)	500 (10µmol)
200mM succinimide	(µL)	50 (10µmol)
200mM EDC	(µL)	50 (10µmol)
DMF	(µL)	400
Total	(µL)	1000

5) Add 2.5 mg of NH_2 beads (TAS8848N1130) into each of four 1.5 mL micro-tubes.
6) Centrifuge at 15,000 rpm for five minutes at room temperature, and discard the supernatant.
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) to 8) two more times. (Wash the beads three times in total.)
10) Add DMF for each ligand immobilization concentration.
11) Add the prepared activated 10mM ligand solution, and disperse the beads with an ultrasonic device as below.
12) React for 16 to 20 hours at room temperature by using a microtube mixer.

Concentration	(mM)	0	0.4	2	10
NH_2 beads	(mg)	2.5	2.5	2.5	2.5
DMF	(µL)	500	480	400	0
Activated 10mM ligand	(µL)	0	20	100	500
Total	(µL)	500	500	500	500

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

13) Centrifuge at 15,000 rpm for five minutes at room temperature, and discard the supernatant.
14) Add 500 µL of DMF, and disperse the beads with an ultrasonic device.
15) Centrifuge at 15,000 rpm for five minutes at room temperature, and discard the supernatant.
16) Repeat the above 14) to 15) two more times. (Wash the beads three times in total.)
17) Resuspend in 430µL of DMF by ultrasonic waves.
18) Add 50µL of triethylamine and 20µL (0.2mmol) of acetic anhydride.
19) Mix for two hours at room temperature by using Microtube Mixer.
(Masking of ligand-unreacted amino groups)
20) Centrifuge at 15,000 rpm for five minutes at room temperature, and discard the supernatant.
21) Add 500 µL of DMF, and disperse the beads with an ultrasonic device.

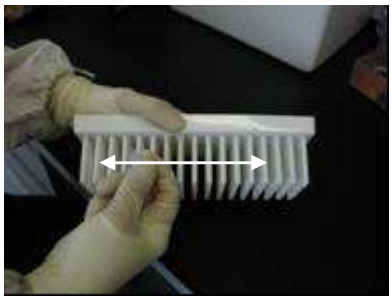
22) Centrifuge at 15,000 rpm for five minutes at room temperature, and discard the supernatant.
23) Repeat the above 21) to 22) two more times. (Wash the beads three times in total.)

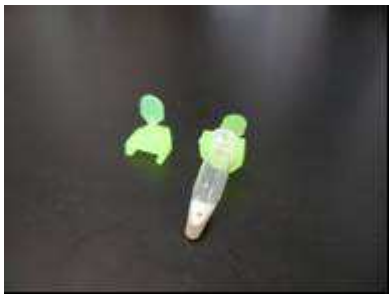
If the chemical compound has functional groups to be acetylated by acetic anhydride such as an OH group, perform a deacetylation process, after masking, by resuspending in 500µL of 0.1 M sodium hydroxide, and by mixing for 30 minutes at room temperature by using Microtube Mixer. After then, repeat the resuspension by centrifugation and ultrasonic waves, and wash with 500µL of ultrapure water three times.

24) Add 500 µL of 50% MeOH, and disperse the beads with an ultrasonic device.
25) Centrifuge at 15,000 rpm for five minutes at room temperature, and discard the supernatant.
26) Repeat the above 24) to 25) two more times. (Wash the beads three times in total.)
27) Resuspend in 100 µL of 50% MeOH, and store 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 : http://www.magneticnanoparticle.jp/en/htdocs/af-notes.html for moving pictures.)





・ Recover beads dispersed in DMF or 50% MeOH by centrifugation because the magnetic separation takes a longer time.
・ Use DMF which is hydrated with a molecular sieve, or a low-moisture solvent.
・ 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.