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Lab & Production Materials

Application Note

Microsphere Coupling—Two-step EDC/Sulfo NHS Covalent Coupling Procedure for Estapor® Carboxyl-modified Dyed Microspheres

Introduction

Proteins can be covalently coupled to the surface of carboxylated microspheres through activation of the carboxyl groups with water-soluble 1-ethyl-3-[3-dimethylaminopropyl] carbodiimide (EDC). The reaction forms an active intermediate (O-acylisourea) that reacts quickly with primary amines (nucleophile) to form a stable amide bond.

The active intermediate formed by the EDC reaction is unstable and readily hydrolysed by water; this competing reaction with water can cleave the active intermediate to regenerate the carboxyl group. To prevent rapid hydrolysis of the active intermediate, N-hydroxysulfosuccinimide (Sulfo-NHS) can be added to the reaction to form a more stable Sulfo-NHS ester intermediate that reacts slowly with primary amines to form a stable amide bond.

We present here a general two-step covalent coupling protocol for conjugation of proteins to Estapor® carboxylmodified microspheres using EDC and Sulfo-NHS.

General Considerations

When to use the Two-step Coupling Protocol

It is best to use the two-step covalent coupling protocol when the molecule being coupled, such as a protein, contains both carboxyl and primary amine groups. As the two-step method forms a more stable Sulfo-NHS ester intermediate, excess EDC can be removed before addition of the protein. This prevents the activation of carboxyl groups on the protein and the possibility for protein cross-linking.[1] It also facilitates buffer exchange, if needed, and has been shown to improve coupling yields.[2,3]

The two-step coupling protocol can be used for both high density (COOH >100 µeq/g) and low density (COOH <100 µeq/g) carboxyl-modified microspheres.

Activation and Coupling of Carboxyl-modified Microspheres

Activation using EDC and Sulfo-NHS is most efficient between pH 4.5 and 7.2. Therefore, it is often preferred to use MES (2-(N-morpholino) ethanesulfonic acid)) buffer at pH 6 for the activation reaction.[4] The activation buffer should not contain any primary amine or carboxyl groups, as they will compete with the activation reaction. Phosphate and acetate buffers may also reduce the reactivity of the EDC. MES works very well as a coupling buffer but can be exchanged for an alternate buffer at a different pH if required for coating optimization.

When conjugating protein to carboxyl-modified microspheres, the following tips may be useful to avoid any issues with the conjugation procedure.

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Considerations before Starting the Coupling Process

1. EDC should be stored at –20 °C in a desiccated container and allowed to equilibrate to room temperature before use. EDC is extremely sensitive to moisture and should not be used if damp or clumped. EDC solutions should be prepared immediately before use.
2. Sulfo-NHS should also be prepared immediately before use.
3. All reagents should be at room temperature before use.
4. Resuspension of the microspheres can be made easier by forming the loosest pellet possible. Below are recommended centrifuge cycles based on microsphere size.

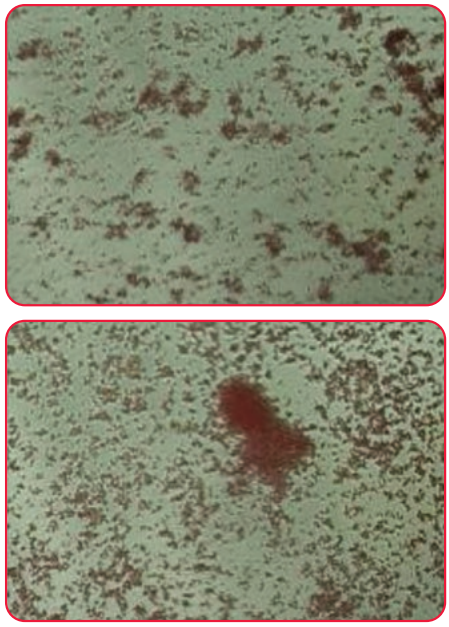
   0.2 µm–17,000 rpm (~28,500 g) for 9 minutes

   0.3 µm–14,000 rpm (~19,300 g) for 7 minutes

   0.4 µm–12,000 rpm (~14,200 g) for 7 minutes

   0.5 µm–12,000 rpm (~14,200 g) for 5 minutes
5. It is important that the microspheres are completely resuspended between washes and especially before ligand coupling.
6. Microsphere pellets should be resuspended using repeated pipetting first to break up any large clumps and then sonicated if necessary.
7. An immersible ultrasonic probe is most effective to resuspend microspheres to a monodisperse state. Repeated pipetting, vortex mixing, and bath sonication are often insufficient to resuspend microsphere pellets fully.
8. Avoid overheating the sample when sonicating. If required, sonicate the sample with the tube immersed in ice water.
9. Visually, the microsphere solution should look slightly "milky," indicating no major issues with aggregation.
10. Aggregation can be assessed using 400X magnification. Monodisperse microspheres are difficult to see under 400X magnification and will appear as a hazy sea of microspheres (**Figure 1**). Aggregated microspheres, however, are easily observed under 400X magnification (**Figures 2 and 3**).

Figure 1. Monodisperse microspheres



Figures 2 and 3. Aggregated microspheres

11. Use Milli-Q® water when preparing buffers and washing microspheres to avoid any issues that may be caused by contaminants.
12. The antibody concentration will need to be optimized for specific test requirements. For coupling of expensive antibodies that may be unavailable in enough quantity to reach the optimal molar ratio, a nonspecific protein such as bovine gamma globulin or BSA may be added to occupy any remaining reactive sites.

Two-step EDC/Sulfo-NHS Coupling

This is a general two-step EDC/Sulfo-NHS covalent coupling protocol. Optimization may be required, depending on the type and molecular weight of the protein and the carboxyl charge density on the microspheres.

Microspheres

Estapor® Carboxyl-Modified Dyed Microspheres
Product Number e.g. K1-020, K1-030, K1-040, K1-050

Buffers

Activation/Coupling Buffer—50 mM MES, pH 6.0
Blocking Buffer—50 mM Tris, pH 8.0, 0.5% (w/v) casein

Microsphere Activation & Quenching

EDC (Thermo Scientific, 22980)
Sulfo NHS (Thermo Scientific, 24510)
Ethanolamine (Cat. No. 1008452500)

Equipment Used

Eppendorf—5430 Centrifuge with FA-45-24HS rotor
Hielscher Ultrasonics—UP50H Compact
Lab Homogenizer

Health & Safety

Ear protection must be worn when sonicating samples. All hazardous chemicals (e.g. ethanolamine) should be used in a fume hood with appropriate safety precautions.

Conjugation Protocol

Microsphere Washing and Activation

1. Aliquot 100 µL of Estapor® microspheres (at 10% w/v) into a low-protein binding Eppendorf tube.
2. Add 1 mL of activation/coupling buffer and mix thoroughly.

NOTE: The components of the activation/coupling buffer should not contain free carboxyl groups, primary amine groups, or thiols. These functional groups interfere with the cross-linking chemistries.

3. Spin the microspheres at the correct centrifuge cycle for their size.

   0.2 µm–17,000 rpm for 9 minutes

   0.3 µm–14,000 rpm for 7 minutes

   0.4 µm–12,000 rpm for 7 minutes

   0.5 µm–12,000 rpm for 5 minutes

4. Decant the supernatant.
5. Repeat steps 2 through 4 twice. It is important that the microspheres are completely resuspended between washes and before progressing with ligand coating. Repeated pipetting works very well but sonication may also be required, sonicate using an ultrasonic probe.

Optional: Examine the microspheres under a microscope to ensure that they are monodisperse.

6. After the final wash, resuspend the microspheres in 1 mL of activation/coupling buffer. It is important to ensure the microspheres are monodisperse.
7. Prepare activation reagents immediately prior to use.
8. To prepare 200 mM EDC solution, add 19.2 mg of EDC at room temperature to 500 µL of Milli-Q® water in a clean microfuge tube.
9. To prepare 200 mM Sulfo-NHS, add 21.7 mg of Sulfo-NHS to 500 µL of activation/coupling buffer in a clean microfuge tube.
10. To 1 mL of washed microspheres (from Step 6 above) quickly add 24 µL of 200 mM EDC and 240 µL of 200 mM Sulfo-NHS.
11. Vortex and incubate by mixing on a rotary wheel for 30 minutes at room temperature.
12. Spin the tube to pellet the microspheres.
13. Decant the supernatant.
14. Add 1 mL of activation/coupling buffer to wash the microspheres and mix thoroughly.
15. Spin the tube at the identified spin cycle to pellet the microspheres.
16. Repeat steps 13 to 15 twice.
17. suspend the microspheres in 700 µL of activation/coupling buffer (See antibody coupling section). It is important that the microspheres are monodisperse before adding antibodies/ligand. Sonicate, if necessary.

Antibody Coupling

18. Prepare the antibody at a concentration of 2 mg/mL in activation/coupling buffer.
19. Based on a coating concentration of 60 mg antibody per gram of microspheres, add 300 µL of antibody (at 2 mg/mL) to the 700 µL of microspheres. Mix thoroughly. The total liquid volume will now be 1 mL.

Please see **Table 1**. below for a guide on other coating concentrations. Note: The optimal coating concentration will need to be investigated on a case by case basis.

Coating Concentration	Volume of Coupling Buffer	Volume of 2 mg/mL Antibody Solution
20 mg/g	0.9 mL	0.1 mL
40 mg/g	0.8 mL	0.2 mL
60 mg/g	0.7 mL	0.3 mL
80 mg/g	0.6 mL	0.4 mL

Table 1. Antibody Coating Guide

20. Mix the suspension for 2.5 hours on a rotary wheel at room temperature.
21. For 1 mL of 1% microspheres, add 30 µL of ethanolamine (in a fume hood). Vortex and mix on a rotary wheel for 30 minutes to quench the microspheres.
22. Spin the tube at the correct centrifuge cycle to pellet the microspheres.
23. Decant the supernatant. (Optional: Keep the supernatant for protein content analysis using a BCA assay.)
24. Resuspend the microspheres in 1 mL of blocking buffer. Sonicate and mix for a minimum of 2 hours on a rotary wheel at room temperature. Note: The microspheres can also be left mixing at room temperature overnight.
25. Spin the tube at the correct centrifuge cycle to pellet the microspheres.
26. Decant the supernatant.
27. Add 1 mL of blocking buffer and resuspend the microspheres by repeated pipetting and sonication, if necessary.
28. Spin the tube at the correct centrifuge cycle to pellet the microspheres.
29. Repeat steps 26 to 28 once more.
30. After the final wash step, remove the supernatant and add 1 mL of blocking buffer. The microspheres are now at 1% w/v.
31. Resuspend the microspheres using repeated pipetting and sonication. Optional: Examine the microspheres under a microscope to ensure that they are monodisperse.
32. Store the suspension at 4 °C until ready to use. Use within approximately 5 days; conjugate-specific stability will need to be independently assessed.

Troubleshooting Protein Coupling

Problem No. 1

Microsphere aggregation prior to use.

Solution No. 1

Vortex and sonicate the microspheres before use.

Problem No. 2

Microsphere aggregation during or after conjugation.

Solution No. 2

Identify the point at which aggregation occurs.

Aggregation occurs after:

1. EDC Addition
   - Add EDC slowly.
   - Mix the microspheres well after EDC addition.
   - Decrease the concentration of microspheres in solution to 0.5%.
2. Protein Addition
   - There may be insufficient protein; therefore, increase the protein concentration.
   - Try an alternate coupling buffer.
   - Aggregation may be reversible aggregation. Sonicating the sample to break up any aggregates.
3. Centrifugation
   - Pay attention to resuspension of the pellet.
   - Decrease cycle speed or time so as to form the loosest pellet possible.
   - Pipette the pellet more aggressively to resuspend the microspheres.
   - Increase sonication time and/or ultrasonic wave amplitude.
   - Reduce the quantity of microspheres.
4. Washing
   - Add a small quantity of surfactant (e.g. 0.025% SDS).

Problem No. 3

Low binding of antibody or ligand to microspheres.

Solution No. 3

- The EDC may be inactive; use fresh EDC.
- The Sulfo-NHS intermediate may be hydrolyzing. Prepare EDC and Sulfo-NHS immediately before use.
- There may be insufficient protein, requiring an increase in the coating concentration.
- Add protein directly after COOH activation.
- Avoid competing proteins and compounds with amine groups.
- Change the coating buffer and/or pH.

Problem No. 4

Variable coating.

Solution No. 4

- Mix microsphere suspension thoroughly at each step and use fresh reagents.

Problem No. 5

Nonspecific binding of conjugates.

Solution No. 5

- Use an alternate blocking reagent (BSA, glycine, casein, fish skin gelatin).
- Increase the blocking time.
- Reduce the quantity of ethanolamine in the quenching step.
- Reduce the quantity of coating protein.
- Use an alternate Estapor® microsphere. Try using a microsphere with a higher COOH charge density.
- Aggregated microspheres resulting in nonspecific binding. See Solution No. 2 for suggestions.

References

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To discuss your specific product needs, please contact your local dedicated Account Manager

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