SPHERO™ Technical Note **STN-20 Rev A 032320**

ACTIVATION TECHNIQUES FOR HYDROXYL MAGNETIC PARTICLES

Hydroxylic particles are used to facilitate the development of immunoassays, as well as, quantitate, isolate purify and characterize proteins, cells, or various target analytes. In order to provide the benefits of hydroxyl groups, Spherotech offers SPHERO™ Hydroxyethyl (HEMA)/Polystyrene Copolymeric Magnetic Particles. These copolymeric particles provide a hydrophilic surface which reduces denaturation of immobilized protein. In addition, the hydroxyethyl groups hydrogen bonds with a layer of water in an aqueous solution preventing aggregation of the particles. These two characteristics translate into longer ligand stability, lower nonspecific binding potential, and a more stable particle solution compared to particles of more hydrophobic surfaces.

Although hydroxyl groups do not spontaneously reactive toward functional groups on biomolecules, they can be activated for covalent coupling by a number of known reaction mechanisms. The reactions result in covalent attachment of ligands and can be performed under aqueous or nonaqueous conditions. By utilizing nonaqueous conditions the hydrolysis of activating agents and the intermediate groups is prevented.

In order to facilitate the conjugation to the hydroxyethyl surface, cross-linkers can be used to form spacer arms between the particle and the ligand being covalently coupled. Various cross-linkers have become integral tools for the preparation of coated beads used in many immunoassays, and protein isolation and characterization protocols. The most common and efficient cross-linkers are heterobifunctional, which present two reactive groups. The heterobifunctional cross-linkers provide better control and orientation during the conjugation of molecules to reduce the creation of dimers and polymers. The careful choice of ligand, immobilization chemistry and ligand density will aid in creating a selectivity and sensitivity appropriate for the bioreactivity required for specific applications.

PMPI, or p-maleimidophenyl isocyanate is heterobifunctional cross-linker with an isocyanate group on one end and a maleimide group on the other end. Thus, it is used to facilitate the conjugation of a thiolated ligand to the hydroxyethyl bead surface. Traditionally, PMPI is used to cross-link thiolated protein or peptide to carbohydrates, steroids, or vitamins[1]. PMPI is very moisture sensitive and needs to be dissolved in nonaqueous systems. It cannot be used with buffers, but is soluble in toluene and hexane and also can be reacted in methylene chloride. Herein we present a method we adapted to use PMPI to cross-link a thiolated ligand to SPHERO™ Hydroxyethyl (HEMA)/Polystyrene Copolymeric Magnetic Particles.

Other convenient methods for activation form a reactive carbonyl group on the hydroxyl particle using compounds such as carboxyl diimidazole (CDI) or disuccinimidyl carbonate (DSC). These activation agents create imidazole carbamates (CDI) or NHS-carbonates (using DSC), which are then spontaneously reactive toward amines. In the case of CDI, the imidazole carbamate is also spontaneously reactive to thiol groups. Since no portion of these activation reagents becomes part of the final bond between conjugated molecules and the hydroxyl bead, they are considered zero-length crosslinkers[2,3]. Detailed coupling protocols using CDI and DSC are described herein. However, Spherotech recommends that optimization of quality reagents and coupling conditions be performed since both effect the quality of the coupling of the ligand to the functionalized bead surface.

Ultlization of a Maleimide-and-Isocyanate Crosslinker for Attaching Hydroxyl Magnetic Particles to Compounds with Sulfhydryl Groups from Cysteine

MATERIALS:

1. HEMAM-300-10 (HEMA/Polystyrene Copolymeric Magnetic Particles, 1% w/v, 25-37 um, 10 mL)
2. p-maleimidophenyl isocyanate (PMPI)
3. Phosphate Buffered Saline, 0.1M, pH 7.4 (PBS)
4. Dimethyl Sulfoxide (DMSO)
5. Acetonitrile
6. Compound with Thiol (–SH) reactive chemical group

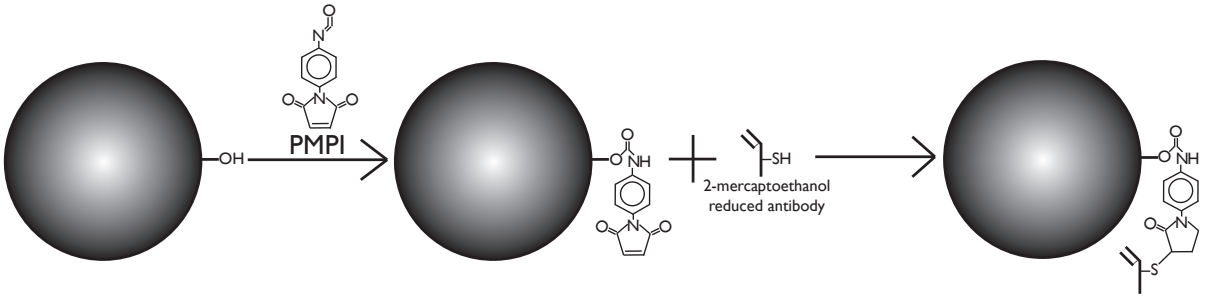
EQUIPMENT:

1. Eppendorf® Thermomixer®

CONJUGATION PROCEDURE:

1. Dry the magnetic beads in the oven at 60°C overnight and determine mass
2. Resuspended the 15µg of beads in 1mL of dry acetonitrile
3. Dissolve1.4mg of PMPI in 1ml of dry DMSO (6.5mM)
4. Add 250µL of this PMPI solution to beads from Step 1
5. Keep at room temperature under rotation for 5 hours and removed the supernatant using magnet
6. Wash the beads with acetonitrile, one time.
7. Dissolved 1mg of compound with thiol (–SH) reactive chemical group in dry DMSO
8. Add 100µL of the diluted sulfhydryl containing compound solution to the PMPI linked beads
9. Mix the beads overnight at room temperature
10. Next day, wash with 0.1 M PBS buffer 3x before use.

Conjugation of 2-mercaptoethanol reduced antibody to Hydroxyl Magnetic Beads using PMPI heterobifunctional crosslinker



Activation of Hydroxyl Magnetic Particles using Imidazole Carbamestes for the spontaneous binding to proteins

MATERIALS:

1. HEMAM-300-10 (HEMA/Polystyrene Copolymeric Magnetic Particles, 1% w/v, 25-37 um, 10 mL)
2. Cabonyl Diimidazole (CDI)
3. Phosphate Buffered Saline, 0.1M, pH8.2 (PBS) or 0.1M Sodium Carbonate, pH 9.5
4. Milli Q Water
5. Tetrahydrofuran (THF)
6. Ligand with a reactive amine group or thiol group
7. Ethanolamine

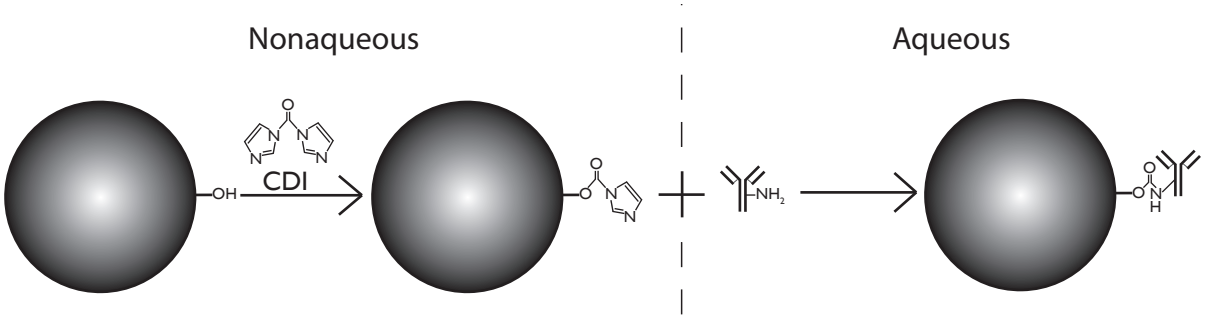
EQUIPMENT:

1. Eppendorf® Thermomixer®

CONJUGATION PROCEDURE:

1. Exchange the storage solution to dry THF by washing, agitating, centrifuging, and sequential exchange
2. Resuspend the particles at a concentration of 5% w/v as a suspension in THF containing CDI at 50 mg/mL(0.3-M)
3. Mixing for 2 hours at room temperature
4. Wash activated particles 3x with THF to removed excess CDI and byproducts
5. After the third wash, perform a wash with ice-cold Milli Q Water to remove any solvent
6. Resuspend the particles at 10 mg/mL in cold coupling buffer, such as 0.1M PBS, pH 8.2, or 0.1M Sodium Carbonate, pH 9.5 (The higher pH coupling buffer will result in greater reactivity of the imidazole carbamate and greater coupling yield for proteins.)
7. Add 1 to 10 mg of protein or antibody containing a reactive amine or thiol group (The optimal amount of ligand will need to be determined experimentally.)
8. React by mixing for at least 18h at 4°C.
(Longer reaction times may be necessary with pH 8.2 coupling buffer; Room temperature reactions will increase rate of reaction)
9. Add 0.1M ethanolamine and mix for 2 hours to quench any remaining active groups
10. Centrifuge and wash the particles at least 3x with 0.1 M PBS to remove unreacted protein and ethanolamine
11. Suspend the particles in 0.1 M PBS prior to use.

Conjugation of a amine-containing ligand to CDI activated HEMA/Polystyrene Copolymeric Magnetic Particles



Activation of Hydroxyl Magnetic Particles using Disuccinimidyl Carbonate to create a NHS-carbonate particle

MATERIALS:

1. HEMAM-300-10 (HEMA/Polystyrene Copolymeric Magnetic Particles, 1% w/v, 25-37 um, 10 mL)
2. Di(N-succinimidyl) carbonate (DSC)
3. Phosphate Buffered Saline, 0.1M, pH8.2 (PBS) or 0.1M Sodium Carbonate, pH 9.5
4. Milli Q Water
5. Acetone, dioxane, acetonitrile, THF, or DMF
6. Ligand with a reactive amine group or thiol group
7. Ethanolamine or Tris buffer

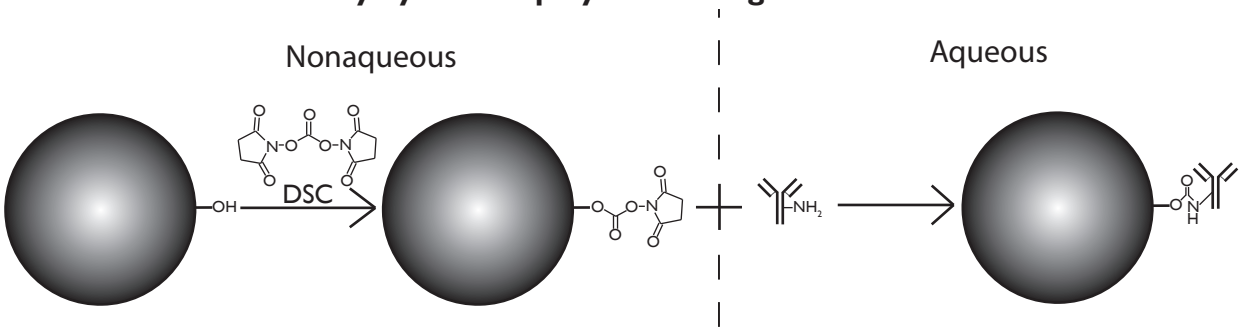
EQUIPMENT:

1. Eppendorf® Thermomixer®

CONJUGATION PROCEDURE:

1. Exchange the storage solution to dry acetone, dioxane, acetonitrile, THF, or DMF(with low amine content) by washing, agitating, centrifuging, and sequential exchange
2. Resuspend the particles at a concentration of 5% w/v as a suspension in anhydrous solvent containing DSC at 50 mg/mL(0.2 M)
3. Mixing for 2 hours at room temperature
4. Wash activated particles 3x with anhydrous solvent, to removed excess DSC and byproducts
5. After the third wash, perform a wash with ice-cold Milli Q Water to remove any solvent
6. Resuspend the particles at 10 mg/mL in cold coupling buffer, such as 0.1M PBS, pH 8.2
7. Add 1 to 10 mg of protein or antibody containing a reactive amine or thiol group (The optimal amount of ligand will need to be determined experimentally). Vortex to mix
8. React by mixing for at least 4 hours at 4°C or 2 hours at 2°C
9. Add 0.1M ethanolamine and mix for 2 hours to quench any remaining active groups
10. Centrifuge and wash the particles at least 3x with 0.1 M PBS to remove unreacted protein and ethanolamine
11. Suspend the particles in 0.1 M PBS prior to use.

Conjugation of a amine-containing ligand to DSC activated HEMA/Polystyrene Copolymeric Magnetic Particles



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