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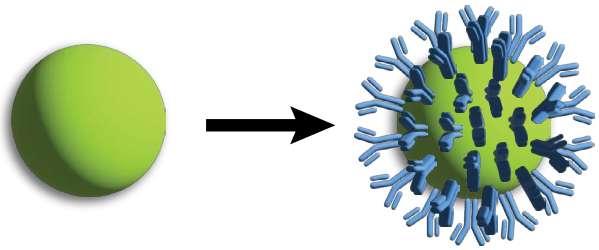
BEADS ABOVE THE REST

Covalent Coupling

CONTENTS



Related Technical Literature

1. PDS 546 – *BioMag® Magnetic Immobilization Kit & BioMag® Amine*
2. PDS 617 – *BioMag®Plus Amine and BioMag®Plus Amine Protein Coupling Kit*
3. PDS 618 – *BioMag®Plus Carboxyl and BioMag®Plus Carboxyl Protein Coupling Kit*
4. PDS 644 – *PolyLink Protein Coupling Kit*
5. TSD 0300 - *Buffers*
6. PDS 738 - *Bangs Bead Buffers and Suspending Solutions*
7. PDS 740 - *Accessory Reagents*

Ordering Information

1. Functionalized Polystyrene Microspheres
2. Non-, Carboxyl-, and Amine-Functionalized Silica Microspheres
3. Magnetic Microspheres & Particles
4. Click Chemistry Microspheres
5. Coupling Kits
6. Buffers and Solutions
7. Coupling Reagents

Please visit bangslabs.com for the full product offerings for covalent attachment chemistries.

I. INTRODUCTION

When adding your molecule of interest to microspheres, there are several strategies that may be considered, including covalent coupling, adsorption, and affinity binding. Each has its benefits and drawbacks, which should be considered in the context of research objectives, reagent requirements, technician/laboratory expertise, timetable, etc. Our TechNote 201, Working with Microspheres, provides a discussion of these different approaches. TechNote 204, Adsorption to Microspheres, and TechNote 101, ProActive® Microspheres, also provide information and sample protocols that may be of help as binding strategies are considered.
Covalent coupling is often employed for the immobilization of biomolecules when a stable microsphere reagent is required. Some of the advantages of covalent conjugation include:

- Biomolecules will not desorb / leach over time;
- Elimination of "crosstalk" between microspheres permits multiplexed tests and assays;
- Ligands can be favorably presented on the surface of the bead such that binding moieties are available for interaction with target molecules;
- Binding kinetics can approach those of solution-based binding (Meldal & Schoffelen, 2016)

The pages that follow contain sample coupling protocols, suggest potential points for you to optimize your reaction, and provide additional information resources. As it would be impossible to adequately address the optimal coupling of every type of biomolecule to every type of microsphere within the scope of this TechNote, we aim to provide the investigator with a foundation that will aid in the development of a high-quality microsphere reagent.

II. BINDING PROTOCOL DESIGN AND OPTIMIZATION

Although the general covalent coupling protocols that follow will typically result in some level of bead coating, it is expected that you will need to optimize the reaction to achieve desired activity, performance, stability, etc. There are several factors and points of optimization to consider, some of which are presented in the list that follows. The amount of optimization that is deemed reasonable or necessary will likely depend upon availability of published coupling protocols, early successes/failures from use of a 'generic' protocol and protocol objectives (required activity, stability, etc.).

A. Reactive Groups and Coupling Chemistries

Chemistry of Bead and Ligand

Ligand
As activity and binding kinetics are highly dependent upon orientation of the immobilized molecule, reactive groups that are available for coupling or modification should be carefully considered (Meldal & Schoffelen, 2016; Steen Redeker *et al.*, 2013).

Microsphere
Biomolecules may be coupled to polymeric, silica, or magnetic microspheres through a variety of surface chemistries (Huang *et al.*, 2022; Parker & Pratt, 2020; Walsh *et al.*, 2001).

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Covalent Coupling

Microsphere Composition	Microsphere Functional Group	Molecule of Interest (MOI) Functional Group	Conjugation Chemistry	Final Covalent Bond
Polymeric	Carboxyl	Amine	EDC (EDAC)	Amide
	Amine	Amine	Amine-reactive homobifunctional crosslinker (e.g. glutaraldehyde)	Amide
	Azide	Alkyne	CuAAC (Click chemistry reaction)	1,5-disubstituted 1,2,3-triazole
	Alkyne	Azide	CuAAC	1,5-disubstituted 1,2,3-triazole
	DBCO	Azide	SPAAC	1,5-disubstituted 1,2,3-triazole
Silica	Silanol	Amine	Silanization	Aminosilane
	Amino	Amine	Amine-reactive homobifunctional crosslinker (e.g. glutaraldehyde)	Amide
	Carboxyl	Amine	EDC	Amide
Magnetic	Carboxyl	Amine	EDC	Amide
	Amino	Amine	Amine-reactive homobifunctional crosslinker (e.g. glutaraldehyde)	Amide
	Azide	Alkyne	CuAAC	1,5-disubstituted 1,2,3-triazole
	Alkyne	Azide	CuAAC	1,5-disubstituted 1,2,3-triazole
	DBCO	Azide	SPAAC	1,5-disubstituted 1,2,3-triazole

Abbreviations include EDC: 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride; CuAAC: copper-catalyzed alkyne-azide cycloaddition reaction; SPAAC: strain-promoted alkyne-azide cycloaddition; DBCO: dibenzocyclooctyne. General coupling protocols are provided in Section V of this TechNote. See Section VIII, Recommended Reading, and Section X, References, for additional sources of information.

Microsphere Composition

The specific composition of the microsphere will determine characteristics, such as hydrophobicity or hydrophilicity, positive or negative charge, surface charge density, etc. These characteristics will have some influence on the loading capacity, i.e., how efficiently the biomolecule will come into proximity of the chemical group so that coupling may occur. They will also affect nonspecific binding characteristics, although nonspecific binding may be addressed with blockers, buffers, test / assay conditions (e.g. sample dilution), etc.

Cross-Linking Reagent (Linker, Spacer, Activator)

There are several chemical compounds that may be employed to modify or bind to the available reactive group on the bead or biomolecule. Cross-linking reagents may be used to 'activate' groups that exhibit low reactivity in an aqueous environment (e.g. carbodiimide for binding to COOH groups), or to join groups that are simply not reactive toward one another (e.g. NH_2 to NH_2). Certain types of linkers function as spacers, extending the biomolecule from the solid surface (often used in the coupling of small molecules or oligonucleotides to address steric effects) (Carmon *et al.*, 2002; Hermanson, 2013). Linkers can also simplify conjugations (e.g. photoreactive) or confer cleavability (Härtner *et al.*, 2007).

Reagent Quality

The quality of reagents is central to successful covalent coupling, and to the ultimate performance of the coated microsphere. Manufacturer's guidelines for reagent preparation, use, storage, and expiration should be observed to ensure activity and stability, and safeguard against contamination. For example, proper storage of EDC (EDAC) is important since it is hygroscopic, and improper storage and handling will inactivate the reagent. The quality (purity, affinity, cross-reactivity) of antibody or other capture biomolecule should be considered prior to covalent immobilization. (does it perform well enough to warrant design and optimization of a coupling protocol?)

Reagent Concentration

Determining appropriate concentrations of different reagents, such as ligand and linker, will be important in controlling surface density. Using too little may result in sub-optimal coating and low activity. Using too much may cause bead 'overload' (steric effect, with diminished activity) or may simply waste expensive reagent. Some guidance is provided below (see equation in next section) and in the general covalent coupling protocols in Section V.

III. LIGAND TYPES

A. Protein

Covalent coupling protocols have historically focused on the binding of a monolayer of protein. The amount of protein that will comprise a monolayer will depend upon factors such as the molecular weight (MW) of the protein and its relative affinity for the bead. This amount may be estimated using the following equation:

$$S = (6 / \rho S d)(C)$$

Where,

S = amount of representative protein required to achieve surface saturation (mg protein/g of microspheres)

ρS = density of solid sphere (g/cm^3)

d = mean diameter (μm), and

C = capacity of microsphere surface for a given protein (mg protein/ m^2 of sphere surface)

Cantarero *et al.* (1980) provide capacity data (C) for bovine serum albumin (BSA, MW 65 kD) and bovine IgG (bIgG, MW 150 kD). By comparing the MW of your ligand to that of BSA and IgG, surface saturation of other ligands can be approximated.

We base our calculations, as well as the reagent volumes listed in the coupling protocols, on microspheres with a mean diameter of 1.0µm. Therefore, the calculation is carried out as follows:

For BSA: C = ~ 3 mg/m^2, so:

S = (6 / ρSd)(C)

= (6 / [1.05 g/cm^3 • 1.0µm])(3 mg/m^2)

~18 mg of BSA to saturate 1 gram of 1µm polystyrene-based microspheres.

For bovine IgG: C = ~ 2.5 mg/m^2, so:

S = (6 / ρSd)(C)

= (6 / [1.05 g/cm^3 • 1.0µm])(2.5 mg/m^2)

~15 mg of bIgG to saturate 1 gram of 1µm polystyrene-based microspheres.

It is important to note that the resulting calculated value is a starting point in the determination of optimal protein concentration. The optimal protein concentration may be substantially higher than the 'monolayer' value (up to 10X) if coupling efficiency is not high, or if high coating density is required (e.g. sample contains only a trace amount of the target molecule). Conversely, much lower amounts of protein may be appropriate if high activity is not required (e.g. flow cytometric assay with abundant target) or desired (e.g. steric effects or increased nonspecific binding).

B. Nucleic Acid

There are special considerations for the immobilization of single-stranded nucleic acid probes. As the full length of the oligonucleotide will participate in the hybridization event, high probe density on the bead surface has been associated with reduced hybridization efficiency due to steric effects (Beaucage, 2012; Devor *et al.*, 2005; Fraser *et al.*, 2015; Guo *et al.*, 1994; Steel *et al.*, 2000). Figure 1 illustrates the relationship between probe length and packing configuration / surface density. The authors envisioned short probes packing in extended configurations, with long probes existing in more flexible, polymeric-like configurations.

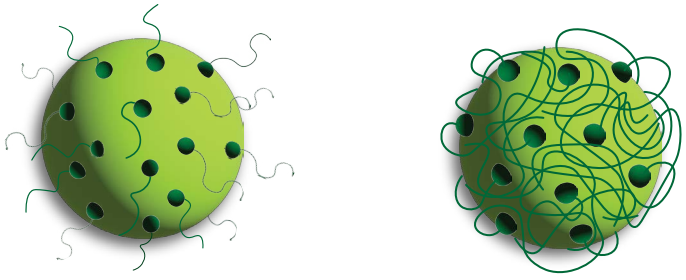


Fig. 1: Probe Length vs Packing Configuration

A generic protocol for the covalent coupling of an aminated oligonucleotide to COOH-functionalized microspheres is provided in TechNote 302, Molecular Biology. Covalent Nucleic Acid Immobilization, and strategies for addressing hybridization efficiency (e.g. use of a linker, controlling probe density), have been documented in the literature. Although it may be necessary to determine optimal probe concentration empirically, the literature holds many references that provide guidelines (Aparna & Tetala, 2023; Sapsford *et al.*, 2013; Scholtz *et al.*, 2023).

C. Other Biomolecules

There are numerous published coupling protocols available for a variety of biomolecules, including peptides, glycolipids, glycoproteins, extracellular vesicles, haptens, hormones, drugs, etc.
Investigators are encouraged to refer to the literature for guidelines on suitable biomolecule and reagent concentrations. Use of the equation for determination of a protein monolayer may also aid in establishing trial concentrations.

IV. REACTION CONDITIONS

A. Buffers

General Considerations
There are several buffers that have been used successfully in covalent coupling reactions. The protocols that follow typically do not contain recommendations for specific buffers, as there is not a buffer that would be ideal for every scenario (ligand and reactive group). Generally, the pH at which each reaction (activation, binding, quenching, blocking) occurs will be important; recommendations are provided within the coupling protocols that follow. The compatibility of the buffer and ligand (solubility, activity) is, of course, important, and should be considered when selecting buffers.

Additionally, the buffer should not contain certain compounds that will interfere or compete with the reaction or ligand (Buffers for Biochemical Reactions, 2024; Stoll & Blanchard, 2009). For example, phosphate and acetate buffers can reduce the reactivity of carbodiimides and are thus not recommended for use as activation buffers when coupling to COOH-modified microspheres. A popular alternative in this instance is MES. Also, buffers containing free amines, such as tris or glycine, should be avoided when working with amine reactive chemistries. Similarly, click chemistry (azide, alkyne, or DBCO functionalized microspheres) is sensitive to azide in buffers. Carefully evaluate buffer components to determine if there can be any inhibitors.

Recipes for common buffers can be found in the Tech Support Doc 0300, Buffers. Ready to use buffers and solutions are also available. See Buffers and Solutions for more information.

B. Chemical Linkers

Carboxyl- and amine-functionalized microspheres are commonly used for covalent attachment of a molecule of interest. DEPC-carbodiimide (EDAC) and glutaraldehyde crosslink available amine groups on a ligand to carboxyl-functionalized and amine-functionalized beads, respectively. Bangs Labs offers EDAC and glutaraldehyde as individual reagents for coupling reactions. In addition, for quick coupling to magnetic particles, Coupling Kits for BioMag®Plus superparamagnetic particles that include the reagents needed for coupling proteins or oligonucleotides.

C. Antimicrobials

Low concentrations (0.05-0.1%) of antimicrobial agents, such as sodium azide or merthiolate, are often added to the microsphere storage buffer, particularly for long-term storage. Antimicrobials should be carefully selected, as they may exhibit differing stability, involve special disposal considerations, etc. For example, sodium azide may react with lead and copper plumbing to form explosive metal azides. Therefore, upon disposal of materials, large amounts of water must be used to flush the plumbing and prevent azide accumulation. Azide may interfere with the click chemistry conjugation reaction, and therefore, Bangs Labs uses an alternative antimicrobial in the storage buffer of microspheres functionalized with azide, alkyne, and DBCO.

D. Blockers

Blocking agents are often coated on beads (via adsorption) following the coupling reaction. These compounds are used to minimize nonspecific interactions between the coated bead and non-target molecules in the sample (e.g. hydrophobic interaction between proteins and polymer surface). The blocking agent should be selected carefully, to ensure that it is effective in minimizing nonspecific interactions, since certain blockers may interfere with the test / assay or contribute to nonspecific binding. Blocker concentration should be evaluated to ensure adequate blocking without appreciable loss of activity (especially considering the coating level of the capture molecule). Blockers are often added to the storage buffer in varying amounts, standard concentrations being anywhere from 0.05% to 0.1% (w/v). A separate incubation in a higher concentration of blocker (up to 1%) is also recommended before storage to saturate the exposed surfaces of the microspheres. Some commonly used blockers include:

- BSA (bovine serum albumin): Often used alone, but can be combined with other blockers, most commonly surfactants.
- Casein: A milk-based protein, containing indigenous biotin, which should be avoided when working with systems involving biotin to prevent interference (Cui & Ma, 2018).
- Pepticase (casein enzymatic hydrolysate): An enzymatic derivative of casein, which should also be avoided when working with systems involving biotin.
- Non-Ionic Surfactants: Tween® 20 and Triton™ X-100 are typical. When used in combination with another blocker, a common ratio is 1% blocker to 0.05% surfactant.
- "Irrelevant" IgG: Often used when conjugating a specific IgG to microspheres. For example, if coupling mouse IgG, rabbit (or any non-cross-reacting IgG) can be adsorbed as a blocker.
- FSG (Fish Skin Gelatin): Pure gelatin or gelatin hydrolysate may also be used.
- PEG (Polyethylene Glycol): A very versatile blocker, available in several sizes (MW, chain length), configurations, and charges.
- Sera: Non-cross-reacting sera, such as horse or fish serum, are highly inert in terms of cross-reacting with various types of antibodies.
- Commercial Blockers: Many companies offer preparations which are a composite of two or more single blocking substances of various molecular weights, and which may be used effectively over a wide range of conditions. These are sold under various trade names, and most chemical vendors will offer a variety.

There are many additional blockers, and we suggest experimenting with various combinations and concentrations.

E. Bead Handling

Bead handling can also have a significant influence on the outcome of the coupling procedure. Investigators should consider bead washing procedures, which may affect coupling efficiency, bead loss, etc. In-process controls should be implemented to monitor monodispersity (e.g. microscopy, sizing, turbidimetry, etc.), and treat aggregation if observed. See TechNote 202, Microsphere Aggregation, and TechNote 203, Washing Microspheres, for further information.

F. Other Reaction Consideration

There are other parameters that may be evaluated, including incubation times and temperatures, the order and rate of reagent addition, etc. (Aparna & Tetala, 2023; Geißler *et al.*, 2021; Hermanson, 2013; Sapsford *et al.*, 2013). Walsh *et al.* (2001) provide examples of bead coating design and optimization processes.

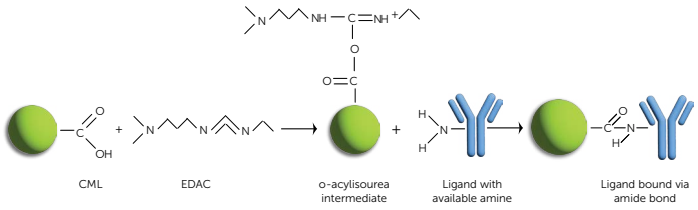
Thoughtful design, optimization, and execution of the coating protocol should result in the development of a high-quality microsphere reagent. However, the parameters that are optimized, and the amount of optimization that is conducted, should depend upon ultimate objectives and required bead characteristics.

V. SAMPLE COUPLING PROTOCOLS

The following protocols are intended to provide general guidelines for the coupling of biomolecules to microspheres bearing different surface groups. Although these generic protocols are likely to result in some level of bead coating, optimization may be required to achieve optimal activity and stability, while minimizing nonspecific binding characteristics.

We strongly encourage the performance of literature searches since published protocols for immobilization of the biomolecule of interest (to beads or other supports, other molecules [e.g. fluorophores], etc.) are likely available, and may dramatically reduce, or perhaps even eliminate, the optimization phase of coating protocol development. Suppliers of reagents (e.g. antibodies, oligonucleotides) may also have proven attachment protocols or troubleshooting guides available.

A. Carboxyl-Modified Microspheres



Carboxyl-modified microspheres can be conjugated to a biomolecule using a one-step or two-step reaction. One-step reactions are often faster, but two-step reactions can enhance the reaction since you can control the speed and pH. The following is a two-step reaction:

Reagents:

- Carboxyl-modified microspheres (often supplied at 10% solids)
- Activation buffer (pH 4.5-7.5)* [MES buffer is a common choice. See Section IV, Reaction Conditions.]
- Coupling buffer (pH 7.2-8.5) [Buffers containing free amines, such as Tris or Glycine, should be avoided.]
- Water soluble carbodiimide (WSC) [e.g. EDC, CMC, etc.]**
- Protein or other biomolecule [See Section III, Ligand Types; see

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TechNote 302 for a general nucleic acid coupling protocol.]

- Quenching solution with primary amine source, 30-40 mM [e.g. hydroxylamine, ethanolamine, glycine, etc.] And 0.05-1% (w/v) blocking molecule (See Section IV, Reaction Conditions).
- Storage buffer (pH 7.0-7.5) with 0.01-0.1% (w/v) blocking molecule

Procedure:

1. Wash 1mL (100 mg/mL) of microspheres 2 times in 10mL of activation buffer.***
2. After second wash, resuspend pellet in 10mL of activation buffer, ensuring that the microspheres are well suspended. (Vortexing, sonication, or rolling should aid in resuspension.)

NOTE: Concentration of microsphere suspension is now 10 mg/mL.

3. While mixing, add 100mg of WSC. (Addition of WSC may cause clumping; this is generally not a cause for great concern and should be resolved by incubation with biomolecule [Steps 6-7].)
4. Allow to react for 15 minutes at room temperature (18-25˚C), with continuous mixing.
5. Wash 2 times in coupling buffer and resuspend in 5mL of same. As much as possible, ensure that the particles are well suspended, as in Step 2.
6. Dissolve protein (1-10X excess of calculated monolayer) in 5mL coupling buffer. Combine microsphere suspension and protein solution.
7. React at room temperature for 2-4 hours with constant mixing.
8. Wash, resuspend in 10mL of quenching solution, and mix gently for 30 minutes.
9. Wash and resuspend in storage buffer to desired storage concentration (often 10 mg/mL).
10. Store at 4˚C until used.

*Reaction rate after addition of WSC is pH dependent (as the pH decreases, the reaction rate increases).

**EDAC or EDC: 1-Ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride

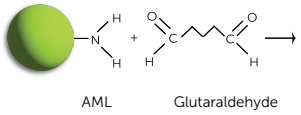
CMC: 1-Cyclohexyl-2-(2-morpholinoethyl) carbodiimide Methyl-*p*-toluenesulfonate

***See TechNote 203 for various washing methods.

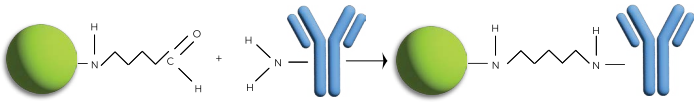
Alternatives:

1. One-step coupling reactions, whereby the carbodiimide, protein, and microspheres are combined in one step are often problematic for coupling larger molecules, but have been used effectively for the coupling of smaller molecules, like steroids and haptens. See Quash *et al.* (1978).
2. Water-soluble sulfo-N-hydroxysuccinimide can be added to increase coupling efficiency. The active ester intermediate formed by the N-hydroxy compound will replace the o-acylisourea intermediate formed by the WSC (unstable), which is more stable to hydrolysis, and yet still highly reactive toward amines on the protein to be coupled. A procedure incorporating N-hydroxysulfo-succinimide is found in Staros, *et al.* (1986).

B. Amino-Modified Microspheres



AML Glutaraldehyde



Aldehyde Activated Microsphere | Ligand with available amine | Ligand bound via amide bond

Reagents:

- Amino-modified microspheres (often supplied at 10% solids)
- Amine reactive homobifunctional cross-linker (e.g. glutaraldehyde, imidoesters, or NHS esters)
- Wash / coupling buffer (pH 6.0-9.0) (See Section IV, Reaction Conditions.)
- Protein or other biomolecule (See Section III, Ligand Types.)
- Quenching solution with primary amine source, 30-40 mM [e.g. hydroxylamine, ethanolamine, glycine, etc.] And 0.05-1% (w/v) blocking molecule [See Section IV, Reaction Conditions.]
- Storage buffer (pH 7.0-7.5) with 0.01-0.1% (w/v) blocking molecule

Procedure:

1. Wash 1mL (100 mg/mL) of microspheres 2 times in 10mL of wash/coupling buffer.*
2. After second wash, resuspend pellet in 10mL of glutaraldehyde solution (glutaraldehyde dissolved in wash / coupling buffer to a final concentration of 10%)**, ensuring that the microspheres are completely suspended. (Vortexing, sonication, or rolling should suffice.)

NOTE: Concentration of microsphere suspension is 10mg/mL.

3. Allow to react for 1-2 hours at room temperature (18-25˚C), with continuous mixing.
4. Wash 2 times, resuspend in 5mL wash / coupling buffer, and ensure that the particles are completely resuspended, as in Step 2.
5. Dissolve protein (1-10X excess of calculated monolayer) in 5mL wash / coupling buffer. Combine microsphere suspension and protein solution.
6. React at room temperature for 2-4 hours with continuous mixing.
7. Wash, resuspend in 10mL of quenching solution, and mix gently for 30 minutes. Wash and resuspend in storage buffer to desired storage concentration (often 10 mg/mL).
8. Store at 4˚C until used.

*See TechNote 203 for various washing methods.

**Glutaraldehyde should be added in large excess so that amino groups on microspheres will be saturated, thus avoiding cross-linking between microspheres

Prior to ligand attachment. The amount added will require optimization, as too much glutaraldehyde may alter the native conformation of the protein, thereby reducing its biological activity.

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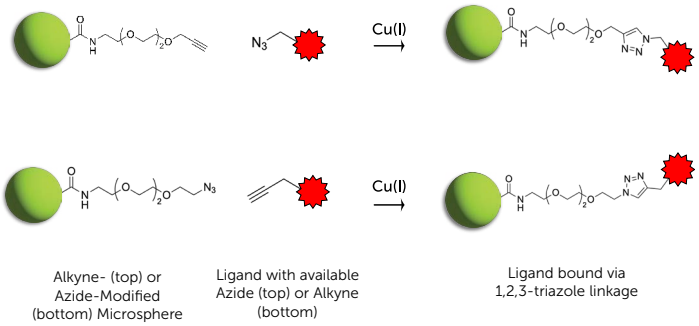
Alternatives:

1. Amine-reactive homobifunctional cross-linkers of varying lengths, other than glutaraldehyde, can be used to form spacer arms, allowing the covalently coupled proteins to be set off from the surface by varying lengths.
2. The bond formed between an amino group and an aldehyde forms a reversible Schiff base, which must be reduced by a process called reductive alkylation for the bond to be covalent. Examples of commonly used reducing agents are sodium cyanoborohydride, amine boranes, and pyridine boranes (Raso & Basala, 1984b, 1984a). However, because several amino groups on each protein are interacting with the aldehyde groups on the microspheres, it is sometimes considered unnecessary to reduce these bonds when coupling most large proteins, like antibodies.

C. Alkyne- or Azide-Modified Microspheres



Copper-catalyzed azide-alkyne cycloaddition (CuAAC), commonly referred to as Click reaction, requires a functionalized protein or biomolecule that has either an azide or alkyne. Be sure to analyze the buffers used for this reaction to ensure they do not have azide since this can interfere with the reaction.

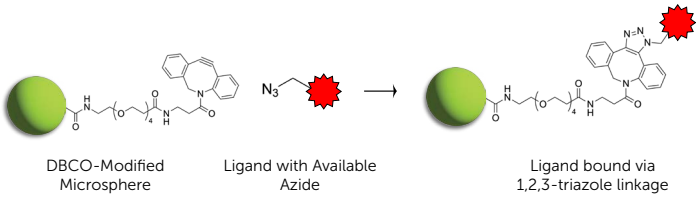
Reagents:

- Click Beads functionalized with azide or alkyne
- Copper sulfate solution (1mg/mL)
- THPTA cross-linking reagent (1mg/mL)
- Sodium ascorbate (NaAsc) (1mg/mL)
- Protein or other biomolecule (See Section III, Ligand Types)

Procedure:

1. Pipette 0.5mL of Click Beads into 1.5mL centrifuge tube.
2. Add the copper sulfate solution to equal ~25% of the functional groups on the beads.
3. Add THPTA reagent so it is 4X the copper sulfate solution.
4. Add the protein or other biomolecule to the desired concentration.
5. Vortex briefly to mix.
6. Add sodium ascorbate so it is 4X the copper sulfate solution.
7. Vortex briefly to mix.
8. Cover centrifuge tube with aluminum foil and roll end-over-end for 4 hours to overnight.
9. Separate beads, remove supernatant, resuspend in water.

D. DBCO-Modified Microspheres



For biological applications where copper has a deleterious effect, DBCO-modified microspheres can be attached to a protein or other biomolecule that has an azide group using strain-promoted alkyne-azide cycloaddition (SPAAC) click chemistry reaction.

Reagents:

- Click Beads functionalized with DBCO
- Protein or other biomolecule (See Section III, Ligand Types)

Procedure:

1. If Click Beads were not stored on a roller, allow the material to roll for at least 30 minutes at room temperature.
2. Examine the microspheres with a microscope to ensure there are no aggregates. If minor aggregation is observed, briefly sonicate the material in a bath sonicator and recheck. Repeat this process as needed until the particles are acceptable.
3. Add 0.5mL of Click Beads to a 1.5mL centrifuge tube.
4. Add the azide tagged protein or other biomolecule to the desired concentration.
5. Vortex briefly to mix.
6. Cover centrifuge tube with aluminum foil and roll end-over-end for 4 hours to overnight.
7. Separate beads, remove supernatant, resuspend in water.

VI. MISCELLANEOUS COUPLING STRATEGIES

A. Coupling to Non-Functionalized Polymeric Microspheres

Polystyrene (PS)
It is possible to covalently couple biomolecules to plain polystyrene microspheres through a four-step process: nitration of surface styrene rings; conversion of nitro groups to aromatic amine groups; diazotization of aromatic amine groups to form diazonium compounds; and coupling to the protein's tyrosine residue (Beaucage, 2012).

Polymethyl methacrylate (PMMA)
PMMA microspheres are not widely used for covalent coupling of ligands; however, the methyl ester groups will react readily with hydrazine, yielding acyl hydrazide reactive sites (Shmanai *et al.*, 2001).

B. Coupling to Non-Functionalized Silica Microspheres

The coupling of silanized nucleic acids (oligonucleotides, PCR products) to unmodified glass surfaces has been reported in the literature (Huang *et al.*, 2022; Kumar *et al.*, 2000). This approach may be modified for coupling of silanized biomolecules to silica microspheres. The procedure typically involves the regeneration of hydroxyl groups followed by silanization. For more information on silica microspheres, please refer to PDS 702, Silica Microspheres.

C. Conversion of Surface Functional Groups

Several linkers may be used to convert one surface functional group on a microsphere to another. For example, amine-modified microspheres may be converted to carboxyl-modified microspheres through a succinic anhydride reaction (Gounaris & Perlmann, 1967). Conversely, carboxyl groups may be converted to amine groups through carbodiimide mediated attachment of a diamine (Kurzer & Douraghi-Zadeh, 1967). Sulfhydryl-modified microspheres may be developed by reacting amine-functionalized microspheres with iminothiolane (Traut's reagent) (Traut *et al.*, 1973). These and other conversion chemistries may be utilized to broaden the attachment strategies for various ligands.

D. Covalent Attachment of Small Molecules

The covalent attachment of small molecules (haptens, hormones, drugs, etc.) can present special difficulties that call for creative solutions, e.g. combinations of carrier molecules and various types of cross-linkers (Shah *et al.*, 1994; Wood & Gadow, 1983). A spacer/cross-linker may be employed to extend the small molecule from the surface of the microsphere, thereby reducing steric hindrance and allowing interaction of the small molecule with target molecules in sample. Carrier molecules with available surface groups (e.g. BSA, polylysine) may be adsorbed to the microsphere surface with subsequent covalent coupling of the small molecule to the carrier molecules' reactive groups. Alternatively, the small molecule may first be covalently coupled to the carrier molecule, followed by adsorption of the carrier / small molecule complex to the microsphere (see TechNote 204, Adsorption to Microspheres). The adsorbed carrier molecules may then be covalently linked to one another, preventing their desorption/loss from the particle.

VII. EVALUATION OF COATING PROCESS

Following the coating procedure, some steps should be performed for quality assurance. There are a number of analytical and functional methods that may be employed to address questions such as:

- Is the bead coated with the biomolecule of interest (not just a blocker)?
- How much of the ligand or probe is specifically (covalently) vs. nonspecifically bound?
- Does bound ligand exhibit expected activity (are there steric effects that should be addressed)?
- Does the coated bead exhibit expected specificity (could the blocker be contributing to nonspecific binding, does the ligand exhibit a high level of cross reactivity)?
- How stable is the coating under ideal (and usual) storage conditions?

There are several methods for determining (or estimating) the amount of bound biomolecule, the details of which may be found in the literature. Anti-biomolecule antibodies labeled with a reporter molecule can provide some information regarding the success of the coupling procedure (Kellar *et al.*, 2001). Total protein assays (e.g. BCA, Lowry) have been utilized to determine the amount of protein bound to beads (Basinska & Slomkowski, 1992; Derango & Page, 1996). The use of fluorescent or radiolabeled probes and nucleic acid dyes have been utilized for detection of immobilized nucleic acid (Andreadis & Chrisey, 2000; Aparna & Tetala, 2023; Beaucage, 2012; Walsh *et al.*, 2001). Combined methods may be employed to determine whether the molecule has been bound, and whether bound molecules exhibit expected activity.

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