TechNote 205

Covalent Coupling

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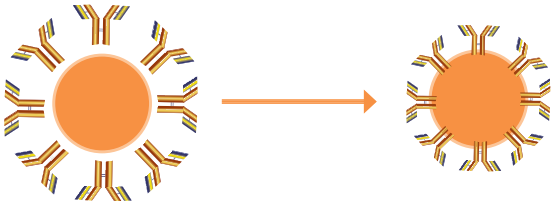
BEADS ABOVE THE REST™



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I. INTRODUCTION

When coating microspheres, there are a number of 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 very active and stable microsphere reagent is required. For example:

- biomolecules are permanently bound, and will not desorb/leach over time;[37]
- elimination of "crosstalk" between microspheres permits multiplexed tests and assays;
- ligands are 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.[14, 28, 47]

The pages that follow contain sample coupling protocols, suggest potential points of optimization, and identify 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 basic 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 optimization will be required in order to achieve desired activity, performance, stability, etc. There are a number of 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

1. 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.[7, 12, 16, 44]

2. Microsphere

Biomolecules may be coupled to polymeric or silica microspheres through a variety of surface chemistries. Surface functional groups that are available include: polymeric - carboxyl and amino (greatest availability), as well as hydroxyl, hydrazide, and chloromethyl; and silica - silanol, carboxyl. General coupling protocols are provided in Section III of this TechNote. See Section IV, *Recommended Reading*, and Section VIII, *References*, for additional sources of information.

3. *Cross-Linking Reagent (Linker, Spacer, Activator)*

There are a number of 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).[10, 20, 21, 32, 37, 38] Linkers can also simplify conjugations (e.g. photoreactive) or confer cleavability.[32]

B. 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.

C. 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. 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?)

D. Reagent Concentration

1. General

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 (equation, Section VIII, *References*) and in the general covalent coupling protocols in Section III.

2. Protein[9]

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 through the use of the following equation:

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

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.[9] 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:

$S = (6 / \rho Sd)(C)$
$= (6 / [1.05\ g/cm^3 \bullet 1.0\mu m])(3\ mg/m^2)$
~ 18 mg of BSA to saturate 1 gram of 1µm polystyrene-based microspheres.

For BIgG: C ~ 2.5 mg/m^2, so:

$S = (6 / \rho Sd)(C)$
$= (6 /[1.05\ g/cm^3 \bullet 1.0\mu m])(2.5\ mg/m^2)$
~ 15 mg of BIgG to saturate 1 gram of 1µm polystyrene-based microspheres.

It is important to note that the resulting calculated value is considered to be 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).

C. 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.[31, 41] Figure 1[41] 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.

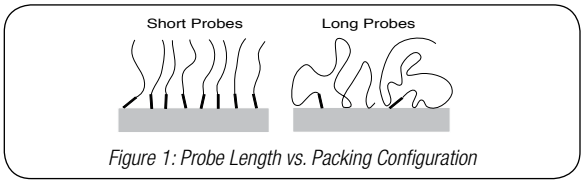


Figure 1: Probe Length vs. Packing Configuration

Most recently, probe density has been demonstrated to affect polymerase activity.[10]

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. (See references 1, 2, 4, 17, 23, 27, 28, 31, 39, 42 and 45.)

D. Other Biomolecules

There are numerous published coupling protocols available for a variety of biomolecules, including peptides, 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.

E. Buffers

1. General

There are a number of 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.[8] 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.

2. Recipes for Common Buffers

A listing of common biological buffers (with recipes) is provided below. Ionic strength should be adjusted as appropriate (often ~25-100mM for coupling reactions[7]). Please note that the list is not all encompassing, and investigators are encouraged to look to the literature and experiment with the use of different buffers.

a. *Phosphate Buffered Saline (PBS); pH 7.4*
   - i. Potassium Phosphate dibasic: 1.82 g/L (MW 174.2)
   - ii. Sodium Phosphate monobasic: 0.22 g/L (MW 120.0)
   - iii. Sodium Chloride: 8.76 g/L (MW 58.4)

   • Bring to a final volume of 1L using deionized water. Adjust pH to 7.4 using either 1 N HCl or 1 N NaOH.

b. *Borate Buffer; pH 8.5*
   - i. Boric Acid, H3BO3: 12.4 g/L (MW 61.8)
   - ii. Sodium Tetraborate: 19.1 g/L (MW 381.4)

   • Add 50mL of (i) to 14.5mL of (ii). Bring to final volume of 200mL using deionized water. Adjust final pH to 8.5 using 3 M NaOH.

c. *Acetate Buffer; pH range 3.6 to 5.6*
   - i. 0.1 M Acetic acid: (5.8mL made to 1000mL)
   - ii. 0.1 M Sodium acetate: 8.2 g/L (anhydrous, MW 82.0)

   • Mix acetic acid and sodium acetate solutions in the proportions indicated below and adjust the final volume to 100mL with deionized water. Adjust the final pH using 1 N HCl or 1 N NaOH.

mL Acetic Acid	46.3	41.0	30.5	20.0	14.8	10.5	4.8
mL of Na Acetate	3.7	9.0	19.5	30.0	35.2	39.5	45.2
pH	3.6	4.0	4.4	4.8	5.0	5.2	5.6

d. Citrate-Phosphate Buffer; pH range 2.6 to 7.0
   - i. 0.1 M Citric acid: 19.2 g/L (MW 192.1)
   - ii. 0.2 M Dibasic sodium phosphate: 35.6 g/L (dihydrate, MW 178.0)

   • Mix citric acid and sodium phosphate solutions in the proportions indicated below and adjust the final volume to 100mL with deionized water. Adjust the final pH using 1 N HCl or 1 N NaOH.

mL Citric Acid	44.6	35.9	29.4	24.3	19.7	13.6	6.5
mL of Na Phosphate	5.4	14.1	20.6	25.7	30.3	36.4	43.6
pH	2.6	3.4	4.2	5.0	5.8	6.6	7.0

e. Carbonate - Bicarbonate Buffer; pH range 9.2 to 10.4
   - i. 0.1 M Sodium carbonate: 10.6 g/L (anhydrous, MW 106.0)
   - ii. 0.1 M Sodium bicarbonate: 8.4 g/L (MW 84.0)

   • Mix sodium carbonate and sodium bicarbonate solutions in the proportions indicated below and adjust the final volume to 200mL with deionized water. Adjust the final pH using 1 N HCl or 1 N NaOH.

mL Na Carbonate	4.0	9.5	16.0	22.0	27.5	33.0	38.5
mL of Na Bicarbonate	46.0	40.5	34.0	28.0	22.5	17.0	11.5
pH	9.2	9.4	9.6	9.8	10.0	10.2	10.4

f. *MES Buffer; pH range 5.7 to 7.2*

• Dissolve 19.2g of MES free acid (MW 195.2) in ~900mL of pure water. Titrate to desired pH with 1 N HCl or 1 N NaOH and adjust final volume to 1000mL with pure water.

3. Antimicrobials

Low concentrations (0.05-0.1%) of antimicrobial agents, such as sodium azide or merthiolate, are often added to the 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.

F. 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; certain blockers may interfere with test/assay, or actually contribute to nonspecific binding. Blocker concentration should be evaluated to ensure adequate blocking (especially in light of coating level of the capture molecule), without appreciable loss of activity. 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, in order to saturate the exposed surfaces of the microspheres. Some commonly used blockers include:

a. *BSA (Bovine Serum Albumin):* Often used alone, but can be combined with other blockers, most commonly surfactants.
b. *Casein*: A milk-based protein, containing indigenous biotin, which should be avoided when working with systems involving biotin to prevent interference.
c. *Pepticase (Casein Enzymatic Hydrolysate):* An enzymatic derivative of casein, which should also be avoided when working with systems involving biotin.

d. *Non-Ionic Surfactants:* Tween® 20 and Triton™ X-100 are typical. When used in combination with another blocker, a common ratio is 1% blocker; 0.05% surfactant.
e. *"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.
f. *FSG (Fish Skin Gelatin):* Pure gelatin or gelatin hydrolysate may also be used.
g. *PEG (Polyethylene Glycol):* A very versatile blocker, available in a number of sizes (MW, chain length), configurations, and charges.
h. *Sera*: Non-cross-reacting sera, such as horse or fish serum, are highly inert in terms of cross-reacting with various types of antibodies.
i. *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.

G. 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.

H. Other

There are other parameters that may be evaluated, including incubation times and temperatures, order and rate of reagent addition, etc. Lund[28] and Walsh[45] provide examples of bead coating design and optimization processes.

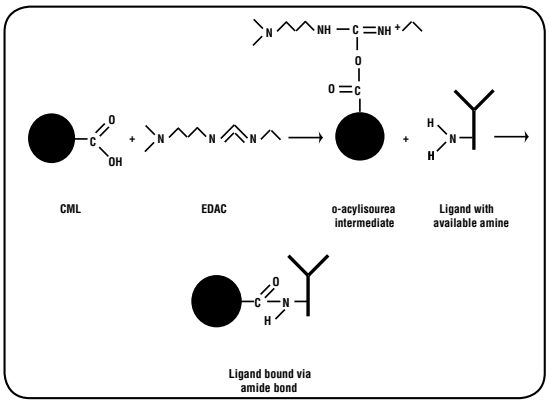
Thoughtful design, optimization, and execution of the coating protocol should result in the development of a high-quality microsphere reagent. However, the parameters that are optimized, and the amount of optimization that is conducted, should depend upon ultimate objectives and required bead characteristics.

III. 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 in order to achieve optimal activity and stability, while minimizing nonspecific binding characteristics.

We strongly encourage the performance of literature searches by the investigator. 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



Reagents:

1. *Carboxyl-modified microspheres* (often supplied at 10% solids)
2. *Activation buffer* (pH 4.5-7.5)* [MES buffer is a common choice. See Section II, *Buffers*.]
3. *Coupling buffer* (pH 7.2-8.5) [Buffers containing free amines, such as Tris or Glycine, should be avoided.]
4. *Water soluble carbodiimide (WSC)* [e.g. EDC, CMC, etc.]**
5. *Protein or other biomolecule* [See Section II, *Reagent Concentration*; see TechNote 302 for a general nucleic acid coupling protocol.]
6. *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 II, *Blockers*).
7. *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. Wash and resuspend in storage buffer to desired storage concentration (often 10 mg/mL).
9. Store at 4˚C until used.

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

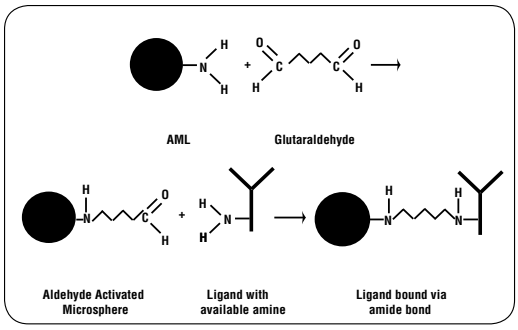
** *EDAC or EDC:* *1-Ethyl-3-(3-dimethylaminopropyl) carbodiimide Hydrochloride*
CMC: *1-Cyclohexyl-2-(2-morpholinoethyl) carbodiimide Metho-p-toluenesulfonate*

*** *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: Nathan,[29] Hager,[19] and Quash.[35]
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), is more stable to hydrolysis, and yet still highly reactive toward amines on the protein to be coupled. Procedures incorporating these compounds are offered by Borque[6] and Staros.[40]

B. Amino-Modified Microspheres



Reagents:

1. *Amino-modified microspheres* (often supplied at 10% solids)
2. *Amine reactive homobifunctional cross-linker* (e.g. glutaraldehyde, imidoesters, or NHS esters. Jones[21] provides further alternatives.)
3. *Wash / coupling buffer* (pH 6.0-9.0) (See Section II, *Buffers*.)
4. *Protein or other biomolecule* (See Section II, *Reagent Concentration*.)
5. *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 II, *Blockers*.]
6. *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 now 10 mg/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.

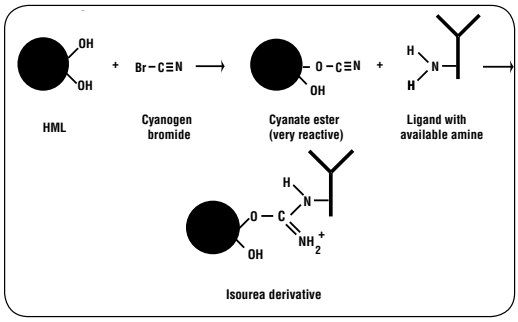
* *See TechNote 203 for various washing methods.*

** *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.*

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, in order for the bond to be covalent. Examples of commonly used reducing agents are sodium cyanoborohydride, amine boranes, and pyridine boranes.[33, 34] 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. Hydroxyl-Modified Microspheres



Reagents:

1. *Hydroxyl-modified microspheres* (often supplied at 10% solids)
2. *2 M sodium carbonate* (activation buffer)
3. *Cyanogen Bromide* (CNBr)
4. *Acetonitrile* (C_2H_3N) [to dissolve CNBr]
5. *Protein or other biomolecule* (See Section II, *Reagent Concentration*.)
6. *Wash / coupling buffer* (pH 8.0-9.0). [Avoid amine-containing buffers such as Tris or glycine, which will compete with the ligand for coupling sites. See Section II, *Buffers*.]
7. *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 II, *Blockers*).
8. *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 in 9.5mL of activation buffer, ensuring that the microspheres are completely suspended. (Vortexing, sonication, or rolling should suffice.)
3. In a fume hood, dissolve 1g of CNBr (or a ratio of 1g CNBr:100mg microspheres) in 0.5mL acetonitrile. (**Warning**: CNBr is highly hazardous, and should be handled under a fume hood, using all appropriate precautions.)
4. Add CNBr solution (dropwise) to the stirring microsphere suspension, and allow the activation reaction to continue for precisely 2 minutes at room temperature (18-25˚C). *Note*: Concentration of microsphere suspension is now 10 mg/mL.
5. Quickly wash the activated microspheres in a large volume of ice-cold

water, then with cold coupling buffer. Resuspend microspheres in 5mL of coupling buffer (4˚C). Dissolve the ligand to be coupled in 5mL of coupling buffer, at a concentration corresponding to a 1-10X excess of calculated monolayer. Combine microsphere suspension and protein solution.

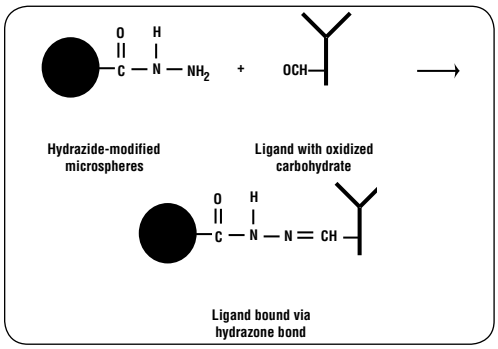
6. Keep suspension at 4˚C for 24 hours, with constant mixing.
7. Wash, resuspend in 10mL of quenching solution, and mix gently for 30 minutes. Wash and resuspend in storage buffer to desired storage concentration (often 10 mg/mL).
8. Store at 4˚C until used.

* *See TechNote 203 for various washing methods.*

Alternatives:

1. 1-cyano-4-dimethylaminopryidinium tetrafluoroborate can be substituted for CNBr, as it is a non-volatile, less toxic chemical with a longer half-life.
2. Tosyl Chloride, Tresyl Chloride[30] or carbonyl diimidazole activation[5] can also be used in place of cyanogen bromide.

D. Hydrazide-Modified Microspheres



Reagents:

1. *Hydrazide-modified microspheres* (often supplied at 10% solids)
2. *Wash / coupling buffer* (pH 5.0-7.0) [See Section II, *Buffers*.]
3. *Protein* (See Section II, *Reagent Concentration*.)
4. *100 mM sodium meta-periodate* ($NaIO_4$)
5. *Storage buffer* (pH 7.0-7.5) with 0.01-0.1% (w/v) *blocking molecule* (See Section II, *Blockers*.)

Procedure:

A. *Oxidation of Protein* (*Note*: The reaction is light sensitive and should be performed in the dark.)

1. Dissolve or dilute 1-10X excess of calculated monolayer of protein in 1mL of wash/coupling buffer.
2. Add the protein solution to an amber vial containing 1mg sodium meta-periodate : 20mg of protein; swirl gently to dissolve the oxidizing agent.
3. Incubate the sample for 30 minutes at room temperature, with constant mixing.
4. Stop the reaction and remove unreacted NaIO4 by passing the mixture through a desalting column (like Sephadex™ G25 or PD10), equilibrated with the coupling buffer.

B. *Coupling to Hydrazide-Modified Latex Microspheres*

1. Wash 1mL (100 mg/mL) of microspheres 2 times in 10mL of wash/ coupling buffer.**
2. After second wash, resuspend microspheres in 9mL of wash / coupling buffer, ensuring that the microspheres are completely suspended. (Vortexing, sonication, or rolling should suffice.)
3. Mix 9mL suspension of microspheres with 1mL of oxidized protein suspension. (*Note*: Concentration is now 10 mg/mL.) React with mixing for a minimum of 6 hours at room temperature (18-25˚C).
4. Wash, resuspend in 10mL wash / coupling buffer with 0.05-1% (w/v) blocking molecule, mix gently for 30 minutes.
5. Wash, and resuspend in storage buffer to desired storage concentration (often 10 mg/mL).
6. Store at 4˚C until used.

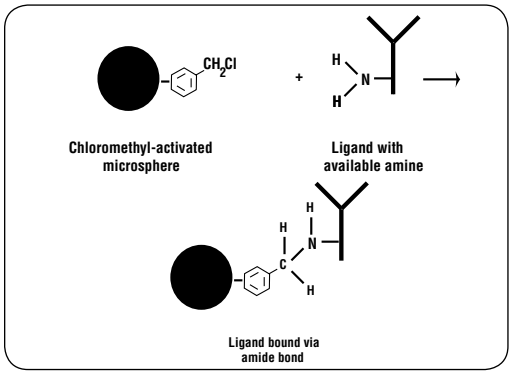
* *This oxidized carbohydrate method binds IgG specifically at the Fc region.*

** *See TechNote 203 for various washing methods*

Alternatives:

1. Another option is to activate the microspheres with glutaraldehyde, using the same protocol as for amino-modified microspheres.

E. Chloromethyl-Modified Microspheres



Reagents:

1. *Chloromethyl-modified microspheres* (often supplied at 10% solids)
2. *Protein* or other biomolecule (See Section II, *Reagent Concentrations*.)
3. *Wash / coupling buffer* (pH 7.5) [See Section II, *Buffers*.]
4. *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 II, *Blockers*.)
5. *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 in 5mL of wash / coupling buffer, ensuring that the microspheres are completely suspended. (Vortexing, sonication, or rolling should suffice.)
3. Dissolve protein (1-10X excess of calculated monolayer) in 5mL wash / coupling buffer. Combine microsphere suspension and protein solution. *Note*: Concentration of microsphere suspension is now 10 mg/mL.
4. React at room temperature (18-25˚C) for 2-4 hours, with constant mixing.
5. Wash, resuspend in 10mL of quenching solution, mix gently at room temperature for 30 minutes.
6. Wash and resuspend in storage buffer to desired storage concentration (often 10 mg/mL).
7. Store at 4˚C until used.

* *Note: Chloromethyl microspheres can be classified as pre-activated, as the chloromethyl groups on the microspheres will react with available amino groups directly, with no pretreatment steps required. Because of the high reactivity of these*

chloromethyl groups, they will dehydrohalogenate in an aqueous suspension over time, and therefore have a limited shelf life after synthesis. Once the ligand has been coupled, however, their stability matches that of any other ligand coated microsphere.

*** See TechNote 203 for various washing methods.*

IV. MISCELLANEOUS COUPLING STRATEGIES

A. Coupling to Non-Functionalized Polymeric Microspheres

1. Polystyrene (PS)[43]

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.

2. 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. A patent exists in which the inventor describes the reaction of PMMA methyl ester groups particles with amine-derivatized dyes by transacylation at elevated pH.[11]

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.[25] This approach may be modified for coupling of silanized biomolecules to silica microspheres.

C. Conversion of Surface Functional Groups

A number of 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.[18] Conversely, carboxyl groups may be converted to amine groups through carbodiimide mediated attachment of a diamine.[26] Sulfhydryl-modified microspheres may be developed by reacting amine-functionalized microspheres with iminothiolane (Traut's Reagent).[24] These and other conversion chemistries may be utilized to broaden the attachment strategies for various ligands.

D. Covalent Attachment of Small Molecules[36, 48]

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. 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.

V. EVALUATION OF COATING PROCESS

Following the coating procedure, some steps should be taken down the path of 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?

For example, there are a number of 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.[22] Total protein assays (e.g. BCA, Lowry)[3, 13] have been utilized to determine the amount of protein bound to beads. The use of fluorescent or radio labeled probes and nucleic acid dyes have been utilized for detection of immobilized nucleic acid.[12, 25, 28, 45, 46] Combined methods may be employed to determine whether the molecule has been bound, and whether bound molecules exhibit expected activity.

VI. RECOMMENDED READING

Bioconjugate Techniques
G.T. Hermanson, 1996, Academic Press
www.piercenet.com

Immobilized Ligand Affinity Techniques
G.T. Hermanson, 1992, Academic Press
www.piercenet.com

Bioconjugation: Protein Coupling Techniques for the Biomedical Sciences
M. Aslam, A.H. Dent (eds.), 1998, MacMillan
www.macmillanonline.net/science

Chemistry of Protein Conjugation and Cross-Linking
S.S. Wong, 1991, CRC Press
www.piercenet.com

Buffers: A Guide for the Preparation and Use of Buffers in Biological Systems
EMD-Calbiochem, 1999
www.emdbiosciences.com

Suggested websites for performance of literature searches may be accessed through the *Links* section of the Bangs Laboratories' website, **www.bangslabs.com**.

VII. REAGENT SUPPLIERS

There are many suppliers of reagents used in covalent coupling reactions. A few suppliers are noted below; others may be located through an Internet search or *Materials and Methods* sections of pertinent papers.

Pierce, www.piercenet.com
Sigma-Aldrich, www.sigma-aldrich.com
EMD-Calbiochem, www.emdbiosciences.com
BioMedTech Laboratories, Inc., www.biomedtech.com

VIII. REFERENCES

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