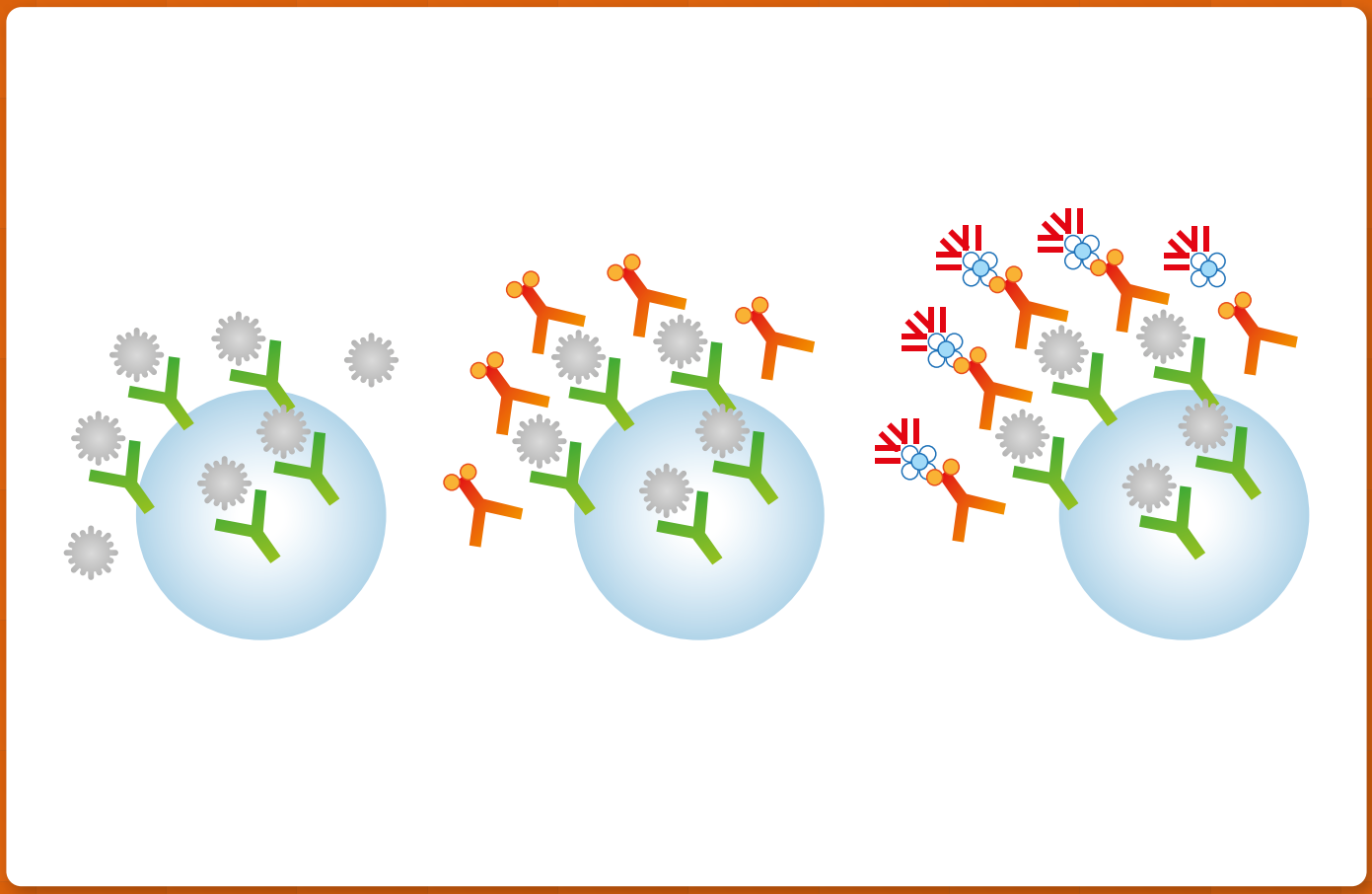
The advanced guide for the use of magnetic beads in ChemiLuminescent ImmunoAssays

Learn how to successfully develop a Chemiluminescent Immunoassay for a biomarker of interest

by Dr. Sergi Gassó



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SUMARY

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Dr Sergi Gassó has more than 11 years of experience in the medical device sector, with the majority of his career spent in progressively senior management positions in the R&D field of IVD reagents. Prior to founding Pragmatic Diagnostics, he has served as Alere Toxicology plc (UK, former Concateno), Spinreact (Spain, now part of Toyobo group) and Future Diagnostics BV (The Netherlands) in charge of the development of several immunoassays and biomarkers. Sergi holds a PhD in Biochemistry from the Universitat Autònoma de Barcelona and a Master in Business Administration within the Pharmaceutical Industry from the Universitat de Barcelona.

For more information visit: pragmaticdiagnostics.com

Introduction

Bio-functionalized magnetic beads are widely used for capturing specific molecules or cells thanks to their super-paramagnetic properties. They are typically used for two main purposes in the Biotech field. They act as the solid phase for both separation processes such as purification of proteins/molecules and for in vitro diagnostics (IVD) reagents.

In order to be able to bind and capture the desired target molecule from the sample, the magnetic beads have to be coated with a ligand that specifically binds the target. The choice of the type of Ligand will entirely depend on the target molecule that has to be captured. The classic and most common Ligands are the antibodies, which are used for capturing a broad range of molecules. Nucleic aptamers can also be used in the same way than antibodies. There are a number of alternatives to bind the target molecules, such as Protein A/G, Streptavidin/biotin system, specific proteins or antigens with high specificity and avidity for the target…

The selection of the appropriate type of magnetic particles is key for the success of the project. There are several types of magnetic particles in the market that have different physical and chemical properties. The type of magnetic particles we use will have a big impact on the performance of the binding and the manufacturing and/or reproducibility of different batches of the product. These aspects are also affected by the quality of the magnetic separation process. It is important to work with a separation process that allows in process control and homogeneous separation to assure scalability and reproducibility at big scale.

From the physical properties point of view, the main parameters to consider for the selection of magnetic particles are the particle size or diameter, the size dispersion of the suspension and their magnetic charge. The particle size will determine the surface area available for coating the ligand and the force by which the particles are attracted by the magnet system during separation. Consequently, the homogeneity of the particle dispersion becomes a critical parameter, as it will have a direct impact on the reproducibility of the performance of different batches of product. The magnetic charge affects the density of the particles and plays a role in the speed of the separation process, which will affect the performance and the manufacturing processes of the product.

The conformation and orientation of the ligand, as well as its density or parking area onto the surface of the particles determines the capacity of the particles to capture the target molecule. The chemical link between the Ligand and the surface of the magnetic particles use to be through covalent binding. There are several commercial magnetic particles available, which are activated with different chemical groups such as carboxylated or amino that allow a covalent binding to the ligand. The most popular ones for chemiluminescence immunoassays are the tosyl-activated particles, which don't need of pre-activations steps and help to get a reproducible product with low non-specific binding. However, the control of the orientation and conformation is still a challenge for some proteins. There are new technological approaches that help to solve this issue. For instance, metal polymer chemistry can be used to attach proteins to synthetic surfaces via chelation and coordination chemistry as an alternative to the classic covalent binding.

This ebook intends to summarize the current approaches for magnetic bead coating as well as the new arising technological solutions that will help to surpass the current technical challenges.

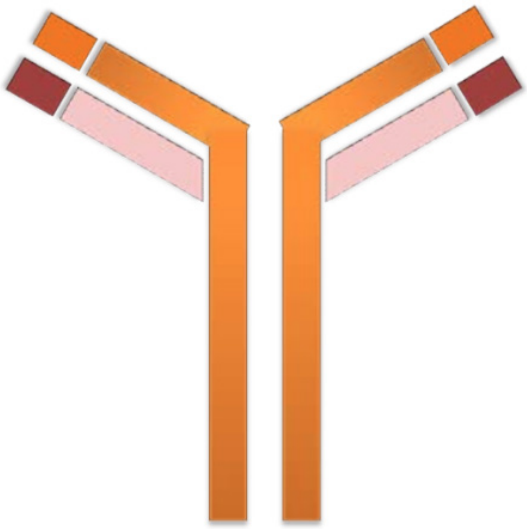
We'd like to thank the contribution in this ebook to some of the best experts worldwide on the present and future of magnetic beads coating.

Chapter 1. How to Decide the Right Platform for a Specific Biomarker

Because of their significantly increased surface area and their ease of manipulation, in vitro diagnostic (IVD) assays commonly make use of nanoparticles, typically utilizing them as solid surface carriers for capture molecules. There are a number of particles that can be utilized in IVD assays. White latex particles, for instance, have traditionally been used in diagnostic tests such as immunoturbidimetry and nephelometry assays. Because of their magnetic properties, magnetic latex particles – latex particles that have been synthesized with iron oxide, thus rendering them superparamagnetic – can serve as a solid phase in chemiluminescence assays and as label or tracer molecules in Point of Care devices capable of detecting their presence. These are also commonly used in chemiluminescence assays., and inorganic particles can also be used in IVD applications. Colloidal gold particles, for instance, have traditionally been utilized in lateral-flow applications as they are particularly useful in immuochromatography tests.

In general, the use of inorganic particles in IVD lab-automated platforms is rare. The majority of IVD platforms utilize polymeric organic or organic-based particles. The decision to use one particle over another will depend on several factors, including the biomarker sensitivity required, feasibility and ease of use, and the overall aim of the process.

When deciding which platform to use with a specific biomarker, it is best to use a push approach. In other words, it is best to allow the biomarker to drive the decision. This is in contrast to a pull approach, where the impetus for choosing a particular platform would be that platform's general availability, rather than its overall suitability to the biomarker. The decision to use a particular technology should be based on factors such as its sensitivity or its detection system, and these factors should be dictated by the biomarker. As such, the biomarker should ultimately be the guiding factor when determining what platform to use for a process.



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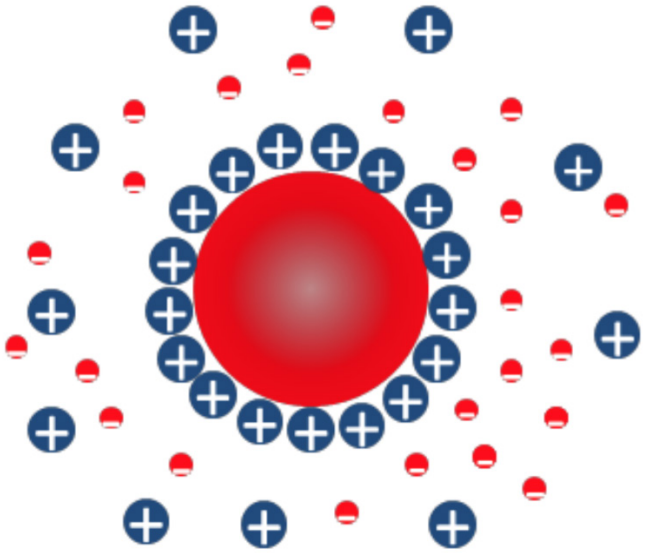
Chapter 2. Difference Between Coating Latex, Magnetic Latex, and Colloidal Gold Particles

In vitro diagnostic (IVD) applications frequently make use of nanoparticles as solid-phase carriers for a given capture molecule. In order to be utilized in this manner, nanoparticles must first be coated, thereby attaching the capture molecules to the particles. There are notable differences in the way that different types of particles are coated, and this will be a significant factor in the decision to utilize one particle over another in a given assay.



Colloidal gold particles, for instance, traditionally attach capture molecules via passive adsorption. This sort of attachment typically relies on hydrophobic, electrostatic and Van der Waals interactions to bind the molecule to the particle. This type of binding allows for very little control over the final orientation of the attached molecule. It is not uncommon, as a consequence, to have multiple layers of a capture molecule bound to a particle. The first layer of proteins used to suffer from huge conformational changes, as they can be partially or totally denatured. Because of this, problems with stability of the capture molecule may arise as a result, particularly for those type of assays where little loss of capture molecule critically affects the performance of the assays, such as the competitive type. The increased commercial availability of carboxylated colloidal gold particles and gold particles conjugated with molecules such as streptavidin, protein A, and protein G, broadens the range of applications with which these particles can be utilized.

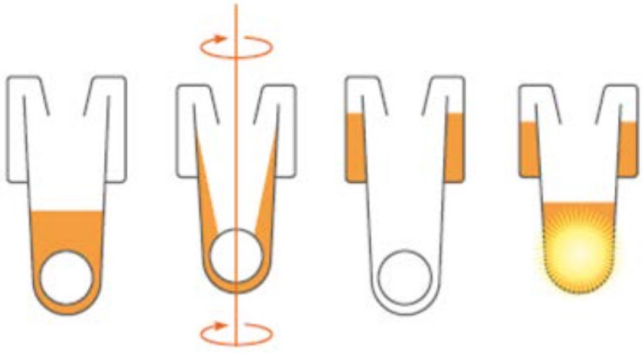
Latex and magnetic particles, however, have historically had a wider spectrum of activations available because of their polymeric nature. This makes this particles capable of attaching molecules via a greater variety of covalent bond types besides passive adsorption. As such, the amount of protein required for coating these particles is often less than what is required to coat colloidal gold particles. Additionally, the capture molecule is oriented in a predictable way, because the site by which it is bound to the particle is specific and well-defined.

The covalent binding allows for maximal exposure of the capture molecule's reactive site, a more uniform coupling, better lot-to-lot reproducibility and the use of chemical linkers or spacers, among other advantages.

Chapter 3. Moving from Gold Particles to Magnetic Beads

There are a number of reasons why a lab might want to switch from colloidal gold particles to magnetic beads in an in vitro diagnostic assay, such as swapping from optical to magnetic detection in a lateral flow format or charging the format from lateral flor to a lab automated platform. Making the shift from utilizing one nanoparticle to another, however, is not a straightforward process. There are several areas where problems may arise. There is a considerable difference, for instance, in the way gold particles and magnetic beads bind a molecule. Moreover, the methodology for applications utilizing gold particles is significantly different from that of processes that make use of magnetic beads.

The procedure to wash the gold particles and remove unreacted ligands is typically caried out using centrifugation. Biomagnetic separation processes, on the other hand, require a magnetic bead separator. As such, a magnetic separator will have to be acquired. Ideally, this would be a homogeneous separator in which the particles are exposed to a constant magnetic force. It is important to note that magnetic beads tend to fall out of suspension in a much higher degree than gold particles because of their higher density. As such, they must be mixed consistently and sonicated prior to use to ensure proper dispersion.

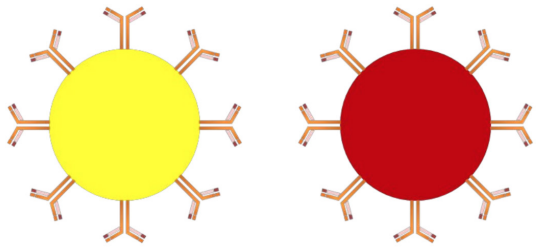


Traditionally, gold particles bind a molecule via passive adsorption, though there are now some alternatives such as protein A/G, streptavidin or carboxylated activated particles. The particle will bind to a hydrophobic area of the protein in a non-specific way. In contrast, magnetic beads attach via a great variety of covalent bond types. As such, when switching from gold particles to magnetic beads, the nature of the molecule to be bound will have to be examined and well-understood in order to determine the best site for covalent attachment. A suitable binding site will allow for attachment to the bead while still orienting the protein in such a way that the probe site remains unobstructed.

In general, magnetic separation processes are gentler, faster, more efficient, and more cost-effective than gold particle processes. Assuming that proper steps are taken to ensure a successful switch, there are numerous benefits to moving a process away from gold particles and toward magnetic particles.

Chapter 4. Moving from Latex Particles to Magnetic Latex Particles

The main reason for changing from latex to magnetic latex particles is the need for a change from an homogeneous to an heterogeneous immunoassay. The latter allows washing steps, which potentially help to improve analytical sensitivity and to reduce interference from sample components. The main consideration in shifting from a process that utilizes latex beads to one that uses magnetic latex beads will be the physical separation process itself. Applications that utilize latex beads traditionally make use of a centrifuge or, alternatively, tangential filtration. In contrast, processes that use magnetic latex beads are carried out in a biomagnetic separator. As such, it is necessary to acquire an adequate separator for the process. Ideally, this would be a homogeneous separator.



Practical concerns when working with magnetic beads include being aware that they have a stronger tendency than latex particles to fall out of solution because of their higher density. Thus, when using magnetic beads, it is important that the suspension undergoes constant mixing. Typically, this is done by placing the bead suspension on a mixer to insure that the beads remain evenly dispersed. Differently to latex particles, a ready to use magnetic bead suspension needs to be resuspended prior to use.

The chemistry of attachment to the particle is not an issue when switching from latex to magnetic latex beads, as it would be when switching over from gold nanoparticles. Both latex and magnetic latex beads use the same types of covalent bonds to attach molecules, so there is no need to seek out alternate attachment sites on the protein. A better quality in the covalent binding can be achieved with magnetic particles because of their faster separation or washing steps.

The main issue when making a switch from latex particles to magnetic latex particles is the difference in the techniques that utilize the two types of particles. Once the protocols are in place, magnetic separation processes prove to be faster and more efficient than protocols that utilize non-magnetic latex particles.

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Chapter 5. Finding the Know-How Necessary for In-House Expertise

In order to ensure the success of a protocol, it is essential to have a clear and unbiased knowledge base and a reliable source of reference material. When trying to decide the best platform or application to use for a process, it is critical to ensure that the information on which the decision will be based is generic and factual, and not propagated as promotional data.

Academic publications are a highly recommended source of knowledge. Texts such as “Bioconjugate Techniques” by Greg T. Hermanson, for instance, serve as technical guides while also providing an impartial overview of commercially available products. Such references can be used to better inform any decisions that will have to be made regarding the choice of technique or material to be utilized, as well as to troubleshoot some processes.

Another way to broaden one’s knowledge base is to develop a good relationship with suppliers who are capable of answering questions in an informed manner. While there is some inherent bias associated with this approach, that bias can be overcome by gathering information from a number of different suppliers and then sifting through the acquired data. There are a number of independent IVD Assay Development companies that offer Consultancy and/or reagent development. They can be of help from very early stage and through all stages of the development. Once the decision to work with a particular process and platform has been made, any questions that arise in the course of developing an assay can be answered by the company that manufactures the equipment and/or the reagents being utilized. Additional assistance in this area might be available from suppliers, if necessary.

It is important, when researching a technique, to cull the information once it has been gathered. Sorting the biased and self-promotional data from the impartial data is essential to ultimately ensuring well-informed decisions, successful protocols, and glitch-free assay development.

Chapter 6. Magnetic Beads: What is the Right Coupling for a Specific Biomarker?

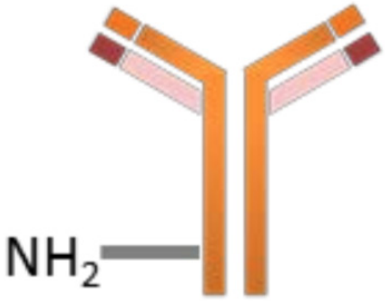
Preparing magnetic beads for a particular assay requires the beads to be functionalized. The beads need to be attached to the biological material that will serve as a capture molecule in the application. The particular type of attachment by which a molecule is linked to the bead will depend primarily on two things: the molecule being bound and the aim of the process.

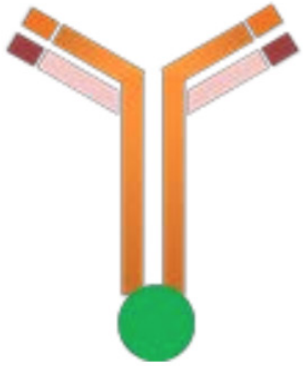
Bead surface linkages fall into two basic types, covalent and non-covalent. Covalent linkages generally restrict binding to a specific, defined site on the molecule. As such, the manner in which the protein adheres is predictable. The attachment site can be chosen such that the molecule is oriented to maximally expose the desired site to the sample. Covalent binding offers advantages when the capture molecule is small or very expensive, and also when coupling uniformity and stability is key. There are a broad range of covalent linkages available to choose from, yielding some degree of flexibility when it comes to designing the assay.

Surface functional groups that bind via non-covalent attachments include streptavidin, Protein A and Protein G. These groups bind a protein with a lesser degree of stability and with less specificity. Of these, the strongest linkage will be formed by streptavidin, though the attachment will still be weaker than that of a covalent bonding group.

your variables before you dive headlong into production. Small scale testing to determine what type of bead to use will save you a lot of trouble in the future when you decide to scale up your assay production.

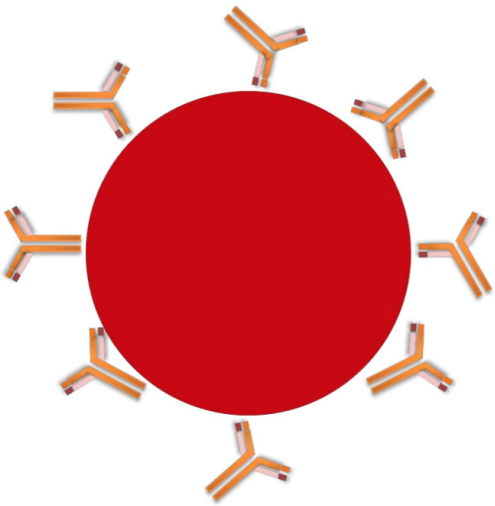
The decision to use one type of binding over another should be based on the manner in which you want to present the bound molecule to the sample. If the molecule must be oriented in a very specific way, then a covalent attachment would be the most suitable. For some applications, specificity might not be a concern. In these cases, non-covalent binding groups might be better suited. The driving forces that guide the selection should ultimately be the particular characteristics of the molecule to be bound and the overall aim of the application.





Chapter 7. Is There any Advantage to Using Beads with Plain Coatings?

Attaching a protein to a bead can be a detailed process that requires forethought and careful planning. Generally, a molecule is attached to a particle through a surface group available on the coating of that particle. In cases where the attachment is covalent, it is essential to choose a binding site on the molecule that will allow for proper orientation, maximally presenting the desired site to the sample while still retaining a strong attachment between the molecule and the bead.



In some cases, it might be more advantageous to utilize beads that have plain coatings, i.e., beads that do not contain any surface functional groups or biolinks. Situations that might warrant utilizing beads with plain coatings include cases where the orientation of the protein is not an issue. Alternately, if some loss of protein due to stability issues can be afforded, or if the amount of conjugate is not a limitation, it might be beneficial to forego the development of a specific binding strategy and simply allow the bead to attach a molecule through passive adsorption. This approach is generally much simpler, requires less planning, and necessitates less troubleshooting.

There are limits to passive adsorption, however. While using beads with a plain coating might be acceptable for certain applications, attaching a molecule to these beads yields no control over the final orientation of the bound molecule. Moreover, the binding is non-specific and, as such, the attachment protocol is less efficient. The decision to utilize beads with a plain coating will ultimately have to be made by weighing the simplicity and ease of passive adsorption against more time-consuming, but ultimately more specific and efficient types of attachments.

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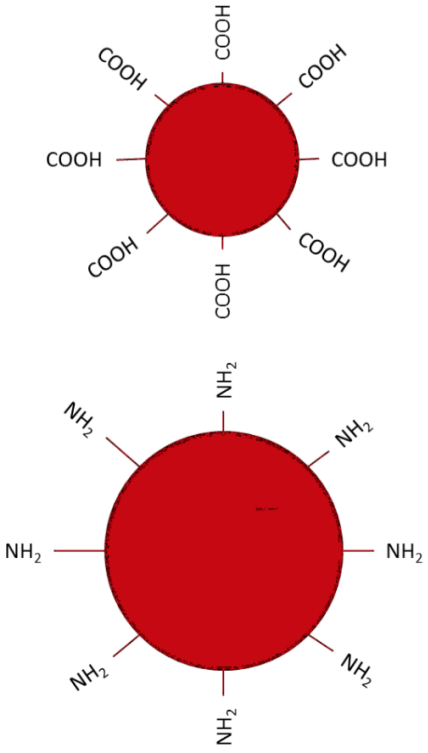
Chapter 8. Pros and Cons of Classical Covalent Links (-COOH, -OH, -NH2)

There are a number of ways by which a molecule can be covalently bound to a nanoparticle. Classical covalent links through carboxyl, hydroxyl, and amino groups have specific advantages and disadvantages inherent to each group. It is important to be aware of these when deciding on a particular functionalized particle for an assay.



Of the aforementioned, the most commonly used is the carboxylated particle. These particles require activation of the surface carboxyl group, typically done by adding carbodiimide (EDC), and optionally N-Hydroxysuccinimide (NHS) or sulfo-NHS and ethyl (dimethylaminopropyl). This activation is performed in order to yield intermediate esters that will then bind to the amino groups in the protein being conjugated to the particle. In contrast, particles functionalized with surface amino groups require activation of the carboxyl groups on the protein to be attached. These particles have the added advantage of utilizing optional bifunctional crosslinkers, small molecules with an active group present at either end. Crosslinkers can serve as spacers between the bound protein and the particle, or they can serve to expand the repertoire of molecules capable of being conjugated to the particle.

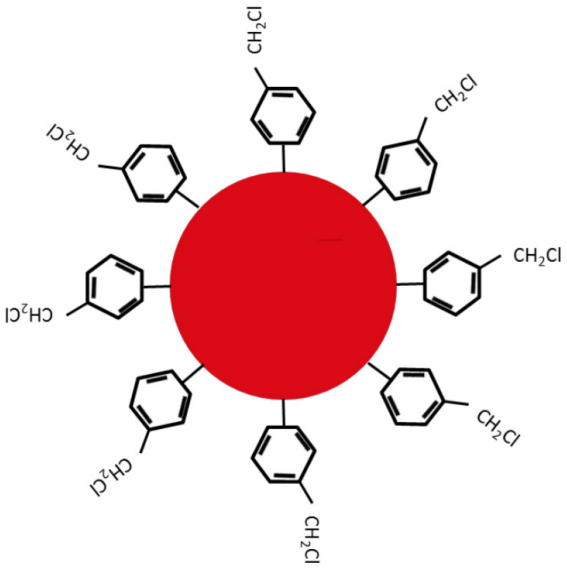
Particles containing surface hydroxyl groups are hydrophilic due to their inherent negative charge. One advantage associated with the hydrophilic nature of these particles is that there will be less aggregation. Additionally, there will be fewer problems that occur with non-specific binding. These particles are more complicated to work with, however, because they require activation in non-aqueous solution to avoid hydrolization of any intermediates. Once activated, however, they can bind a number of different groups, making these particles quite versatile.

Commercially available particles contain a variety of surface functional groups, making it possible to choose between different types of covalent linkages when developing a protocol. Ultimately, the decision to use one functionalized particle over another will depend on the application.

Classical covalent links through carboxyl, hydroxyl, and amino groups have specific advantages and disadvantages inherent to each group. It is important to be aware of these when deciding on a particular functionalized particle for an assay.

Chapter 9. Pros and Cons of Classical Covalent Links (Tosyl, Epoxy, Chloromethyl)

Magnetic latex particles can be covalently attached to a protein through functional groups present on the surface of the particle. There are a number of linkages to choose from, as beads with different types of coatings are commercially available. Some of these surface functional groups require activation before they can bind a molecule. Others, such as tosyl, epoxy, and chloromethyl groups, do not require any preliminary activation. The advantage of these types of surface groups is that they will attach a molecule as long as the conditions (pH, temperature) are favorable for binding.

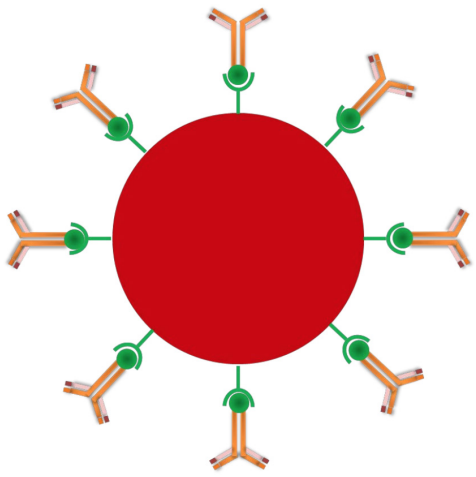


Tosyl groups, for instance, will bind to amino and sulfhydryl groups in a protein at a neutral pH and a temperature of 37ºC. Epoxy groups can bind a number of different groups, depending on the pH of the binding reaction. If the pH is slightly basic, epoxy groups will bind thiol groups. At higher pH conditions, the epoxy group will bind to amino groups. Finally, at very high alkaline conditions, epoxy groups can bind to hydroxyl-containing ligands. Of the three groups listed previously, however, the easiest to work with is the chloromethyl group. These groups will bind amino groups at room temperature and at a neutral pH.
The advantage to using beads containing the covalently bonding tosyl, epoxy, and chloromethyl surface functional groups is that functionalizing the beads is more straightforward. There is no preliminary activation that needs to be carried out prior to attaching a molecule to the bead. The binding, however, occurs with a lesser degree of specificity than is seen with covalent bonding functional groups that require activation, such as carboxyl, amino, and hydroxyl groups, and some passive adsorption may occur.

The advantage to using beads containing the covalently bonding tosyl, epoxy, and chloromethyl surface functional groups is that functionalizing the beads is more straightforward. There is no preliminary activation that needs to be carried out prior to attaching a molecule to the bead.

Chapter 10. Pros and Cons of Classical Biolinks (Streptavidin, Biotin, Protein A, Protein G)

Magnetic beads are available with a variety of surface coatings. Beads can be endowed with a range of functional groups that enable them to covalently attach a particular molecule. Alternatively, a bead can contain a surface biolink such as streptavidin, biotin, protein A, and protein G. These biolinks have unique and specific properties that govern their use, rendering beads coated with these types of groups suitable for a number of different applications.



Unlike surface functional groups that bind covalently to a protein, biolinks attach molecules in a non-covalent manner that is governed by their affinity for said molecule. Protein A and protein G, for instance, are small proteins originally derived from bacteria. These proteins bind certain immunoglobulins subtypes with a very high degree of affinity. Although each of these two proteins has a unique antibody binding profile, there is some degree of overlap in the antibody fractions that are recognized and bound.
Streptavidin is another small, bacteria-derived protein that is utilized as a biolink on bead coatings. Streptavidin has an extraordinarily high affinity for biotin. The strength of binding between streptavidin and biotin is such that it can withstand high temperatures, a wide range of pH values, variations in buffer salts and the presence of detergents. As such, these links are ideal to use in cases where a sample might require extreme conditions. The biotin-streptavidin link can be disassociated with a short 70ºC incubation without denaturing the streptavidin.

The disadvantage of working with these biolinks is that there can be issues with non-specific binding. Biotin, on the other hand, is a naturally occurring molecule and, as such, the highly circulating endogenous biotin present in samples may interfere with an assay. Consequently, when working with beads containing surface biolinks, it is important to modify the protocol to minimize the effects of any non-specific binding.

Chapter 11. Scaling up Coating Protocols

Once a protocol for coating beads is developed and put in place, it will need to be scaled up in order to meet demand. Scaling up a process, however, requires careful attention to ensure that the details of the protocol are replicated for larger volumes.
It is essential, for instance, to ensure that the raw materials can retain their quality when utilized in higher quantities. Similarly, it is important to verify that any equipment utilized in the original protocol is transferable to the scaled-up version. There are some instances where this will not be the case, and adjustments will have to be made. In cases where centrifugation plays a part in the process, for example, this is typically an issue. Materials used for small volume processes such as centrifuge tubes, are not capable of accommodating the same centrifugation velocity for larger volumes. As such, alterations will have to be made to the protocol to ensure that the same conditions are being maintained.

For protocols that involve magnetic particle separation, it is essential that the same conditions are maintained for larger batch volumes or significant problems may arise.

If the same system is not utilized for the larger volumes, or if care is not taken to ensure that the same conditions are maintained throughout, the force experienced by the beads will be altered, and issues including non-reversible bead agglutination may arise.

Scaling up a protocol is not always straightforward. Problems may be avoided, however, if proper measures are taken to ensure that the conditions of a protocol are maintained when moving from a smaller to a larger volume. It is important, therefore, that the research and development department that establishes the protocol and the manufacturing department charged with scaling up the protocol maintain good communication to avoid any complications during a scale up.

Chapter 12. Transferring the Coating Process from R&D to Production: How Can a Magnetic Separation Rack Influence Development?

The key issue when transferring a bead coating process from the Research and Development department to the manufacturing department is scalability. It is essential to ensure that the system being utilized for a particular protocol is adaptable to larger volumes. Ideally, any scale-up would be carried out with the use of a homogeneous biomagnetic separator, as this would ensure that the conditions of the protocol are well-defined and able to be reproduced for a larger volume. Beads that undergo a separation process in a homogeneous separator experience a constant force regardless of the distance from the magnet. In contrast, scaling up a process in a non-homogeneous separator volume results in the beads located at a greater distance from the magnet experiencing a decreased magnetic force. This necessitates a disproportionately large increase in separation time. Problems that arise as a result include non-reversible bead agglutination, consequently leading to decreased and inconsistent yields.

It is crucial that departments involved in scaling up a process remain in constant communication in order to ensure a successful transfer of a coating protocol. The research and development department charged with establishing a procedure should play a hand in the manufacturing process, and vice versa. If the equipment used to develop a protocol can also be used to scale it up, then any problems associated with applying a procedure to a larger volume can be minimized. Similarly, if the conditions of the initial protocol can be clearly defined, as they are when utilizing a homogeneous magnetic separator, then the transition of the process to an increased batch volume will be relatively straightforward and the quality of the final product will be maintained.

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