# Magnetic bead coatings: Today and tomorrow

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# Chapter 1
# 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.

## About the author

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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**

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 2
# The 4 today's surfaces for magnetic beads coating

The use of magnetic beads in IVD is not new. Recent developments –as the described in the next chapters- promise easier and better coating procedures where the orientation and the availability of the capture molecule can be controlled. However, most of the current applications are still using the classical surfaces.

The way of couple your protein or antibody to the surface of the magnetic bead depends basically on its nature and the assay you are working on.

The simpler alternative for coating your beads is the plain surface. Your antibody is coated to the beads surface by passive adsorption. This sort of attachment typically relies on hydrophobic interactions to bind the molecule to the bead. It 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. Problems with specificity and stability of the capture molecule may arise as a result.

## Modified surface (Carboxyl, amino, hydroxyl and sulfates)

To minimize non-specifity problems and reduce the background, you may covalently and stably couple your antibody to the magnetic beads. The typical groups added to the surface are carboxyl, amino or hydroxyl.

Carboxylated particles require activation adding carbodiimide (EDC), N-Hydroxysuccinimide (NHS) or sulfo-NHS and ethyl (dimethylaminopropyl). This activation yield intermediate esters that will then bind to the amino groups in the protein being conjugated.

Magnetic beads functionalized with surface amino groups require activation of the carboxyl groups on the protein to be attached. Utilizing cross-linkers 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, and show less aggregation and fewer non-specific binding. These particles are more complicated to coat and 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.

Some problems can occur such as aggregation and nonspecific binding. If these problems are an issue, you may need to use pre-activated surfaces.

## Pre-activated surface (i.e. tosyl, epoxy, and chloromethyl groups)

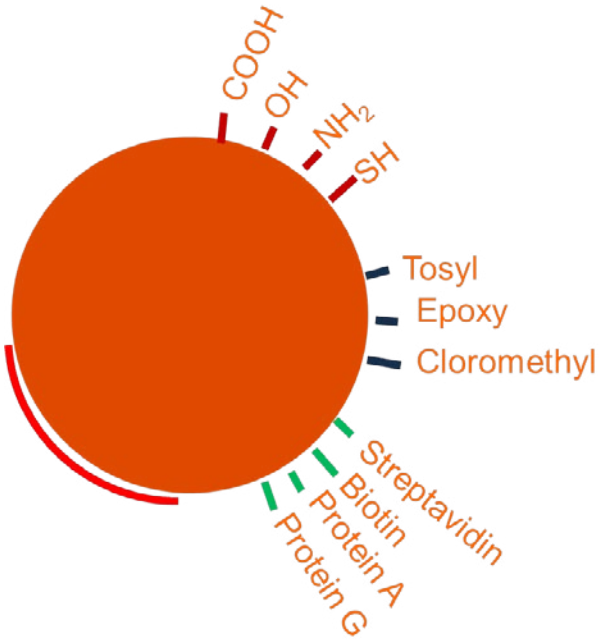
There are a number of commercial magnetic beads pre-activated or functionalized with different chemical groups on their surfaces, such as tosyl, epoxy, or chloromethyl groups. The main advantage of using this type of beads is that coating the beads covalently with the capture protein is more straightforward than with the typical groups mentioned in the previous point. There is no preliminary activation that needs to be carried out prior to attaching a molecule to the bead.

These surfaces allow for very stable covalent coupling of antibody to the beads. The coupling is usually simple: it just needs incubating the pre-activated beads in the correct buffer, pH and temperature.

The Tosyl groups will bind to amino or to sulfhydryl groups in a protein depending on the pH during the coating process. Neutral pH is used for sulfhydryl groups, whereas a more basic pH is used for binding amino groups. Chapter 3 will discuss in detail this pre-activated surface.

Epoxy groups can bind a number of different groups, again 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.

The third group, the chloromethyl, is probably the easiest one to work with: at room temperature and neutral pH it will bind amino groups.




The binding, however, might occur with a lesser degree of specificity than covalent bonding functional groups that require activation, such as carboxyl, amino, or hydroxyl groups.

## Bio-activated surface. (protein A, protein G, streptavidin, biotin)

A third family of surfaces are the bio-activated ones. This is an expensive but highly effective option. The beads contain a surface biolink such as streptavidin, biotin, protein A/G or others. 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

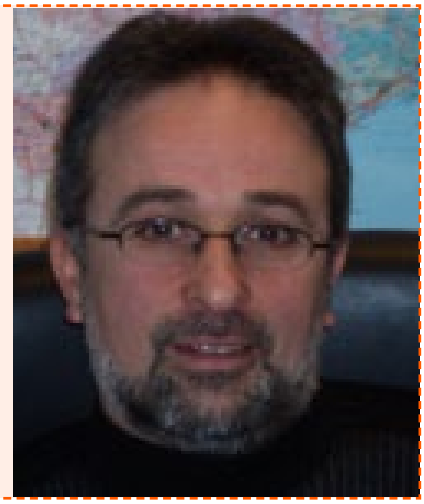
biotin. The strength of binding between streptavidin and biotin is such that it can withstand high temperatures, a wide range of pH values, variations in buffer salts and the presence of detergents. As such, these links are ideal to use in cases where a sample might require extreme conditions. The biotin-streptavidin link can be disassociated with a short 70ºC incubation without denaturing the streptavidin.

The use of these biolinks might result with issues of non-specific binding. For instance, biotin, 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.

## About the author

**Dr. Fabrice Sultan - Merck Chimie**

Fabrice Sultan obtained his PhD in Life Sciences from the Biomedical Institute in Paris in 1990. He joined the B.Braun Medical as product manager in charge of the parenteral nutrition product line of the company. After 8 years he started as sales and marketing manager with Prolabo, a french company of the Merck group. In charge of the Estapor Microspheres product line as sales & marketing manager, Fabrice Sultan combines his background in biology, biochemistry and immunology, together with his experience in sales & marketing and his knowledge in international business. Fabrice Sultan has gained a solid reputation in Microspheres Technologies and provide useful technical trainings in bead-based immunoassays.



# Chapter 3
# Using Tosyl activated magnetic beads in chemiluminescent immunoassays

Magnetic beads are available with a large variety of surface coatings. One of the coatings are the Tosyl activated beads. This post is describing the handling and advantages of the use of Tosyl activated magnetic beads in chemiluminescent immunoassays.

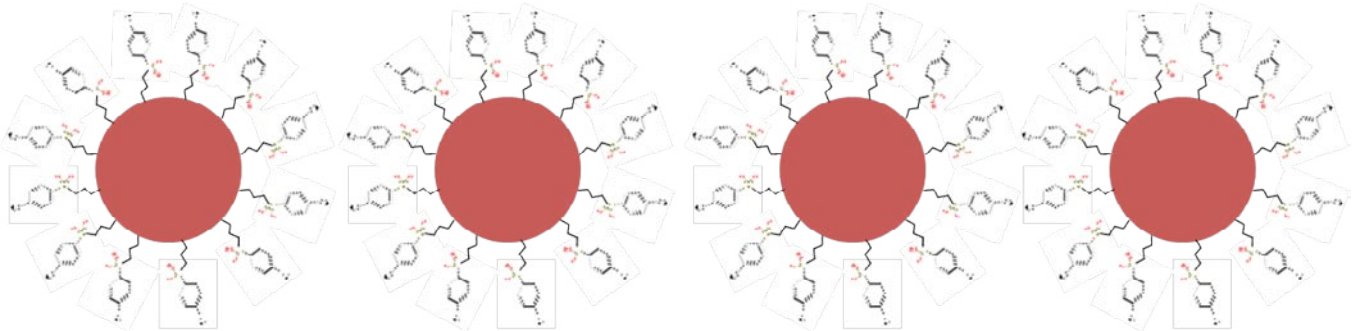
At this moment Tosyl activated beads are available from different companies. Tosyl activated beads can be ordered in different sizes, from 1.0 to 5.0 µm with different functional group densities. The handling of Tosyl activated beads is easy and the beads are ready to use; no pre-activation is necessary. Furthermore, the Tosyl activated beads are known for an excellent reproducibility when producing different batches.

Tosylactivated groups on the magnetic beads can react with sulfhydryl groups and also with amine groups. At pH values around 7.0 to 8.0 the surfhydryl group reaction occurs. At higher pH values (e.g. 8.5 to 9.5) the reaction with amine groups can occur. The high pH coupling is the method that is used the most when developing beads for immunoassays. A typical example of a protocol of coupling antibodies to Tosyl activated beads is described below. Besides antibodies tosyl activated beads can also be coupled with other proteins.

The preparation phase before using the Tosyl activated beads is easy; beads are washed 2 or 3 times with the coupling buffer. Examples of coupling buffers are 100 mM phosphate buffer (PB) pH 7.4 for surfhydryl group coupling or a 100 mM Borate or Carbonate buffer pH 9.5 for amine group coupling. Washing of the beads should be done using a good quality magnetic separator and sonication can be used when re-suspending the beads by using e.g. a sonication bath.

When the coupling process starts the beads should have a concentration preferably higher than 10 mg/mL. Our lab routinely uses a concentration 30 mg/mL beads during the coupling phase. Coupling of the antibodies is done overnight at 37 degrees C using a continuous rotation (overnight incubation is 12 to 18 hours, but needs to be established in a much tighter time period when developing beads for your application). Note that the rotation speed or using a rocking roller can cause influence the coat process. At lower temperatures the coupling time will be longer; 24 to 48 hours. The coupling of the antibodies is a stable covalent bond.

The coat concentration of the antibodies needs to be optimized when developing an immunoassay; normal coating concentrations range from 5 to 20 µg/mg of beads. The high capacity for binding antibodies, or other proteins, is an advantage of the Tosyl activated beads. After the coupling period the beads can be washed 2 or 3 times with a washing buffer containing 0.5% BSA, but this is not obligatory. Blocking of the beads is the fastest at 37 degrees C for 1 hour using a blocking buffer that preferably has the same base composition as the storage buffer. PBS pH 7.4 containing 0.5% BSA is used for many immunoassays as blocking buffer.



When blocking at e.g. 4 degrees C the incubation time is recommended to be overnight. A major advantage of the Tosyl activated beads is the low non-specific binding of proteins to the beads.

The last step after blocking the antibody coated beads is the addition of the storage buffer. This buffer can be the same as the blocking buffer, but a preservative such as sodium azide or MIT/CMIT should be added to extend the shelf life of the stock preparation. Standard storage concentration of the antibody coated beads is 10 mg/mL. In immunoassays beads are diluted to a working concentration using a dilution buffer. Again this buffer can be the same as the blocking or storage buffer, but this needs to be established. Working concentration of beads coated with antibodies typically range from 0.5 to 2.0 mg/mL.

It is clear that working with Tosyl activated magnetic beads really could improve the development of your magnetic particle based immunoassay.

## About the author

**Dr. Michael Jansen – Novel Biomarkers Catalyst Labs**

Dr. Michael Jansen has more than 11 years of experience in the medical device industry. In 2012 Michael founded NBCL together with two partners; NBCL is focused on the development and commercialization of novel biomarkers. Before starting NBCL Michael was Senior Project Leader R&D at Future Diagnostics, heading a team within the R&D department responsible for assay development.

Michael was responsible for the development and validation of assays for the detection of different analytes in biological matrices, on large fully automated immunoassay analysers, point of care platforms and manual micro titer plate assays (ELISA). The development of assays was in the area of fertility, infectious diseases, autoimmune, diabetes, cardiac, thyroid and bone metabolism. Michael held two post-doc positions; one at Wageningen university at the Industrial Microbiology group and at the University of Groningen at the high-throughput screenings facility. Michael holds a PhD in Biochemistry from the University of Groningen.

**For more information visit: nbcl.nl**

# Chapter 4
# Surface Attenuation for High Sensitivity Assays

Designing binding surfaces with optimal ligand (e.g. antibody, antigen or protein) functionality is required for ultra sensitive assays. However, classical solid phase chemistry approaches for conjugating or binding ligands to surfaces do not control the density or parking area of the ligand, nor do they provide control over ligand conformation and orientation.

In fact, the majority of surface conjugation processes used today such as carbodiimide, tosyl chloride, epoxy, and passive absorption typically input a molar excess of ligand based on the total available surface area and ligand molecular weight and/or hydrodynamic radius (Stokes radius of the macromolecule) to improve process Robustness, Repeatability, and Reproducibility (the 3 R's). While this approach will increase total ligand load and theoretical binding capacity, the surface will also be sterically crowded. Moreover, chemistries targeting amino and/or sulfhydryl groups do not necessarily differentiate between available functional groups on the ligand, and subsequently the ligand may be conjugated in a multitude of orientations on the surface depending on the number and location of targeted functional groups. In the case of an antigen down assay, the epitope of interest or binding site(s) may be hidden, and in the case of an antibody down assay the Fab(s) may be unavailable. Since conventional solid phase chemistry approaches to bind ligands may not result in a desired "high sensitivity " binding surface due to steric hindrance (surface crowding), misorientation, and/or ligand inactivation (no longer in a native or active orientation), there has been a need for new conjugation processes that maximize ligand functionality and actual binding capacity, while improving the 3 R's.

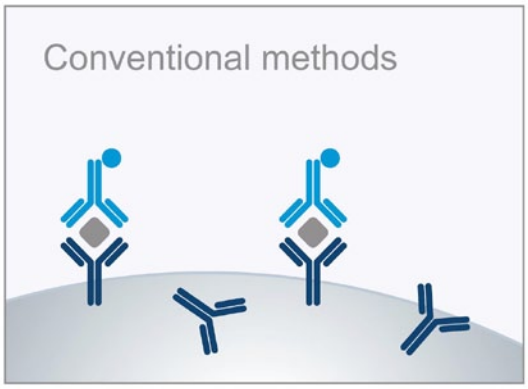
In 2013, Beckman Coulter, Inc. patented such a process whereby they tightly controlled the spatial and conformational orientation of antigens and antibodies on binding surfaces (Soldo J and Sackrison J. Binding Surfaces for Affinity Assays. U.S. Patent 8,518,714, August 27, 2013). In this patent they teach methods and compositions for non-saturated and non-saturated and orientated binding surfaces for affinity assays utilizing a 3-layer approach of biotinylated BSA (a Poisson distribution of biotin), streptavidin (SA) and biotinylated ligand to control the density of biotin, SA, and immunoglobulins on the surface of microparticles for affinity assays. They demonstrate a significant improvement in assay signal-to-noise and dynamic range for assays (AccuTnI, BNP) using non-saturated and orientated microparticle surfaces as compared to conventional microparticles.

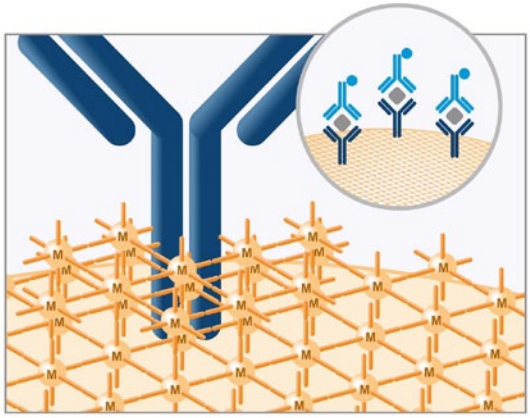
More recently, the group of Philips Research (High Tech Campus, 5656 AE Eindhoven, The Netherlands) published an article that further demonstrates the importance of antibody-capturing activity of antibody-coated nanoparticles (Saha B, Evers T, and Prins M. How Antibody Surface Coverage on Nanoparticles Determines the Activity and Kinetics of Antigen Capturing for Biosensing. Anal. Chem., 2014, 86(16):8158-8166). In this paper, they demonstrate that cardiac troponin I (cTnI) immunoassay response scales with the number of active antibodies, increasing initially but saturating at higher antibody densities. However, in this study the antibodies were non-directionally immobilized on a nanoparticle surface, and they were not able to fully realize the optimal immunoassay response if the antibodies would have been both non-saturated and orientated.

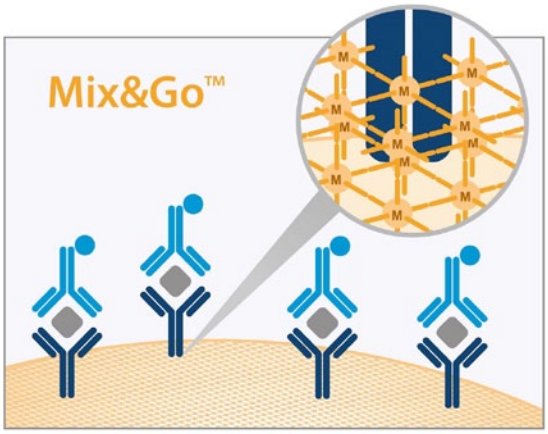
While Beckman Coulter has developed a specific approach for non-saturated and non-saturated and orientated binding surfaces, this process is patented, and not commercially available. In addition, while Philips Research utilized conventional EDC/NHS carbodiimide chemistry to immobilize antibodies on carboxylic acid functionalized nanoparticles, they were only able to control antibody density and not improve orientation. However, there is novel conjugation chemistry commercially available today using polymeric metal ions based around Cr(III). (Mix&Go™, Anteo Technologies, Brisbane, Australia) enables the activation of a variety of different surfaces (magnetic and non-magnetic microparti-

cles and nanoparticles, ELISA plates, glass slides, membranes, gold colloids, etc.) for subsequent immobilization of ligands that are non-saturated and functionally active (native conformation). Most recently, metal polymer chemistry has been successfully used to functionalize and bind a variety of antibodies to cyclic olefin copolymers (COC sensor surfaces) for use in assay systems (Ooi H, Cooper S, Huang CY, et al. Coordination complexes as molecular glue for immobilization of antibodies on cyclic olefin copolymer surfaces. Analytical Biochemistry 456 (2014): 6-13).

High sensitivity assays require sterically assessable and conformational active ligand immobilization on the solid phase surface to increase assay signal-to-noise and sensitivity. While classical approaches to ligand conjugation often result in non-optimized binding surfaces due to crowding, conformational change, and/or poor orientation, there are new approaches available today to support the development of ultra sensitive assays such titrating the amount of ligand per unit surface area instead of using molar excess, or metal polymer chemistry by Anteo Technologies. With these new approaches scientists should be able to more readily and efficiently develop the next generation of immunoassays using their test system and surface(s) of choice.



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Josh has industry reputation as a subject matter expert in assay development and solid phase chemistry. He was a Senior Scientist at Beckman Coulter from 2001-2007, and Product Development Group Manager for DiaSorin Inc. and Corporate Director Scientific Affairs for DiaSorin S.p.A., from 2007-2014.

He joined Anteo Technologies in August 2014 as VP Scientific Affairs.

# Chapter 5
# Rapid biofunctionalization of magnetic beads with function-spacer-lipid constructs

KODE™ Technology is based on novel water-dispersible self-assembling molecules, called a function-spacer-lipids or KODE™ constructs (Figure 1) that are able to coat virtually any biological or non-biological surface with almost any biological or non-biological material [1-10]. The primary coating method of live cells, organisms, bacteria and viruses or solid surfaces (glass, metals, plastics, etc.) is achieved by simple contact with a solution containing one or more FSL KODE™ constructs. Upon contact the FSLs spontaneously and harmlessly create a stable and novel surface coating. Essentially the spontaneous self-assembling process is driven by the need of the constructs to "exclude water". Because the constructs are able to bind to virtually any surface, be it hydrophobic or hydrophilic the mechanisms of action are multiple and complex and include hydrophobic interactions (via lipid tail), hydrophilic interactions (via the head group and spacer), micelle entrapment, encapsulation, bi/multi layer assembly, and other factors such as hydrogen bonding, van der Waals forces, electrostatic and ionic interactions and combinations of all the above on complex surfaces.

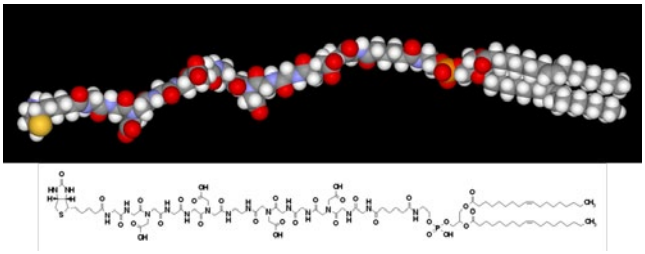


*Figure 1. Schematic of FSL-biotin. Upper image show one possible conformation of the FSL-biotin construct while the lower image shows its schematic formula. The spacer of FSL biotin is a partially carboxymethylated oligoglycine while the lipid tail is 1,2-dioleoyl-sn-glycero-3-phospho ethanolamine (DOPE).*

To-date a large range of peptides, simple and complex carbohydrates (including sialic acids and hyaluronin), peptides, fluorescent markers, reactive functional groups, biotin (Figure 1), oligonucleotides, radiolabels, chelators, and other functional moieties have been created as FSL constructs [1-10]. The key advantages of KODE™ Technology over other conjugation techniques are that it allows the user to create bespoke novel surfaces on demand, and it can also harmlessly modify live cells to facilitate their attachment to beads. Because multiple different FSL constructs can be added simultaneously to a bead, and in a controlled manner (by simply altering relative concentrations of FSLs in the mix), users can build on the surface of the bead a variety of complex multi-ligand biofunctional surfaces. Furthermore, the technology is compatible with existing functionalized beads and would allow users to add further features, such as fluorescent labels, or other enhancing or blocking components.

## Methodology

The use of FSL constructs with magnetic beads has multiple different approaches. The primary approach is to simply modify the magnetic bead with a FSL construct such as biotin or an antigen to facilitate direct binding. This approach can be modified by use of a secondarily active component such as streptavidin or an antibody. A further approach is to also modify the cell/virus with FSLs to facilitate their attachment to the magnetic beads. These approaches to capture live cells or other biological material onto magnetic beads are described below.

## Preparation of biotin koded microspheres

Wash 1g of magnetic microspheres (e.g. Millipore Estapor® Magnetic microspheres) with water and remove most of the supernatant. Add 2 mL of FSL-biotin (187786-1-R&D) diluted to 100 µg/mL (50µM) in PBS and vortex briefly. Incubate at RT for 1 hour and wash once with storage buffer (PBS containing

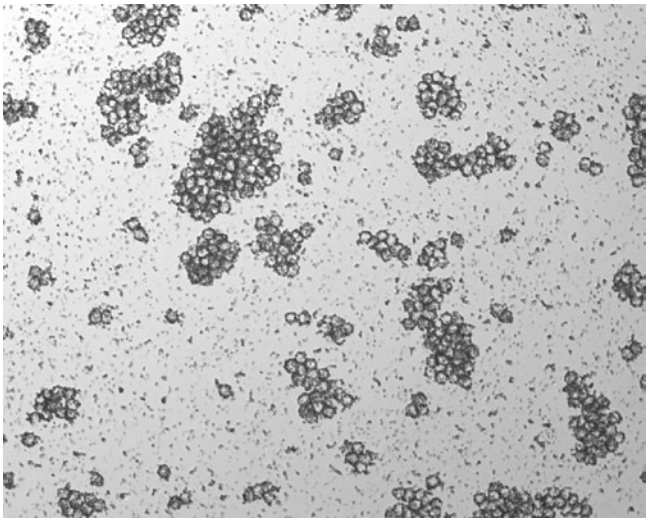
0.1% Tween® 20, 0.5% BSA, 0.05% sodium azide) and then store in buffer at 4°C for up to 1 year. These biotin microspheres can be use to capture avidinylated biological or non-biological material.

## Preparation of streptavidin koded microspheres

Decant supernatant from biotin koded microspheres and add a solution of streptavidin (1 mg/mL) in a ratio of 2 mL per gram of microspheres. Vortex briefly and incubate at RT for 1 hour, with mixing after 30 minutes. Wash twice in storage buffer, then store in buffer at 4°C for up to 1 year. Note: Streptavidin and neutravidin coatings perform better than avidin. These biotin-avidinylated microspheres can be use to capture biotinylated biological or non-biological material.

## Capturing biotin kodecytes onto streptavidin koded microspheres

Prepare kodecytes by contacting cells (red cells, culture lines, sperm, embryo, bacteria, etc) with a 50 µM solution of FSL-biotin, or FSS-biotin (416662-1-R&D) for 1 hour at 37°C. Capture the biotin kodecytes onto streptavidin koded microspheres by simply bringing them into contact (Figure 2). The attachment is sufficiently robust to magnetically isolate the kodecytes captured on the koded magnetic microspheres from other cells in a mixture.



## Capturing cells onto antibody koded microspheres

Preparing antibody koded microspheres is essentially the same method as preparing streptavidin koded microspheres except the FSL-biotin is exchanged for an FSL–antigen and the streptavidin is replaced with a high-titre high-affinity IgM antibody. Capture of antigen positive cells requires simple contact with the antibody koded microspheres.

## Release of captured intact cells from koded microspheres

Dilute the recovered cell covered microspheres about 10-fold with PBS. Vortex for 1 minute to release most of the microspheres from the kodecytes/cells, and immediately magnetically separate the beads and decant the released kodecytes/cells. Large microspheres are required to obtain the shear-forces required for full release of all microspheres from kodecytes/cells. Released kodecytes/cells will be functional, but will contain traces of FSL-constructs and/or related material on their surfaces. These surface preparation remnants should be lost within 48 hours on cells with an active membrane.

## Summary

In summary within a few hours a magnetic bead (or any surface) can be modified with an appropriate FSL construct and used to specifically capture live cells, virions, particles, or other biological or non-biological material.

FSL-biotin and other research related FSL-constructs are available from Sigma-Aldrich and KODE Biotech Materials Ltd. Further information on KODE™ Technology can be found at www.kodecyte.com

*Figure 2. Typical results of cells (7 µm red cells) being coated with magnetic microspheres (1 µm ) biofunctionalized with FSL-constructs. In this example the magnetic microspheres are functionalized with FSL-biotin+streptavidin and the red cells are functionalized with FSL-biotin. Photo (200×) shows cells post magnetic isolation, but before being released from the microspheres by vortexing.*

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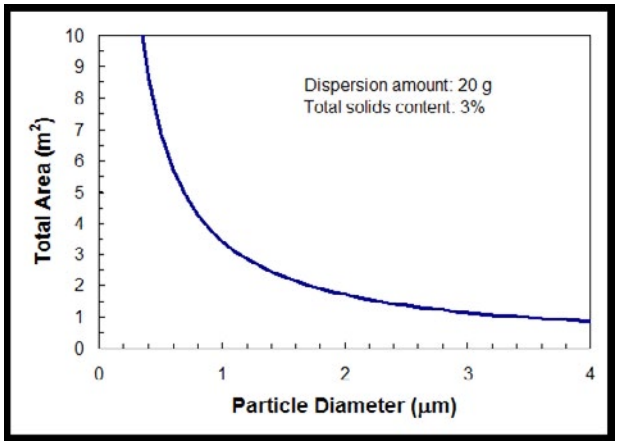
CEO/CSO of KODE Biotech Limited, inventor of the biosurface modification KODE™ Technology and Professor of Biotech Innovation in the School of Engineering at Auckland University of Technology, Auckland, New Zealand. Steve has more than 30 years experience in blood and biotechnology research and has published or presented more than 180 scientific articles and is an inventor on more than 120 patents/applications.

# Chapter 6
# Importance of physical properties of magnetic dispersions during protein coating

During protein (or other kind of molecules) coating onto magnetic particles, there are two main parameters that govern the success of the process: the physical and chemical properties of the protein itself and the magnetic particle dispersions. For this reason, the correct selection of these components is the key for an excellent coating. In this article the importance of physical properties of magnetic dispersions is discussed.

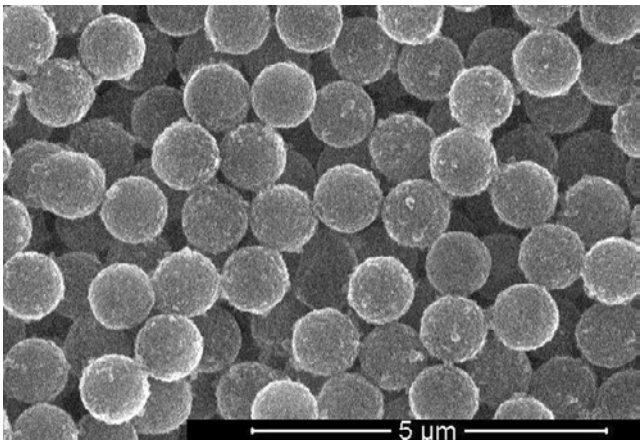
We can say that among others, mean particle size is the parameter that mostly affects the behavior of the dispersion. As a key rule, for the same solid content, the higher the particle size of the particles, the lower the total area available by the particles. For this reason, the amount of protein that can be incorporated to the surface of the particles decreases as the particle size of the dispersion increases. Clearly this effect will determine how the conjugate will work. As an example, the figure below shows the effect of particle size on the total area of 20 g of dispersion at 3% solids content.




Furthermore, mean particle size will determine how fast conjugates will be attracted by the presence of a magnetic field: the higher the particle size, the faster they will be attracted. More than often, there is a compromise between the amount of protein that is wanted to be coupled to the surface of the particle and the separation speed that is desired to obtain under the presence of a magnet.

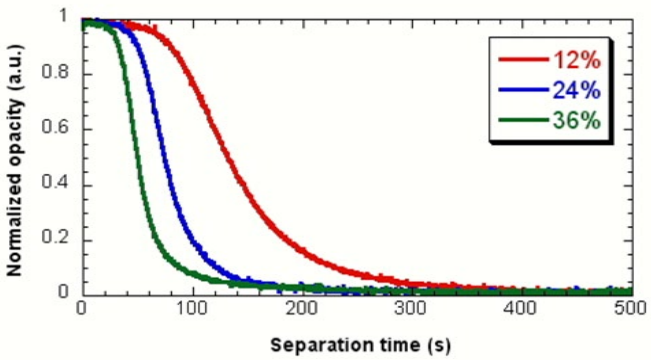
Regarding the particle size, there must emphasize that not only mean particle size has an influence on the behavior of the coating; in addition, the particle size distribution (PSD) is an important parameter. Dispersions with narrow PSDs will offer much better reproducibility since chances to obtain a homogeneous coating (amount of particles incorporating the same amount of protein) increases. Besides, having all particles nearly the same particle size, magnetic force under each particle will be nearly the same, and thus the separation will be homogeneous and reproducible.

The picture below shows a sample of 1 µm magnetic particles synthesized at IKERLAT Polymers, obtained with a Scanning Electron Microscope (SEM). There can be seen that particles maintain their identity after the incorporation of the magnetic compound. Regarding the PSD, the picture seems to show a monodisperse sample. Even the presence of aggregates like doublets and triplets are not detectable by this technique, measurements done with a Disc Centrifugue Photosedimentometer (DCP) confirmed the presence of a unique population of single particles in the sample.



Other parameter that must be taken into account is the amount of magnetic compound that incorporates the dispersion, what we will call the magnetic charge. We can say that this value can vary from 10 to 60%. The most evident consequence of increasing this percentage is that a faster separation is obtained under the influence of a magnetic field, which can be very desirable in time depending processes. However, it should be kept in mind that an increase in the magnetic charge implies an increase of the particle density, which can reach values up to 1.9 g/cm3. Sometimes this is a handicap since combining large particles with high particle densities gives high sedimentation speeds, which is not recommended for prolonged processes and it complicates the complete dispersion of the beads.

These are the reasons for which it is recommended to consider the magnetic charge as a variable that can be tuned on demand. As an example, the figure below shows the variation of the separation speed (measured with Sepmag's QCR) for three magnetic dispersions having different magnetic charges and the same mean diameter.

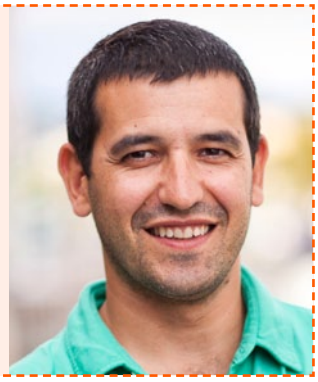



The arguments exposed in this article evidence the impossibility of having a unique reference of magnetic dispersion that will work on every process. In contrast, the physical (and chemical) properties of the dispersion must be adjusted in order to fulfill your process requirements. For this reason, when selecting a magnetic particle supplier, it is important to check the flexibility they can offer, as this can be the key for your process success. At IKERLAT Polymers, the product personalization becomes our leitmotiv, and we will be very glad to assist you in any subject concerning magnetic particles.

## About the author

**Dr. Sergio Rubio - IKERLAT Polymers researcher**

Dr. Sergio Rubio obtained his PhD in Chemisty at the Chemical Engineering Group of the University of the Basque Country (UPV-EHU). Since 2008, Sergio forms part of the R&D department of IKERLAT Polymers, offering solutions to companies and research centers interested in polymer particles, especially for biomedical applications. Before, Sergio has worked as researcher in the UPV-EHU in different projects centered in polymerization in dispersed media.



# Chapter 7
# The 2 critical points of using biomagnetic separation for washing coated magnetic beads

Coating your magnetic beads with biomarkers is the most critical step during the development and production of Chemiluminescence Immunoassay (CLIA). Attaching the antibody (or any other protein) to the bead's surface requires incubating both materials together, using the right buffer and temperature, gently mix and homogenize the suspension. Once the process is completed, it is necessary to separate the solid phase (the magnetic beads with the attached biomolecule) from the rest of the suspension and, once washed, re-suspend the reagent in a new buffer for avoid biomarker reaction and beads aggregation.

Depending on the selected surface chemistry the process coating process may require several conjugation steps. In a typical process, the washing process would need to be repeated a few times to ensure that there are no unbounded biomarker molecules in the final suspension. One option would be using non-magnetic techniques to separate the beads from the suspension, centrifugation, or filtration; but the most popular approach would be to use magnetic separation. This process is faster, cleaner and cheaper. It takes advantage of the superparamagnetic properties of the beads (i.e. when the magnetic field is removed, the beads loss ALL their magnetic moment). Only when beads are ferromagnetic (i.e, remain magnetized after removing the field) is the use of non-magnetic separation techniques plausible.

Even if magnetic separation provides big advantages, it is not a risk-free technology. When it comes to the non-magnetic techniques there is a lot of cumulated know-how, while biomagnetic separation is usually a new subject for the company. Unfortunately, the biomagnetic separation process is governed by physics laws not included in the curriculum of the technicians and scientists of an IVD company. When the inefficiencies of the magnetic rack generate losses and/or in-lot inconsistency, it is not easy for the involved team to realize that the problem can be the magnetic separation process itself.

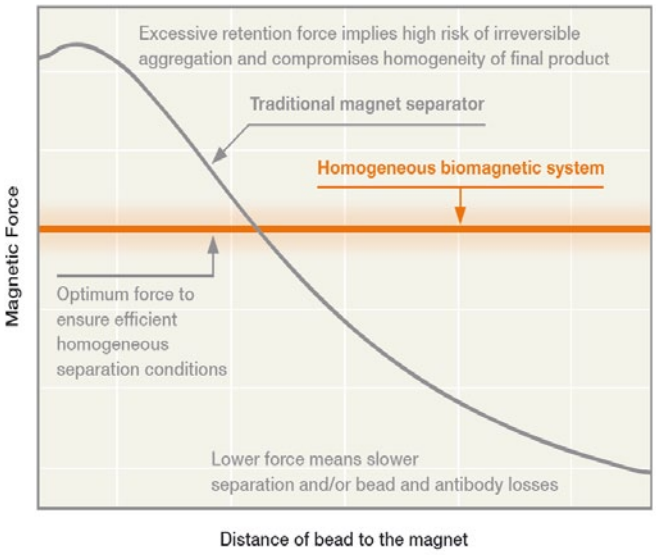



*Diagram showing the variable force a bead experiences in a conventional magnet (grey) compared with the homogeneous force experienced in a Sepmag homogeneous biomagnetic system (orange).*

When problems are detected at small scale –R&D team works usually with volumes of few ml-, they are usually attributed to quality problems of the magnetic beads or to bad coating protocols. But, in most cases, the problems are not so evident at small volumes for the early validations are passed. When the protocols are scaled up the losses increases and become evident. The problems generated by the in-lot inconsistencies also increase in number. Again, the first reaction is to blame the magnetic beads or the coating protocols. The time and resources invested trying to fix the wrong cause can be painful expensive and useless.

What is the real problem? To understand the problem we should realize that most of the magnetic separation racks generate a magnetic force stron-

gly dependent on the distance. Then the farthest beads experience a very weak force. The solution at small scale is to increase the separation time, since passing from few seconds to a couple of minutes is not a big problem. However, when the suspension volume increases, so the distance of the farthest beads to the retention area, the separation time increases exponentially. In realistic separation times (up to about one hour), losses are appreciable, and then repeated washes can reduce the throughput and efficiency. Even small losses of the magnetic beads in a single separation step, would generate a significant reduction after the repeated process. A loss of 5% of the beads by per step will become a cumulative loss of the 23% just during 5 separation steps. If the losses are not constant, there would be other factors contributing to compromise the lot consistency.

The first solution seems to be increasing the magnetic force over the farthest beads. However, this would imply the increase of the force at the retention area several times. This even more excessive force would crush the beads generating irreversible aggregates ('clumps') that would reduce the exposed surface to the analyte or to the next step of the coating protocol. Since the whole processed volume should be divided into small single-doses (typically few microliters), the clumps imply a large variability in the IVD-kit response, even in the same single lot. Re-suspending the magnetic beads would be complicated (sometimes even impossible), and the use of sonication would become increasingly complex and expensive when the volume scales reaches fraction of liter.

Using magnetic separation rack with an inhomogeneous magnetic force profile makes it impossible to deal with both problems at the same time. If you try to reduce losses, you will generate more clumps. If you try to be gentler in the retention area to avoid clumps, then the losses and/or the separation time will increases exponentially.

How can the problem be solved without coming back to the painful centrifugation or filtration techniques? Just by avoiding root cause, which is using a biomagnetic separation system that generates a magnetic force constant with the distance to the wall. If the force is homogenous in all the working volume, you can reduce the retention force and, at the same time, have a stronger force over the farthest magnetic beads. As the beads placed far away from the retention experience the same force nearest, all the beads move at the same speed. The separation time will be much shorter (and proportional to the distance the beads travel), reducing both the losses and the separation time. These two facts, and the gentler retention, which also rewards with no clump formation, allow increasing the number of steps of the washing and coating protocols.

The use of homogenous separation conditions means having a well defined single magnetic force value in all the working volume. It allows selecting the right value for retaining the magnetic beads: just strongly enough to don't be aspired when the supernatant is extracted. No applying an additional and excessive force at the retention area, means being gentler than any classical magnetic separation rack. At the same time, even for the gentler retention, the force at large distance –exactly the same value as in the retention area- would be much higher than an in the inhomogeneous magnetic force devices. The exact value of the force would be defined by the magnetic field profile and the magnetic moment of the beads, and the speed would also depend on the suspensions viscosity and bead diameter. For different combinations of magnetic beads and buffer, the magnetic field profiles of the separation system should be different (as is the case of the SEPMAG® LAB, A and Q series).

Using the same force value, changing scale would simply need to extend the separation time proportionally to ration between the vessel diameters. By reducing the retention force and the time the beads remains in the retention area, we reduce the risk of irreversible aggregation. As consequence, the coating protocols developed at small volumes can easily be scaled up. The well established conditions (value of the magnetic force, homogenous in all the working volume) ensure the washing steps can be reproduced regardless the volume without clumps formation or losses increase. As the separation speed is constant, the separation time would need to be increased just proportionally to the vessel diameter.

The use of the right homogenous magnetic force would simultaneously eliminate losses, avoid the limitation on the number of steps for your coating protocol and improve the in-lot consistency -avoiding irreversible aggregation and simplifying the re-suspension-.

Having all the magnetic beads surface exposed to linking the biomarkers and keeping the same amount of magnetic beads during all the steps are basic requirements to compare the performance between different coating protocols. By being assured that your biomagnetic separation steps (washings) do not loose beads nor form clumps, you will be able to focus on optimizing your coating procedures

## About the author

**Lluís M. Martínez - Chief Scientific Officer at SEPMAG®**

Founder of SEPMAG®, Lluís holds a PhD in Magnetic Materials by the UAB. He has conducted research at German and Spanish academic institutions. Having worked in companies in Ireland, USA and Spain, he has more than 20 years of experience applying magnetic materials and sensors to industrial products and processes. He has filled several international patents on the field and co-authored more than 20 scientific papers, most of them on the subject of magnetic particle movement.

# Chapter 8
# Conclusions

The goal of the present eBook was to review the state-of-the-art of magnetic beads coatings. The contributors have reviewed the classical surfaces, but also the new approaches to improve and simplify the process. Last but not least, the physical aspects of the magnetic beads and the separation process were discussed, as they have a critical impact on the success of the coating process.

Dr. Fabrice Sultan (Merk Chimie) has reviewed the most popular surfaces: plain for passive adsorption coatings, modified surfaces (carboxyl, hydroxyl, amino) and pre-activated (tosyl, epoxy, and chloromethyl) for covalent binding and bio-activated (protein A, protein G, streptavidin, biotin) for non-covalent binding. Today, the most common surfaces are still the covalent bindings, but as the use of magnetic beads is expanding, it is necessary to work with surfaces that allow rapid development of the coatings and control of the orientation and coating-efficiency of the capture molecule.

This is the reason why Tosyl-activated magnetic beads are becoming a popular alternative. Dr. Michael Jansen (NBCL) describes in chapter 3 the handling and advantages of the use of Tosyl activated magnetic beads in chemiluminescent immunoassays. As he explains with detail, this surface is ready to use and does not require pre-activation. In addition, Tosyl activated beads show an excellent lot-to-lot reproducibility.

Looking for tomorrow' solutions, we asked experts working at companies that are introducing new products for surface coating with capture biomolecules. Josh Soldo (Anteo Dx) focuses his chapter on the limitations of classical surfaces to meet the requirements of ultrasensitive assays, paying special attention to the density or parking area of the ligand and to the ligand conformation and its orientation. He proposes new approaches, such as the titration of the amount of ligand per surface area unit (instead of using molar excess) or the metal polymer chemistry. He concludes that these new approaches will be the key for the development of the next generation of immunoassays.

Another innovative approach has been presented in chapter 5 by Prof. Stephen Henry (Auckland University of Technology and KODE Biotech). He describes novel water-dispersible self-assembling molecules, called function-spacer-lipids, which are able to coat virtually any surface with almost any biological or non-biological material. Using this technology, a magnetic bead can be modified with an appropriate FSL construct and used to specifically capture live cells, virions, particles, or other biological or non-biological material

However, the success of the coating process not only depends on the selected surface and activation protocols. The last two chapters of this eBook pay attention to two factors often overlooked when developing a new test. Dr. Sergio Rubio (Ikerlat Polymers) reviewed the importance of the physical properties of the magnetic beads. The size of the bead will influence the specific area and the separation time, but also the distribution. The effect of the magnetic charge is also analyzed, as higher charge means fastest separation but also higher density. The trade-offs for each development would require the selection, not only of the surface but also of the physical characteristics of the magnetic beads. Dr. Rubio emphasizes the impossibility of having a unique reference of magnetic dispersion that will work on every process, but the need of adjust the physical properties for any single process.

The last chapter analyzes the two main problems that the magnetic separation rack can generate when used for washing the magnetic beads during the coating process. Even if the biomagnetic separation is the faster, cheaper and easier to use technique to separate the solid phase, Dr. Lluis M. Martinez (SEPMAG) point that not paying attention to two critical points may jeopardize all the coating protocols. The use of the wrong magnetic separation rack can imply important losses of materials and irreversible aggregation problems. These problems are not always easy to be detected at small volume and are the main causes of trouble when the protocols are scaled up. Reviewing the physics behind

the separation problems, he encourages the use of biomagnetic separation systems using homogenous force.

As editors of this eBook, we want to acknowledge the effort of all the contributors, specially their quick and enthusiastic response after contacting them to explain this project. Their openness to share their knowledge with the magnetic bead's user community is an encouraging sign of the potential of this technology for helping IVD and biotechnology communities and by extension humankind.

Dr. Sergi Gasso and Dr. Lluis M. Martinez, Editors

## About the authors

**Sergi Gassó (see chapter 1) and Lluis M. Martinez (see chapter 7)**

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