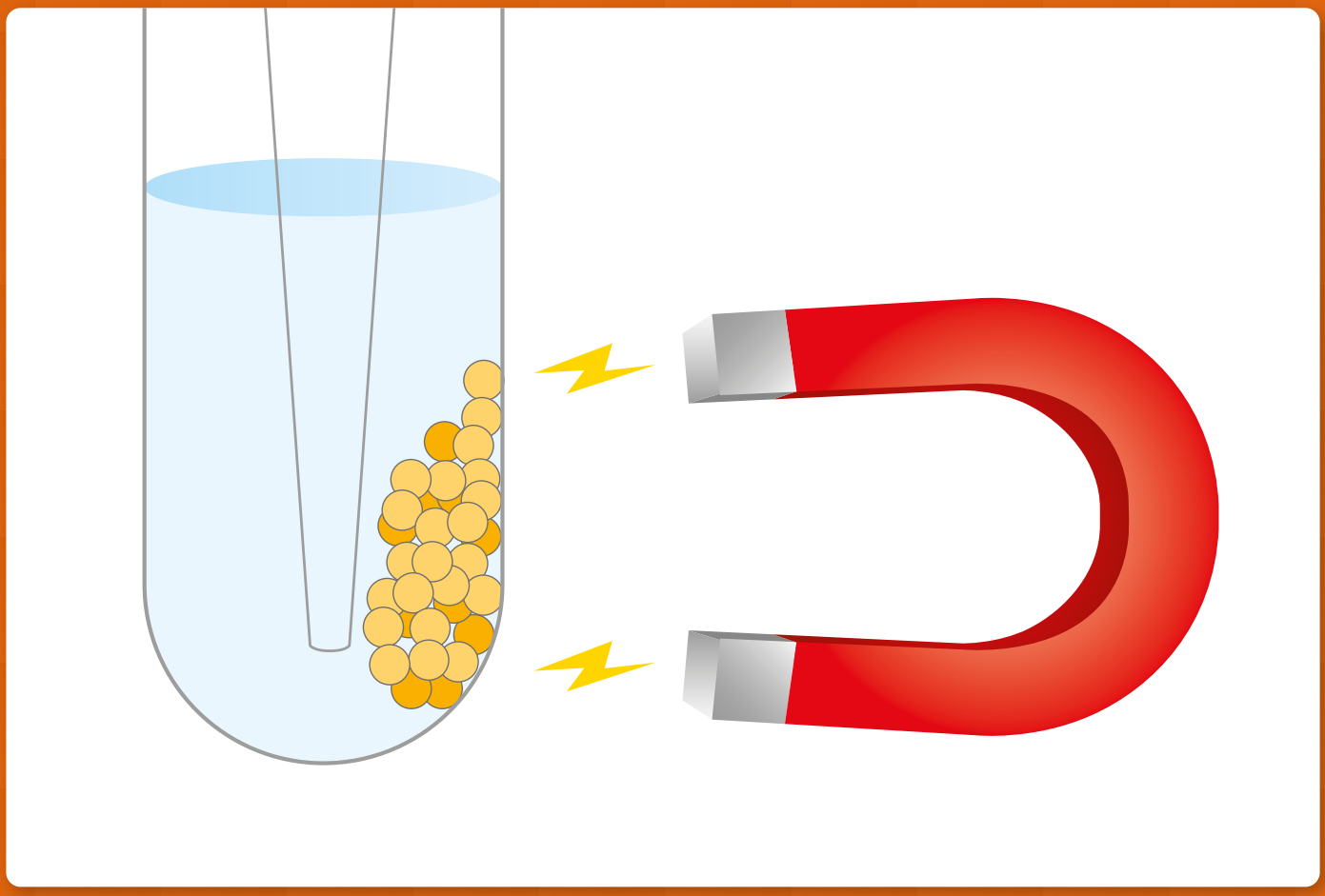
The advanced guide to biomagnetic protein purification

Discover the available tools and techniques for protein purification via biomagnetic separation.

Edited by Dr. Lluis M. Martinez



Sepmag®

SUMARY

Introduction

Limited natural sources and the growing demand for proteins from the health, industrial production and food sectors has led to a burgeoning demand for recombinant proteins. The continuous increase in expression levels (upstream process) is challenging the purification (downstream) process and there is the need to clarify the sample in order to eliminate larger particles or aggregates that may interfere with subsequent steps, a problem which is exacerbated when the target protein is inside the cell fraction.

Magnetic beads-based purification is considered a promising alternative, as it may work with raw suspensions without need for clarification, however, the high price of classic magnetic beads, lack of tools and an insufficient knowledge base has confined Biomagnetic Separation to screening and other low volume applications.

Nevertheless, numerous tools have appeared in recent years, most of which harness technologies which have already become established in other areas of biomedicine. Biomagnetic Separation Systems are used routinely with batches of several liters in production IVD-CLIA-kits, classical chromatographic resins have evolved to become magnetic, new kinds of coatings have been developed and less costly magnetic materials with higher binding capacity have appeared on the market.

This eBook is a compilation of contributions from experts from eight different companies, which offers an overview. These companies range from large multinationals (such as Millipore and GE Healthcare), to new high-tech players (Anteo Diagnostics, ReSyn Biosciences, CubeBiotech), as well as companies with a long history in IVD (IKERLAT, SEPMAG®) and, last but not least, a recombinant-expression protein manufacturer already using magnetic purification in its processes (Algenex).

The first chapters in this eBook review different magnetic beads technologies, dealing with the advantages of magnetic agarose protein purification (chapter 2), the key benefits of magnetic sepharose (chapter 3), how new hyper-porous beads increase capacity and separation speed (chapter 4), and how the size distribution of magnetic beads can affect the protein purification process (Chapter 5).
A second block is devoted to new tools, namely how to use new coatings to functionalize nanoparticles (chapter 6) and how modern homogenous biomagnetic separation systems are able to work with large volume and crude extracts (chapter 7).

Last but not least, the final two chapters give examples of the use of biomagnetic separation on protein purification. Chapter 8 describes a one-step lysis and protein purification process, while chapter 9 looks at how magnetic beads and modern biomagnetic separation systems are able to purify recombinant proteins derived from insect-derived crude extracts.

As editor, I would like to thank Dr Roland Fabis, Dr (GE Healthcare contributor name, pending), Dr Joe Maeji, Dr Isak Gerber, Dr Sergio Rubio, Dr Anja Dedeo and Prof. Jose A. Escribano for their invaluable contributions, patience and diligence throughout the editing process.

If we help readers to understand a little more about the advantages, benefits and challenges of using magnetic beads separation in protein purification, all this effort will have been worthwhile!

I hope you enjoy this eBook. Please feel free to send your feedback to the editor (martinez@sepmag.eu).

Chapter 1. Advantages of Magnetic Agarose in Protein Purification

Most current protein purification methods use agarose beads carrying affinity functionalities such as IMAC, Glutathione, or antibodies. The choice of these functional groups depends on the protein of interest to be purified, and a large variety is available, including pre-functionalized beads that can be coupled to biomolecules (see SEPMAG® protein purification e-book chapter 4 and 5).
Agarose is most frequently used as protein purification matrix for two reasons. First, agarose shows very low non-specific binding to proteins, increasing the purity of the eluted protein fraction. Secondly, agarose particles as a whole consist of a hydrophilic, three-dimensional mesh large enough for proteins to diffuse into. This mesh allows biomolecules to interact with the first micrometers of the outer shell of the agarose particle, providing a large interaction surface between the functional groups and the proteins of interest, thereby increasing the yields that can be obtained in protein purification.

Agarose matrices are available both non-magnetic and with a magnetite (Fe_3O_4) core that renders them magnetic. Non-magnetic agarose beads are typically larger (40-150 µm in diameter) than magnetic beads to reduce back-pressure in chromatography. Magnetic beads are typically smaller (2-40 µm in diameter). Smaller beads provide more surface area and therefore higher interaction with the protein of interest. However, for best magnetic behavior a minimal size of 10 µm should be used, especially when working with ferrimagnetic beads (see SEPMAG® protein purification ebook chapter 12).

For maximum flexibility, the chemistry of the affinity functions, as well as the surface, should be identical between magnetic and non-magnetic materials, allowing for easy switching between the two systems.

The main difference between non-magnetic and magnetic agarose beads is the way they are separated from the surrounding medium. In the first purification steps, this medium can be bacterial or cell lysate, cell culture medium or any kind of physiological buffer. During the wash and elution steps of protein purification, this medium contains buffers of different properties (e.g. salt concentrations, pH) that induce binding of the specific protein, removal of contaminants, and elution of the protein of interest. Viscosity of these solutions can vary, e.g. when substances like glycerol are added to reduce non-specific binding. Every protein purification process requires several separation steps, therefore efficient separation has a large impact both on processing time, and purification success.

For all protein purification experiments, it is important to adjust the amount of affinity beads to the amount of protein in the starting material. This is important not only for cost reasons: too little affinity material will result in incomplete binding of the protein in the solution. Too many affinity binding sites offered, on the other hand, will lead to binding of other proteins, making the purification less specific, so that additional purification steps are required in order to obtain pure protein.

Non-magnetic agarose beads can be separated from the medium by centrifugation, by filtration in gravity columns, or on chromatography systems. Switching between these methods (e.g. when moving to higher purification scales) requires optimization. When protein expression levels are low, large amounts of starting material need to be applied to rather small volumes of agarose beads, which can be a time-consuming process. For very sensitive applications requiring only microliters of volumes, such as immunoprecipitation, separation could be incomplete, leading to either loss of material or contamination with agarose beads.

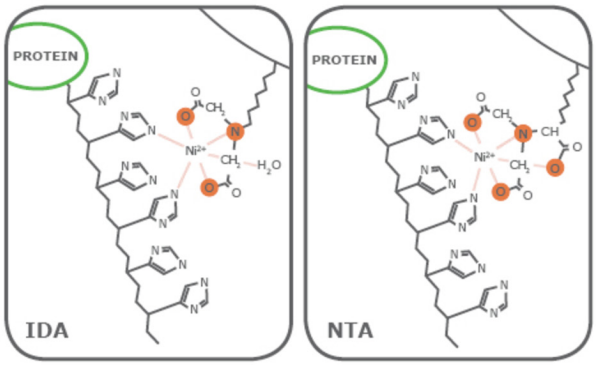


Fig. 2. IDA vs. NTA. Chelating ligands nitrilotriacetic acid (NTA) and iminodiacetic acid (IDA) support similar interaction between Ni2+ and imidazole rings of a polyhistidine tag, but NTA coordinates the Ni2+ with 4 valencies and IDA with only 3 (orange circles). This difference impacts the quality of the resulting purified protein fraction.

With magnetic beads, the principle of separation stays the same regardless of application and volumes. Test reactions or sensitive applications like immunoprecipitation can be performed in few microliters, and even automated on microliter robots. Purification of proteins from liters of medium or cell lysate can be done equally well because the handling volumes are reduced significantly after the first separation step.

Read more at http://www.cube-biotech.com/s-products/mag-beads

Author: Dr. Roland Fabis, Head of Operations at Cube Biotech GmbH, Monheim, Germany
http://www.cube-biotech.com/scompany/management-team

Dr. Roland Fabis holds a PhD in inorganic chemistry from the University of Muenster, Germany, where he worked on silicon-organic compounds, surface modification with silanes, and applications of these substances. Dr. Fabis joined the QIAGEN headquarters in Hilden, Germany in 1995, where he held various positions in basic research, product development and technology transfer, especially in the fields of magnetic particle synthesis and protein purification. He is one of the co-founders of Cube Biotech and responsible for the development and production of protein affinity materials, including manufacturing of magnetic beads.

Chapter 2. Key benefits of Magnetic Sepharose beads

Choosing the right matrix for protein enrichment and purification processes, such as immunoprecipitation or pull-down assays, is an important step in optimizing the efficiency of a protocol. The decision will be based on a number of variables and ultimately depends largely on the nature of the target biomolecule. The goal is to choose a matrix that will not only maximize the final yield, but will also be practical and accessible.

One well-defined approach is the use of paramagnetic beads. Mag Sepharose beads, for instance, are composed of highly cross-linked agarose particles. These beads contain a surface ligand, which captures the target biomolecule within a sample, either directly or through a bound antibody.

There are several advantages to using Mag Sepharose beads in purification protocols. Because Sepharose beads are non-adherent, it is not necessary to add detergents. Aggregation problems that might be encountered with other types of beads are not an issue. Additionally, the high density and visibility of the beads simplify the collection process, ensuring rapid capture of the bound target protein. The beads are collected with a magnetic device rather than centrifugation, which means that the collection time is significantly decreased, taking place in seconds. Isolated in this manner, a target protein can be concentrated into volumes from the milliliter down to the microliter scale.

A wide variety of functionalities are available for use, making these types of beads suitable for purifying a range of proteins. Beads can essentially be divided into two categories: pre-activated beads which bind the ligands covalently and beads with immobilized ligands which are prepared with ligands. The choice to use one group over another will depend on a number of factors including the target protein, the goal of the assay, and any downstream applications.

Pre-activated beads are more flexible with ligand binding occurring at defined sites. N-hydroxysuccinimide (NHS), for example, binds to amino groups in any ligand. Beads containing this surface group are commonly used in pull-down assays. Once an appropriate ligand has been covalently linked to the matrix, the beads can be used to affinity purify the target biomolecules. When the target is eluted, the ligand remains bound to the bead.

Beads with immobilized ligands are more convenient and easy to use. Protein A and Protein G, for example, bind a number of monoclonal and polyclonal antibodies. Each has a distinct affinity profile, but there is some degree of overlap. These types of beads are well-suited for use in immunoprecipitation assays. Antibodies can be cross-linked to the surface ligand so that they remain affixed to the matrix when the target molecule is eluted. Alternatively, the antibody can be eluted along with the target molecule.

The advantage of using Mag Sepharose beads becomes apparent in cases where there the target molecule is present in low relative quantities and where yields are traditionally undetectable by standard methods.

Low abundance molecules such as signaling pathway proteins are usually undetectable via electrophoresis or mass spectrometry (MS) without additional sample preparation. Mag Sepharose beads make it possible to capture such targets from a large starting sample volume, and then subsequently elute it into volumes suitable for SDS-PAGE or MS analysis.

It's important to bear in mind that each assay is different. Every protocol must be reviewed and modified in order to minimize issues such as non-specific binding before proceeding. Buffers should be optimized in order to maximize binding efficiency and

subsequent results. The final parameters of the buffers will depend on the biomolecular composition of the sample. It's also important to choose the surface group that is best-suited for a particular protocol. Given the availability of a wide range of surface ligands, chances are there will be more than one match for every purification and enrichment assay.

Author: Johan Öhman, Senior Scientist at GE Healthcare, Uppsala, Sweden

Johan is a chemical engineer with degree from the University of Uppsala in Sweden. He has more than 20 years professional experience focused on protein purification and analysis. His first role was at the biopharmaceutical company Kabi Pharmacia working with process development of growth hormones. After this, he worked with high-throughput protein production providing various projects with purified target proteins for structural studies and high-throughput screening of small drug compounds. Johan joined GE Healthcare Life Sciences more than 10 years ago, and he has been working as a Research Engineer/Senior Scientist within the R&D department mainly with protein purification and sample preparation products and applications.

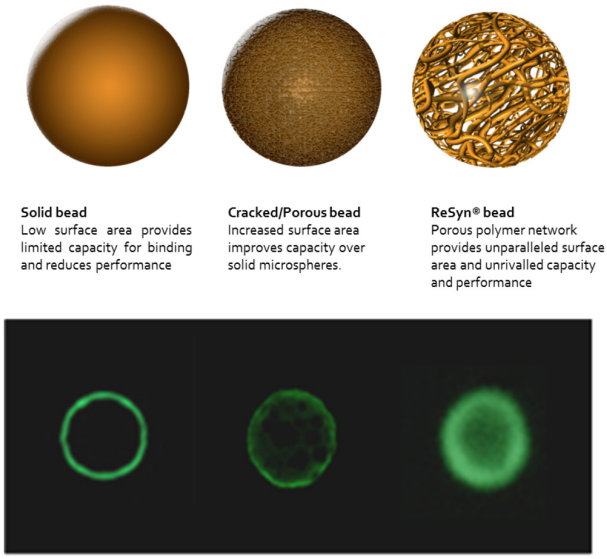
Chapter 3. Novel Hyper-porous Polymer Magnetic Beads as High-capacity/Fast separation alternative

Magnetic beads have several advantages over alternate non-magnetic bead technologies, and are thus finding increasing application in all areas of life-sciences research and development including drug discovery, biomedicine, bioassay development, diagnostics, genomics and proteomics. This is attributed to the numerous advantages of magnetic beads including ease of use; rapid experimental protocols; the amenability and convenience for high-throughput automated and miniaturized processing such as high-throughput screening; and the potential for scalability due to the availability of large homogenous biomagnetic separation equipment. The beads may be functionalized with biomolecules (e.g. proteins or nucleic acids) or chemically derivatized to obtain various functional groups that enable a diverse range of applications. Several different types of magnetic beads are currently available; however, not all magnetic beads are created equally.

Current microsphere technologies (magnetic and non-magnetic) use solid or semi-porous/cracked microspheres which limit binding capacity for target molecules to the surface of the polymer beads. The novel microsphere technology platform, ReSyn®, comprising a hyper-porous polymer matrix, allows penetration of biological and synthetic molecules throughout the volume of the beads, as opposed to limited surface-based binding of conventional bead technologies.

As an example, custom MagReSyn® Streptavidin MAX microspheres (www.resynbio.com) offer exceptional binding capacities of >12 mg.ml-1 (1.2 mg.mg-1 support) biotinylated IgG and >120 000 pmoles.ml-1 (12 000 pmoles.mg-1 support) biotinylated oligo. The exceptional surface area for binding of molecules makes hyper-porous polymer magnetic beads a competitive alternative to slower non-magnetic techniques in applications where volumetric capacity (low microsphere content or concentrated sample preparation) is important. The capacity of a microsphere technology is an important consideration for your potential application, since it may lead to a significant cost-reduction as fewer magnetic microspheres, and less reagent, are required.

The technology allows for high capacity without compromising the speed of magnetic separation, as opposed to attempts to increase capacity by reducing particle size (increasing surface area), where slower bioseparation unfortunately leads to increased protocol times.



A schematic representation of solid, cracked/porous and hyper-porous ReSyn® technology.

Dr. Isak B. Gerber has a Doctorate in Biochemistry from the University of Johannesburg in South Africa, and has amassed an array of experience in protein biochemistry including method and assay development, protein production and purification. He is a co-inventor on two international patents and author on nine international peer-reviewed scientific publications. For the past four years Dr Gerber has been an integral member of the team that developed and patented a novel next-generation microsphere technology platform (ReSyn®) at the Council for Scientific and Industrial Research (CSIR, South Africa). The patented technology is currently being commercialized by a new biotech start-up enterprise, ReSyn Biosciences (Pty) Ltd (www.resynbio.com). The technology was translated into a range of superior performance magnetic microsphere products suitable for life-sciences R&D largely through the efforts of Dr Gerber. Dr Gerber currently serves as Chief Technology Officer for ReSyn Biosciences.

Chapter 4. Effect of Particle Size Distribution of Magnetic Particles in Protein Purification Processes

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.

The use of magnetic particles in protein purification processes presents many benefits compared to classical procedures, being process simplicity and time saving the most relevant. There are many factors and variables that can potentially influence the success of the process. However the properties of the magnetic particles to be used, in addition to the magnetic equipment used for the separation, stand out among them.

It is known that the mean particle size of the magnetic beads mostly governs the purification process. This is because the lower the mean particle size the higher the surface available for protein binding. However, increasing the particle size gives higher magnetic forces and therefore reduces the process. In that sense, there is a compromise between binding capacity and process time.

In order to fulfill the different necessities, magnetic particle suppliers usually offer magnetic dispersions with different mean particle sizes. However, there is another variable that has not had as much attention as the main particle size and in fact can highly affect the behavior of the process: the particle size distribution (PSD). Well known and reported conditions of non-magnetic particle synthesis allow the production of highly monodisperse dispersions with very low variance in particle size. However, the incorporation of an inorganic compound in order to manufacture the magnetic dispersions complicates the production process, and broadening of the PSD is not easy to avoid.

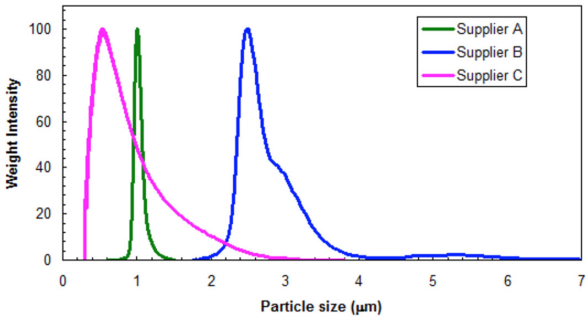
In protein purification it is mandatory to obtain a strict control of the protein binding to magnetic particles in order to obtain satisfactory and reproducible results. For this reason the use of magnetic particles with broad PSD is a focus of variability that affects negatively to the process. The use of magnetic particles with broad PSD gives differences in the amount of ligand incorporated and in the separation speed, which affect directly in the protein purification yield. This variability is critical in screening processes (where small amounts of product are used), but also in industrial scale where a robust lot-to-lot and intra-lot reproducibility is essential. Besides, this variability can be especially important if the proper magnetic separation equipment is not used.

This lack of attention to the PSD from some manufacturers could be related to the difficulty for a proper characterization of the magnetic dispersions. For example, the PSDs obtained by dynamic light scattering differ from real ones when broad or multimodal samples are measured, mainly above micron scale.

In addition, using imaging characterization techniques like transmission or scanning electron microscopes it is really difficult to distinguish between aggregated particles and aggregates formed during the drying step of the sample.

In contrast to the above mentioned techniques, Disc Centrifuge Photosedimentation has been validated as a really powerful technique for a proper PSD characterization. In this technique, particles of different sizes are allowed to reach a photodetector at different times due to the centrifugal force. In this way, the PSD and the number (Dn) and weight (Dw) average particle sizes are obtained. In order to quantify the monodispersity of the PSD, Polidispersity Index (PDI) is defined as the ratio between Dw and Dn, and dispersions with values less than 1.05 are considered monodisperse.

In the figure, the PSDs of 3 commercially available magnetic dispersions are shown.



The table below shows the data corresponding to these distributions:

	Dn (um)	Dw (um)	PDI
Supplier A	1.021	1.037	1.02
Supplier B	2.888	2647	1.09
Supplier C	0.519	0.949	1.83

Attending to these results, it is clear that monodispersity is not always guaranteed when purchasing magnetic dispersions.

Summarizing, we can say that the PSD of magnetic dispersions is a parameter that can highly influence the success of protein purification processes. For this reason it is strongly recommended to assure the supply of magnetic dispersions with narrow PSD.

This article is part of a teamwork of IKERLAT's R&D department formed by Dr. Joxe Sarobe, Dr. Pedro Ilundain, Dr. Sergio Rubio and Dr. Asier Eleta.

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 5. Multi-functional Nanoparticles for Protein Purification

Nanoparticles incorporating different functions are useful and necessary products for assays, drug delivery, and other life science applications. For example, magnetic nanoparticles can be used as contrast agents for magnetic resonance imaging (MRI), to dissipate energy under an oscillating field to locally raise temperature (hyperthermia), or to improve manufacturing of complex nanoparticles via use of magnetic separation. One or more different antibodies and/or fluorescence, luminescence agents as well as other functionalities such as catalytic or enzymatic groups can be attached to nanoparticles.

However, achieving reproducible and controllable addition of just one protein (let alone two or more different proteins) into ever smaller particles is a major challenge - and a technically complicated process.

When additional requirements, such as the need to prevent aggregation and to maintain size uniformity are added, simple procedures that are compatible with many different particles are difficult to find.

Forming Nanometre Glues

Metal polymers - as Mix&GoTM - are able to coat a broad variety of particles including nanoparticles such as bare magnetite (15 nm) and QDots (10 nm). Coating a particle with those polymers effectively addresses the issue of aggregation as charge repulsion prevents clumping of the activated particles. As well, charge attraction in combination with multiple coordination forces give rapid and efficient binding of synthetic or bio-polymers such as proteins to the coated particles.

There are a number of benefits in using metal polymers but for forming multi-functional nanoparticles, two important characteristics are discussed:

Formation of a thin coating. Depending on the surface and the type of metal (cationic) polymer, a “glue” layer as thin as one metal ion in thickness (approx. 0.2 nm) can be formed. On rigid surfaces like injection moulded COC plastics, a product like Mix&Go forms <1 nm films that maintain the pre-existing contours of the underlying material. The surface roughness of injection moulded COC does not change after activation (see Fig. 5.1). On “softer” surfaces such as 200 nm magnetic particles, it forms films of 2 to 3 nm in thickness (established using Zeta-sizer and TEM). In Fig 5.2, 10 nm QDots coated onto these 200 nm magnetic particles are clearly seen as approx. 7 nm dimples on the 200 nm particle surface.

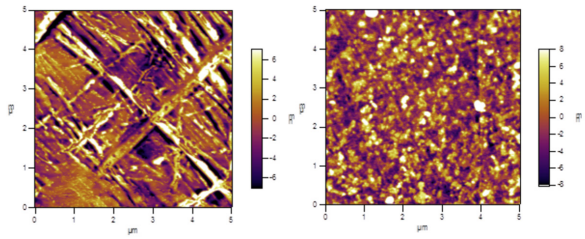


Fig. 6.1. Injection moulded COC (left) has a surface roughness calculated at 4.7 nm ± 0.5 nm by Atomic Force Microscopy (AFM). After addition of aqueous Mix&Go solution and wash, these activated COC surfaces (right) give essentially the same surface roughness at 4.5 nm ± 1.0 nm.

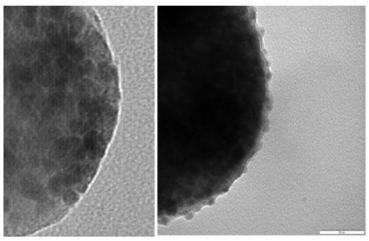


Fig. 6.2. Transmission electron microscopy (TEM) of untreated 200 nm magnetic particles (left) and QDot coupled Mix&Go activated 200 nm magnetic particles (right). QDots are seen as 6 to 8 nm dimples from the background contour of the 200 nm particle.

Rapid kinetics.

On protein binding, using the polymer activated surfaces give high reproducibility of protein density as well as tight particle-to-particle uniformity. Their rapid kinetics means that the amount of protein added correlates highly (R2 of 0.99)

with the amount actually coupled to the particle. As an example, a mixture of two proteins (streptavidin and mouse mAb) was coupled onto 200 nm magnetic particles. As shown in Fig 5.3, antibody titration clearly demonstrates a linear correlation between antibody added and antibody actually loaded onto the particles as determined by anti-mouse detection.

Similarly, streptavidin loading can easily be manipulated allowing two or more distinctly different proteins to be incorporated onto 200 nm magnetic nanoparticles in controllable ratios. In contrast, using passive or amide chemistry it is very difficult to obtain reproducible and consistent ratios of proteins. This difficulty increases with decreasing size of the nanoparticle. Similarly, even more complicated mixtures of different proteins can be assembled on metal (cationic) polymers activated particles in a controllable and predictable manner.

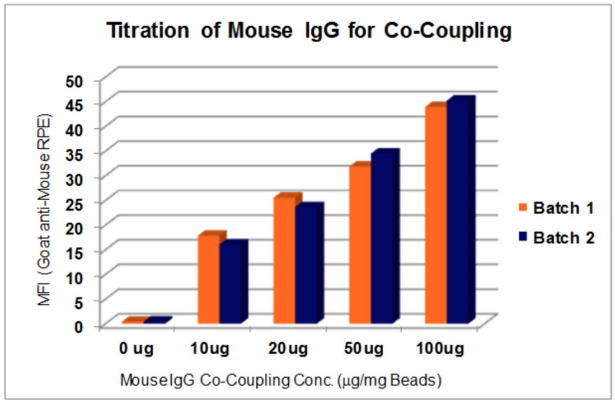


Fig. 6.3. Titration of mouse IgG that was co-coupled with streptavidin on Mix&Go activated magnetic nanoparticles (Merck, 200 nm). Because there is a linear correlation between what is added for binding and what actually binds, it is possible to reproducibly bind two or more proteins in a controlled manner.

Conclusion

Aqueous metal polymers, like the used in Mix&Go, can bind a wide array of different surfaces in as little as a few minutes. Due to its ability to form thin coatings and to its rapid kinetics, these nanometre “glues” can bind proteins and/or other polymers and nanoparticles to create multi-functional and /or multi-layered constructs in a relatively simple and reproducible manner. The technology enables construction of antibody and enzyme conjugates via nanoparticles and similar constructs. It does so while minimising the tendency of nanoparticles to aggregate.

Author: Joe Maeji, Ph.D., CSO Anteo Diagnostics, Brisbane, Australia

Joe’s area of interests has been on the commercialisation of products and technologies at the interface of chemistry and biology for over 20 years.
Joe was a co-founder of the company that became Anteo Diagnostics as well as its founding CEO. Previously, he held senior management positions at Mimotopes Pty Ltd, Melbourne, Australia and at Chiron Corporation, San Francisco, USA. He holds a BSc in chemistry from University of Queensland, Australia, MPhil in enzymology from Griffith University, Australia and PhD in Polymer Engineering from the Tokyo Institute of Technology, Japan.

Chapter 6. Advanced Biomagnetic Separation Systems to Enable Protein Immunomagnetic Purification

The search for alternatives to chromatographic resins is not new. With the continuous increase in expression levels in recombinant protein purification, columns are struggling with crude lysates. The need to clarify suspensions containing high levels of expressed protein for post-purification re-concentration no longer appears to be the most efficient strategy.

Many process development teams have turned their attention to magnetic beads and particles. The use of immunocapture supports which can be made mobile or immobile at will showed great promise. There were two problems with initial attempts: the cost/performance of the magnetic beads and the efficiency of the biomagnetic separation process. Often, however, both problems overlapped and were difficult to distinguish.

Attempts to use the same beads as in IVD-kits were unsuccessful. Even when the immunocapture of the protein can take advantage of the technologies for coating the biomarkers, the diagnostic applications must never elute the protein. Moreover, the IVD beads need to be highly consistent (narrow size dispersion, magnetic content, binding capacity): kit-to-kit consistency must be extremely high, every fraction of the milliliter aliquot must be equal to the rest of the batch. This entails the use of a highly complex manufacturing process (and price by mg) that limits their use to screening and other small volume/high value applications.
But for downstream processes batch-to-batch consistency will suffice (typically measured in liters) and cost is almost always the key issue. Classical IVD-magnetic beads were not a right solution. However, in recent years, breakthroughs in new materials development have put alternative high performance/cost effective magnetic beads on the market.

Nevertheless, selecting the right beads is only half the answer. As IVD-kit producers discovered a decade ago, magnetic bead separation at milliliter scale is easily done even with a simple magnet, however, but when the process is scaled up, matters become more complicated.

The main problem is that magnetic force decreases quickly with distance: when volume grows over tens of milliliters, the beads farthest from the magnet experience very low force. This means, in the best case scenario, very long separation times. In most of cases, the result is high losses.
Classical magnetic racks try to solve the problem by increasing the magnet volume but, if this succeeds, the force in the retention area increases much more quickly. As a result, irreversible bead aggregation becomes an issue and the necessary perfect re-suspension for efficient elution is a major drawback.

The solution is quite simple: generate a homogenous force throughout the entire working volume. Advanced Biomagnetic Separation Systems such as those manufactured by SEPMAG® (www.sepmag.eu) achieve this by generating specific magnetic field profiles. Fast separation/full recovery make it possible to perform stability and re-usability studies without worrying about whether the activity

loss is real or merely because not all the magnetic beads are recovered. The use of homogenous biomagnetic separation systems makes it possible to focus on developing the right capture/elution molecule for the specific protein. The results can easily be transferred to larger volume homogenous force biomagnetic separation systems, making scale-up straightforward.

Some experts have also used this technology to improve other aspects of the downstream process, such as attaining higher concentrations of purified protein in the final suspension volume. Safarik1 and co-workers (disclosure: the author is a co-signer of the article) use the crude extract volume for capturing the protein, and then a 10th time smaller volume for immobilizing the magnetic beads/particles. By passing the suspension through a smaller vessel after the incubation time, they achieve a factor 10 concentration with respect to the crude lysate in a single simple step without losses. Elution than can then be performed and, when the magnetic beads are immobilized again (now without the protein attached), the supernatant would have the captured protein in a tenfold concentration.

Image: Safariket al, AIP Conf. Proc. 1311, 146 (2010); doi: 10.1063/1.3530004

Figure 7. Flow-through magnetic separation system. 1 – mechanical overhead stirrer; 2 – container; 3 – valve; 4 – screw clamp; 5 – tubing; 6 – bottle; 7 – SEPMAG® Q500ml magnetic separator; 8 – bottle for collection of magnetic particles depleted solution/suspension).

Last but not least, biomagnetic separation performance increases with magnetic bead concentration (separation time decreases as the fourth root of the concentration of the beads according to Faraudo & Camacho2). Then, in contrast to what happens with columns, increasing the protein expression level in the upstream process does not hinder downstream performance. Quite the opposite! Adding magnetic beads in excess of the binding requirements also accelerates the biomagnetic separation process.
The Advanced Biomagnetic Separation Systems currently used for manufacturing magnetic beads-IVD kits (at volumes up to tens of liters!) are the second leg of the technological revolution that can finally make immunomagnetic purification a major player in downstream processes.

References
(1) I. Safarik et al, AIP Conf. Proc. 1311, 146 (2010).
(2) J. Faraudo & J. Camacho (2010). Colloid Polym. Sci., 288:207.

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 7. Magnetic bead-enabled one-step lysis and recombinant protein purification

A need for rapid, reproducible, small-scale purification

For many recombinant protein applications, such as expression clone screening and for optimizing expression conditions, there is a crucial need for a rapid, reproducible, small-scale purification process. Traditionally, protein purification from E. coli consists of four distinct phases: harvest, bacterial cell lysis, lysate clarification and protein purification. Bacterial lysis typically requires several time-consuming, potentially harsh, hands-on steps, such as freeze/thaw cycles in combination with sonication or the use of other mechanical disruption techniques such as French Press. Because these harsh lysis techniques may negatively impact protein quality and contribute greatly to sample-to-sample variability, detergent-based lysis buffers and enzymes are routinely used to avoid mechanical extraction, preserving protein activity and integrity.

Regardless of the lysis method used, centrifugation is traditionally required to pellet cell debris and permit recovery of the clarified lysate. The final step, purification, is frequently performed using affinity media specific for expressed epitope tags. Agarose-based media have typically been used, either as slurry in microcentrifuge tubes or packed into gravity-driven or spin columns. While easier to manipulate, columns are greatly affected by lysate consistency and carryover of cell debris, which can lead to detrimental clogging of the column frits. Although spin columns are a convenient solution when processing multiple samples, proteins can easily denature when columns are spun to dryness.

The answer: combining lysis and purification using magnetic beads

A one-step protocol, involving magnetic affinity capture beads, has been developed that condenses the traditional recombinant protein purification workflow by combining enzymatic lysis and purification steps. This results in significantly less hands-on time and a greater than two-hour time savings over the traditional workflow.

Compared to agarose beads, magnetic beads can reduce processing time and increase sample throughput, especially if adapted for automated particle processors.

Certain optimized magnetic beads for capturing histidine-tagged proteins have been successfully used to accelerate both manual and automated purification processes1,2. These beads are ideal for expression clone screening and small-scale purification, providing purified recombinant proteins at high purity. Magnetic beads are generally used in batch mode and are captured by a magnet for simple exchange of sample, wash or elution buffers. This magnetic bead capture allows for unwanted material to be easily washed away.

Protocol for rapid, one-step small-scale purification

The one-step lysis and purification protocol has been validated specifically for PureProteome™ Nickel Magnetic Beads and BugBuster® Master Mix, a lysis reagent that combines detergent-based lysis with a nuclease and recombinant lysozyme for gentle extraction of soluble protein from bacterial cells in as little as 30 minutes. The protocol has not yet been validated for other affinity beads or lysis reagents.

For recombinant protein purification, buffer and magnetic beads are added directly to the E. coli pellet.

After complete re-suspension of the pellet, the mixture is incubated with gentle end-over-end rotation. During this incubation, cells are lysed, nucleic acid is digested and the liberated recombinant protein is immediately captured by the magnetic beads. After removal of the unbound fraction, beads are washed with buffer containing 10 mM imidazole to eliminate loosely bound contaminants, residual lysate and cell debris. The captured proteins are eluted from the beads with high specificity using elution buffer containing 300 mM imidazole. Compared to the results of a traditional purification process involving mechanical extraction (Figure 1, left panel), the one-step protocol delivers increased reproducibility with equal or greater yield (Figure 1, center and right panels).

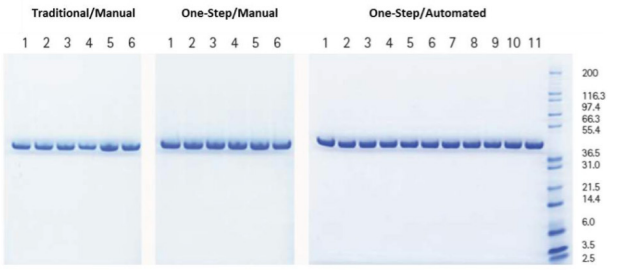


Figure 1. SDS-PAGE analysis of His-tagged GAPDH protein purified using traditional and one-step recombinant protein purification protocols using PureProteomeTM Nickel Magnetic Beads and BugBuster® Master Mix. The one-step protocol was performed manually as well as on an automated magnetic bead handler.

One-step lysis and purification is an easy, flexible technique, whether adapted to a manual or automated processing workflow, requires significantly less hands-on time and a greater than two-hour time savings over the traditional workflow, while reducing overall sample–to-sample variability. An automated approach incorporating the one-step lysis and purification protocol further reduces hands-on time and significantly increase sample throughput.

EMD Millipore Corporation. 2013. Automated IgG Purification Using PureProteome™ Magnetic Beads. Literature No. AN1255EN00.
EMD Millipore Corporation. 2013. Automated purification of proteins from non-clarified E.coli lysate using BugBuster® Master Mix and PureProteome™ Nickel Magnetic Beads. Literature No. AN9377EN00.

Author: Anja Dedeo, Senior Scientist at EMD Millipore

Anja Dedeo is Senior Scientist at EMD Millipore. She has over 20 years experience in research and development of novel filtration devices, protein sample prep solutions and protein detection. She has contributed to the development of ZipTip® SCX tips, Montage® antibody purification kits and the PureProteome™ magnetic bead portfolio. She is currently leading a team supporting technology, product and application development.

Chapter 8. Recombinant protein purification from insect-derived crude extracts using magnetic beads

The business value of potentially large production capacities coupled to lower capital expenditures (CapEx) requirements and manufacturing costs may reduce the gap between production volumes and patient needs for potentially life-saving drugs. This is the reason because pharmaceutical companies are continuously seeking for new technologies. An economically efficient alternative to bioreactor-based technologies is the use of living biofactories such as transgenic animals, plants or insects.

Numerous studies using insect larvae platforms have demonstrated the cost-efficiency and the scalability of production of many recombinant proteins, which include diagnostic reagents, vaccines and therapeutic molecules, among others.

One of the most used technologies based on insects, combines the use of Autographa californica multiple nucleopolyhedrovirus (AcMNPV) vectors with its natural host, the lepidoptera Trichoplusia ni (T. ni).

The company Alternative Gene Expression S.L. (ALGENEX) has optimized the industrial production in T. ni larvae by a proprietary technology denominated IBES(R) (Improved Baculovirus Expression System). IBES® represents one of the best production alternatives based on baculovirus vectors. Baculovirus-infected larvae produce high yields of recombinant protein (frequently more than one milligram per insect) at a reduced cost and working in nonsterile conditions. IBES® technology provides a final product with high biosafety standards as animal compounds are not involved in the production process and neither does cross-infection in mammals occur between pathogens infecting this lepidopter, reducing drastically the serious concern over animal by-product contamination of the final product. However, part of the costs associated to biologics production is related to the purification processes. Larvae extracts are more complex than those obtained from cell cultures and require of additional steps of clarification before conventional affinity purification processes.

Recently, our company has tested the use of magnetic beads in a single step purification process for a nanobody (single domain llama antibody). This nanobody is directed to a conserved epitope of protein VP6 from human Rotavirus A, which is able to recognize most of the viruses belonging to this virus group and can be used both for a universal Rotavirus A diagnostic test and for neutralization of the virus in the intestinal tract (therapeutic molecule). Complex extracts derived from baculovirus-infected T. ni insect larvae expressing the his-tagged nanobody, named 3B2, were mixed with magnetic beads (Dynabeads; Invitrogen), to capture the his-tagged protein.

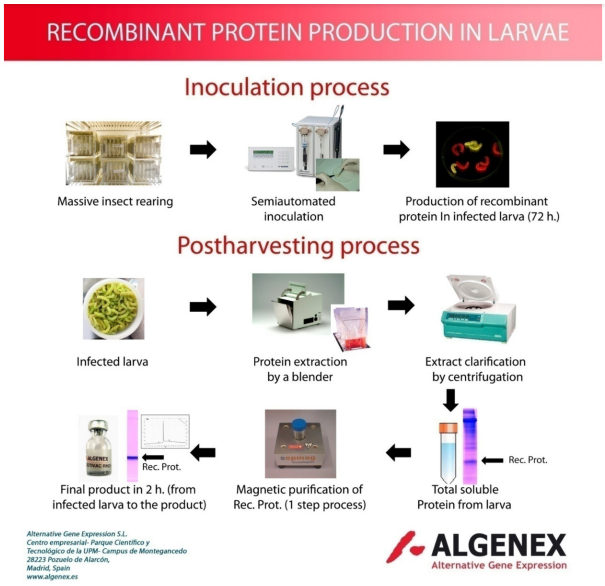


Figure 9. Schematic representation of the purification process of nanobody 3B2 produced in insect larvae (IBES® technology) by magnetic beads and a biomagnetic separator

Figure 9 shows a schematic representation of the purification process followed. We used a biomagnetic separation system provided by SEPMAG® on extract volumes of about 10 ml. Interestingly, in a single step purification process, we were able to reach up to 90% purification of nanobody 3B2, in a quantity sufficient to coat ELISA plates for virus capture purposes. This purification procedure was cost-efficient for the mg scale needed in a diagnostic test production, recovering most of the recombinant protein present in the extracts. In fact, purification yields were higher than those obtained by other conventional chromatographic purification technologies. Most importantly, the content of DNA, particles or lipids in the samples (crude extracts) did not affect the efficiency of magnetic bead-mediated purification.

Next, we will use the biomagnetic separation technology for large-scale purifications needed for in vivo protection experiments with the nanobody (scale of g of purified recombinant protein). The objective will be scaling up sample volumes in order to test the efficiency of bigger biomagnetic separator sizes for the nanobody purification. Additionally, we have tested this purification system with a number of different recombinant proteins obtained by the same methodology. In general, tagged proteins attached very efficiently to the magnetic beads, but high molecular weight proteins presented problems of detachment from the beads, once isolated by biomagnetic separators. We do not know if these problems were due to the protein themselves, the beads used, the complexity of the extracts or the buffers employed.

Future work will focus in improving the purification processes based on magnetic beads and to evaluate the cost-efficiency of this technology for large-scale purifications of biologics derived from insect larvae extracts. Probably, it should be optimized for each individual protein.

Author: José M. Escribano DVM, PhD

Researcher at the Biotechnology Department, INIA. Spain

Co-founder of Alternative Gene Expression S.L. (ALGENEX)
jmescribano@algenex.com
www.algenex.com

Conclusions

This advanced guide reviews the latest breakthroughs in biomagnetic protein purification. The author describes the advantages, benefits and limitations of the development of different aspects of the technology.

The main conclusion is that magnetic separation principles are not dependent on volume.

The same technology can be applied to milliliter tests and screening and to purification of liters of medium or cell lysate. One of the main advantages when considering this technique for large-scale application is the reduction of the handling volumes from the first step.

In the first eBook chapters, contributors discuss different options for magnetic beads. Magnetic agarose and sepharose, new hyper-porous polymer matrix magnetic beads and narrow size dispersion polymer beads are reviewed.

Magnetic agarose is used as a protein purification matrix, taking advantage of its very low non-specific binding, increasing the purity of the eluted protein fraction, and its ability to provide a large interaction surface between functional groups and proteins of interest, increasing the yields that can be obtained.

Magnetic sepharose is an option when the target molecule is present in low relative quantities and for yields which are traditionally undetectable by standard methods. These beads make it possible to capture such targets from a large starting sample volume, and to subsequently elute it into volumes suitable for SDS-PAGE or MS analysis.

Hyper-porous polymer matrix magnetic beads are a new alternative with an exceptional surface area for binding molecules, especially attractive for applications where volumetric productivity (low microsphere content or concentrated sample preparation) is important. They may lead to a significant cost reduction, given that fewer magnetic microspheres and less reagent, is required.

For classical polymeric beads, efforts are focused on obtaining satisfactory, reproducible results. Cost-effective beads with narrow particle size dispersion are reducing the differences in both the amount of ligand incorporated and the separation speed, improving the protein purification yield. This is a key benefit for screening processes (where small amounts of product are used), but also on an industrial scale where robust batch-to-batch and intra-batch reproducibility is essential.

The eBook reviews more than magnetic beads. It also looks at other key aspects of the technology. One critical issue is ligand binding to magnetic bead surfaces. Aqueous metal polymers have demonstrated their ability to form thin coatings and, due to their rapid kinetics, to bind to proteins to create multi-functional and /or multi-layered constructs in a relatively simple and reproducible manner. The technology enables construction of antibody and enzyme conjugates via nanoparticles while minimizing the tendency of nanoparticles to aggregate.

New systems for separating magnetic beads from the supernatant are also reviewed. The use of homogenous biomagnetic separation systems enable fast separation/full recovery of the beads, taking full advantage of new developments in magnetic beads and capture/elution ligands. The results can easily be transferred to larger volumes using the same biomagnetic separation systems currently used for manufacturing magnetic bead-IVD kits at volumes up to tens of liters.

In the final two chapters of the eBook, contributors give examples of how these technologies are already being applied.

A protocol for one-step lysis and purification is described. This purification procedure has proven cost-efficient for the mg scale needed in a diagnostic test production, recovering most of the recombinant protein present in the extracts. In fact, purification yields were higher than those obtained using other conventional chromatographic purification technologies. Most importantly, the content of DNA, particles or lipids in the samples (crude extracts) did not affect the efficiency of magnetic bead-mediated purification.

In a second example, the use of biomagnetic separation technology for large-scale purifications (scale of g of purified recombinant protein) is described. The objective was to scale up sample volumes in order to test the efficiency of larger biomagnetic separator sizes for the nanobody purification needed for in-vivo protection experiments. The purification system was tested with a number of different recombinant proteins: tagged proteins attached very efficiently to the magnetic beads, but high molecular weight proteins presented problems detaching from the beads.

We hope that the described results will encourage readers to apply new biomagnetic protein purification to your own processes. If you need further details or additional information, please feel free to contact the editor martinez@sepmag.eu.

Keep updated on biomagnetic protein purification

SEPMAG's blog is a great way of learning the latest news about biomagnetic separation processes, such as protein purification. You can also find interesting discussions on the subject in the LinkedIn group Magnetic Particles Interest Group.

Join us there to contribute with your expertise to the Magnetic Particles community or to find questions, research, news and notes involving magnetic micro or nano particles and intrinsically magnetic cells.

Lluis M. Martinez, PhD. (CSO, SEPMAG®)
Editor

www.sepmag.eu

sepmag®

Follow us on:

SEPMAG SYSTEMS
contact@sepmag.eu
+34 935 820 161

Parc Tecnològic del Vallès
E-08290 Cerdanyola del Valles. Barcelona