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Magnetic separations in biotechnology

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Magnetic separation techniques provide probably the most rapid and convenient method of separating certain particles from dilute suspensions, especially those that might block columns or filters. This and other applications of magnetism, including cell sorting and product recovery are discussed.

The purification techniques most widely employed in conventional chemistry – distillation and crystallization – are seldom applicable to biological materials. Separations of biological molecules often involve complex mixtures of delicate, charged and chemically similar compounds present in dilute aqueous solutions or suspensions. Biological purifications requiring separations of cells or cell fragments are rendered even more difficult by the fragility and ease of aggregation of these particles. This review outlines the characteristics of a relatively seldom-used class of separations based on magnetic interactions[1,2] and illustrates the potential of these types of separations in biological separations.

The important strength of magnetic separations is that they provide what is probably the most rapid and convenient method of separating appropriate *particles* from dilute suspensions, especially those containing undesired particles which might clog columns or foul filters. The weaknesses of magnetic separations are that they can only be performed on particles (that is, they are not ordinarily directly applicable to individual molecules) and that the components of the system to be separated must have different magnetic susceptibilities. Magnetic separations have been employed to sort cells, to recover from solution antibodies or enzymes attached to magnetic supports, to purify proteins using affinity techniques, and to remove unwanted particles from suspension.

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Magnetic separations fall into two general types: those in which the material to be separated is intrinsically magnetic, and those in which one or more components of a mixture have been rendered magnetic by the attachment of a magnetically responsive entity. Current large-scale industrial magnetic separations are based on the intrinsic magnetic properties of materials. For example, low grade iron ores can be separated magnetically into hematite and waste; iron sulfide is paramagnetic and can be extracted from pulverized coal[1,2]. In biological systems, magnetic separations generally involve conferring magnetism upon a non-magnetic (diamagnetic) molecule by attaching or adsorbing it to a magnetically responsive particle. Red blood cells[3,4] (which contain high concentrations of paramagnetic hemoglobin) and magnetic bacteria[5] (which contain small magnetite particles) provide examples of intrinsically magnetically responsive biological particles.

The magnetic properties of materials

The magnetic properties of matter can be classified in three categories depending upon response to an external magnetic field: diamagnetism, paramagnetism and ferromagnetism[2,6]. Very small particles (<100Å) of normally ferromagnetic materials may be unable to support magnetic domains. Such materials are termed superparamagnetic because they exhibit high magnetic susceptibilities and saturation magnetization but have lost the property of magnetic hysteresis[6]. Superparamagnetic materials offer unique properties which should be widely useful in separations in biotechnology: because they are small and only weakly magnetic in the absence of an external magnetic field, they can be easily dispersed as slowly settling suspensions having very high particulate surface areas. On application of an external field they become magnetic, and interparticle forces cause them to agglomerate readily. In this agglomerated state they can be readily separated magnetically.

In general, forces on particles which can be generated magnetically are relatively weak. A paramagnetic particle in a strong magnetic field gradient

Diamagnetism – reflects the magnetic field induced in all matter by an externally applied magnetic field, and is considered classically in terms of changes in the circulation of paired electrons caused by this field. The induced field of diamagnetic materials opposes the applied magnetic field. Diamagnetic substances (water, sugars, lipids, most proteins) are weakly repelled from regions of high magnetic fields. Diamagnetic materials are commonly regarded as magnetically unresponsive, and paramagnetic and ferromagnetic effects, if present, ordinarily overwhelm diamagnetic effects.

Paramagnetism – results from the independent behavior of unpaired electron spins in an external magnetic field. The size of the magnetic field induced in a paramagnetic substance by an external magnetic field is proportional to the number of unpaired electrons and to certain details of the electronic structure of the sample, to the strength of the applied field, and to the temperature. The induced field *reinforces* the applied field. Typical paramagnetic materials include the organic free radicals, O_2, and transition metal complexes (including many proteins containing iron, copper, vanadium, nickel, and other metals). Paramagnetic particles are attracted to the region of highest magnetic field (typically, close to the pole of the external magnet); the force on the particle is proportional both to the strength of the magnetic field and to that of the field gradient (see Eqn 1). Usually individual *molecules* cannot be manipulated using magnetic forces because thermal effects in solution overwhelm the weak magnetic effects. Paramagnetic *particles* are magnetically responsive at room temperature, and show a range of macroscopic effects in external magnetic fields which can be used in separation schemes.

Ferromagnetism – arises from the *cooperative* interaction and alignment of electronic spins. It is observed only in solids (metals and metal oxides) in which the structure of the solid permits strong interaction between electrons on different atoms. Ferromagnetic materials have three distinguishing properties: First, they have very high magnetic susceptibilities compared to paramagnetic materials; that is, they become magnetic in weak magnetic fields. (For these purposes we consider weak magnetic fields to be several hundred to several thousand gauss). Second, their response to large magnetic fields is finite; that is, in sufficiently strong applied magnetic fields their induced magnetism reaches a maximum. This magnetic saturation results from the complete alignment of electronic magnetic moments. Third, they show magnetic hysteresis; that is, after the removal of an applied magnetic field, their spins remain aligned and they exhibit residual magnetism.

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experiences a force comparable to the force of gravity (itself a very weak force). A ferromagnetic particle can experience a force 10–100 times larger than the 'force of gravity. Thus, magnetic separations of particles can be considered to be based on forces corresponding to those of gravity or moderate centrifugation.

Principles of magnetic separations

A magnetically isotropic material in a perfectly uniform magnetic field experiences no net force and a magnetic field gradient is necessary to effect its movement. The relationship between properties of the particle, the magnetic field, and the force on the particle is described by Eqn 1[1,2].

$$F_x = V\chi_V \mathbf{H} \frac{\partial \mathbf{H}}{\partial x} \qquad (1)$$

Here F_x is the force on the particle in direction x, V is the volume of the particle (assumed to be spherical), χ_V is its magnetic susceptibility per unit volume, $\mathbf{H}$ is the strength of the magnetic field and ($\partial \mathbf{H}/\partial x$) is the magnetic field gradient. The equation indicates qualitatively that the force on a particle is proportional to χ_V, $\mathbf{H}$, V and $\partial \mathbf{H}/\partial x$. It also contains several simplifications – no allowance is made for particle shape or for interactions between magnetic particles. The major implication of Eqn 1 for magnetic separations is that small magnetic particles – which have large surface areas and slow settling rates and are thus particularly suitable substrates for many types of separation schemes – will require large magnetic fields and field gradients to influence their movement. High magnetic field gradients are required for separation in instances in which the material to be collected has low magnetic susceptibility (for example, red blood cells[3,4]) and/or is comprised of small particles, and when extremely high rates of separation are required.

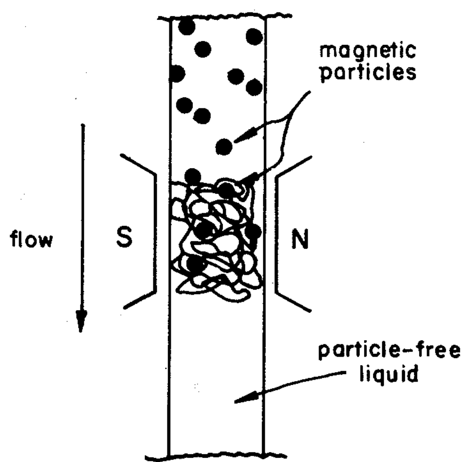

Fig. 1. Schematic diagram of a magnetic filter. Ferromagnetic or paramagnetic particles suspended in a diamagnetic liquid are passed through a tube containing ferromagnetic steel filaments between the poles of a magnet. The particles are retained in the high magnetic field gradients near the surface of the filaments and the particle-free liquid passes through.

The practical limit to the strength of available magnetic fields (10 000–100 000 gauss) is such that it is impossible to generate high magnetic field gradients over large volumes (> several cm), and thus it is difficult to collect magnetic materials from large volumes of solution using only externally applied fields. One approach to this problem is to pass the suspension of magnetically responsive particles through a device called a high-gradient magnetic filter. These filters consist of a ferromagnetic matrix (typically magnetic stainless steel wool) packed into a tube (Fig. 1). The small ferromagnetic fibers of the steel wool distort the externally applied magnetic field strongly in their vicinity, and generate strong local magnetic field gradients (Fig. 2). These gradients attract and hold the magnetic particles in the suspension while the external field is on. The collected particles can be released by removing the external field; any residual magnetism in the steel wool retains particles only weakly, and can be overcome by vibrating the matrix. Because the ferromagnetic matrix can be dispersed over a relatively large volume, the high-gradient filter provides a practical way of generating a relatively large volume of space subject to high magnetic field gradients. Indeed, most commercial applications of magnetic separations to date have been in large-scale processes. High gradient magnetic separators available commercially – those removing colored impurities from kaolin clays or concentrating iron ores – have capacities up to 300 tons/h. Ferromagnetic particles can be removed from water at the rate of 300 $m^3/h/m^2$ of filler face[1,2]. While separations on this scale require the high magnetic fields generated by electromagnets (~10 000 gauss), most laboratory scale and some production scale biotechnological applications should be satisfied by the lower magnetic fields provided by permanent magnets (100–1 000 gauss).

Applications to biotechnology

Magnetic separations provide unique advantages that have been exploited to solve biotechnological problems. Table 1 lists some advantages and disadvantages of magnetic separations.

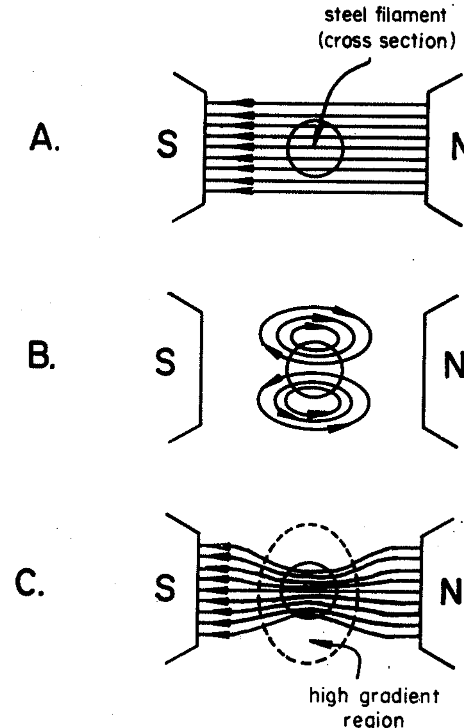

Fig. 2. The origin of high magnetic field gradients around a steel filament. The magnetic field near a steel filament (C), is the sum of the external magnetic field due to the magnet (A), and the magnetic field induced by the external field in the steel filament (B). High field gradients can be generated either by thin filaments or by small features on larger filaments.

Cell fractionation

Existing techniques for cell fractionation – especially fractionation of delicate mammalian cells – have limitations. Random selection and culturing of clones is inefficient and time-consuming. Techniques based on antibiotic resistance or specific nutritional requirements have limited applicability. Immobilization of antibodies to cell surface antigens on appropriate glass or plastic supports and permitting cells to bind to these functionalized sur-

Table 1. Characteristics of magnetic separations

Advantages
Allows the selective separation of solids based on magnetic susceptibilities
High filtration rates; low pressure drop across filter
Applicable to small (< 1 μm) and soft particles; also to liquid droplets
Simpler manipulations make automation feasible
Easy to maintain sterile
Disadvantages
Limited to magnetically responsive systems
Useful only for particles; not applicable to molecules
Magnetic structure can be expensive
Low filter capacity
Aggregation of particles due to residual magnetization can reduce effective surface area

faces – the technique which forms the basis for 'panning' or cell affinity chromatography[7] – is hampered by the non-specific sticking of cells to the surfaces and by the fragility of the bond between the cell and the surface. (Mild shear forces may easily dislodge the bound cell). Fluorescent-activated cell sorting (FACS) is expensive and limited to samples containing only small quantities of suspended debris[8].

The fractionation of cells by labelling their surfaces with magnetic antibodies or antigens is a promising application of magnetic separations. Rembaum and co-workers have shown that human erythrocytes can be labelled with antibodies coupled to magnetic microspheres which are substantially smaller than the cell itself[9]. B and T lymphocytes have been separated from mixed populations using such microspheres[10] and using large polyacrylamide-agarose beads[11]. Choleragen, a protein which binds specifically to neuroblastoma cells possessing the ganglioside GM-1, has been coupled to magnetic particles and used to isolate cells having GM-1 on their surface[12].

Concanavalin A immobilized on magnetic particles has been used to isolate microorganisms[13]. Healthy and leukemic bone marrow cells have been separated based on binding to monoclonal antibodies immobilized on magnetic particles[14]. Cells have also been magnetically sorted according to the presence of paramagnetic molecules intrinsic to the cell. Red cells containing the paramagnetic forms of hemoglobin – the deoxy and met (ferric) forms – can be manipulated using high magnetic field gradients[3,4]. An interesting application involves using sheep blood cells containing methemoglobin to surround the spleenocytes from a mouse immunized with the sheep red cells. Rosettes form around the spleenocytes which can be magnetically separated[15]. Cells can also be rendered paramagnetic by allowing them to adsorb a paramagnetic ion such as erbium[16] or rendered ferromagnetic by inducing them to phagocytize iron particles[17].

Enzyme immobilization

Enzymes attached to magnetic solids have certain advantages over those immobilized to non-magnetic supports. First, they can be readily separated from large volumes of media which may contain particulate matter – especially soft or sticky matter which would clog conventional filters – by use of a high gradient magnetic filter. Polyacrylamide containing covalently coupled invertase and physically occluded particles of magnetite has been used to evaluate the advantages of processes based on magnetic separations[18,19]. Second, the magnetic property allows the particles to be manipulated in a reactor in ways not possible with non-magnetic supports. For example, papain has been attached to magnetic iron oxide and retained magnetically in a fluidized bed reactor[20]. Magnetic particles can be stirred magnetically to minimize physical damage due to mechanical stirring. Magnetic mixing has been demonstrated for immobilized urease in a batch enzyme reactor[21] and also for immobilized antibodies in immunoassays[22].

A wide variety of enzymes have been immobilized on magnetic supports. These include α-chymotrypsin, β-galactosidase[23], trypsin, invertase[24], adenylate kinase, acetate kinase and horseradish peroxidase[18]. Given the ability to magnetize support materials as polyacrylamide, cellulose or silane-functionalized glass, it is probable that any enzyme which can be immobilized can be coupled to a magnetic support. In addition to enzymes, whole yeast cells have been immobilized in calcium alginate gels containing magnetic iron oxides and used for conversion of glucose to ethanol[25].

Magnetic affinity chromatography

Mosbach has shown that Sepharose coupled to ligands such as 5′-AMP or proteins such as human serum albumin can be magnetized by allowing the Sepharose to entrap the iron oxide particles from a water-based ferrofluid[26]. Magnetic 2′-5′-ADP Sepharose prepared in this fashion has been used to achieve an 11 000-fold purification of glucose-6-phosphate dehydrogenase from a red blood cell homogenate in a single step[27]. Magnetic affinity chromatography has also been tested with trypsin[28], antibodies to human serum albumin[27] β-galactosidase, asparaginase[29] and alcohol dehydrogenase[30]. The efficiency of magnetic and non-magnetic solid phases have been considered in some detail in one case, with the conclusion that kinetics of adsorption were better using small, soft, magnetic gel particles than using the same gel supported on a macroscopic screen[31].

Immunoassay

The separation of ligands bound by antibodies from those free in solution is an essential step in many immunoassay methods. Centrifugal separations require a significant amount of time and attention, and while antibody-coated tubes eliminate the need for centrifugation they suffer from a limited surface area and from slow kinetics[32]. Antibodies attached to very small ferromagnetic or superparamagnetic magnetite can be efficiently removed in less than 5 min from 1- to 2-ml volumes of test sample using small permanent magnets. This type of separation eliminates the need for centrifugation while preserving the high surface areas and fast kinetics obtained with antibodies immobilized to non-magnetic particles. Magnetic immunoassays have been recently reviewed[33]. One radioimmunoassay manufacturer is converting a number of controlled pore glass assays to a magnetic solid phase and magnetic separation will be a widely used separation method in immunoanalysis[34].

Extraction of impurities by adsorption of materials onto magnetic particles

Particles of magnetic iron oxide have a useful non-specific capacity to adsorb biologically-derived molecules and particles on their surface. The ability of microorganisms to adsorb on iron oxide has been used to remove coliform bacteria and certain viruses from water[1]. Mixing particles of γFe_2O_3 and activated charcoal in a crosslinked polymer matrix gives a material that can be used to remove bitter-tasting isohumulenes from extracts of brewers' yeast. The charcoal is regenerated by washing with trichlorethylene[35]. The isolation of the phytoplankton *Chattonella sp.* (red tide) from sea water by adsorption on magnetic particles and by high gradient magnetic filtration has been demonstrated[36].

Magnetic supports

Some of the criteria for evaluating the performance of magnetic particles are similar to those for conventional supports – the number of reactive groups on the surface to which proteins can be covalently attached, the surface area, the ability of the particles to permit adsorption with retention of biological activity, the absence of non-specific adsorption effects and the uniformity of particle size. Other properties required for successful magnetic supports are unique. Very small (~50Å) particles are often desirable to ensure the advantages of superparamagnetism: efficient aggregation and collection in the presence of a magnetic field, but uniform stable suspension in its absence. Most work with magnetically responsive particles has been carried out using ferromagnetic materials,

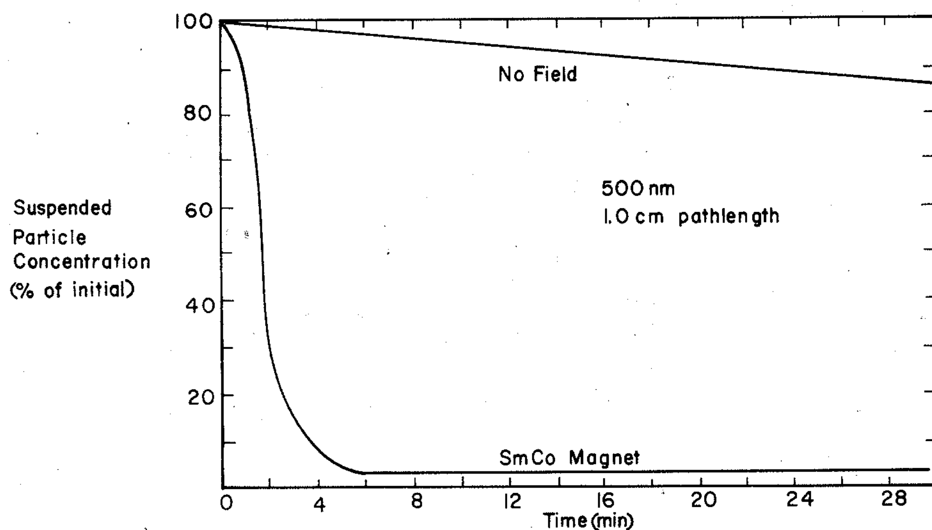

Fig. 3. Stability of aqueous suspensions of the superparamagnetic magnetite particles (Biomag®). The suspension stability is measured by light scattering at 500 nm. In the absence of an applied magnetic field suspensions of particles are stable for several hours (top trace), while aggregation and settling is complete within 5 min upon application of the field from a small permanent magnetic (bottom trace).

and aggregation of the particles has limited their application (Table 1) in separations. In many applications, it is helpful if the particles show minimal settling under gravity to avoid the need for constant stirring or magnetic mixing; superparamagnetic particles suspend well.

The most commonly used procedure for the synthesis of magnetic particles for use in biological systems has been to coat (or embed) a magnetic particle with(in) a polymer matrix containing functional groups permitting the covalent coupling of proteins[37]. Representative polymers and coupling methods for iron oxide particles are: poly-1,3-diaminobenzene activated by diazotization[22] (Enzacryl FEO-M®, Aldrich Chemical Co., Milwaukee, WI), polyacrylamide-agarose activated by cyanogen bromide or glutaraldehyde[10] (Magnogel®, LKB Instruments, Inc., Gaithersburg, MD), polyacrylamide gels containing *N*-hydroxysuccinimide active esters[18,19,31] and magnetite surface-functionalized with reactive amino, carboxyl or aminopropyl groups[34] (Biomag™ BioClinical Group, Inc., Cambridge, MA). Direct adsorption of proteins to iron oxides is also possible, but is limited by the ease of spontaneous desorption, especially under conditions of high ionic strength, or of mild acidity or basicity. Adsorbed materials can be fixed to particles using cross-linking agents such as glutaraldehyde[20,37].

Supports prepared by magnetizing existing non-magnetic supports or by grinding and sieving polymers with entrapped iron oxide are comprised of particles between 20 and 200 μm (Refs 16, 23, 29). These particles settle rapidly, with half times ordinarily less than 5 min. By contrast, a superparamagnetic support (BioMag™, iron oxide surface-functionalized with 0.25 mequiv/g of primary amino groups) comprises particles with sizes between 0.5 and 1.5 μm, and settles slowly in the presence of a gravitational field (half-time ~3 h). These particles can be efficiently retrieved from solution by applying a magnetic field (Fig. 3).

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