1. INTRODUCTION

Nanotechnology comprises technological developments at the nanoscale, typically having dimension ranging from 1 to 100 nm. The increasing interest in nano-sized material results from their potential applications in various fields such as material and biomedical sciences, electronics, optics, magnetism, energy storage and electrochemistry. During recent years, several types of nano-sized particles have been developed, which includes superparamagnetic iron oxide nanoparticles (SPIONs), gold nanoparticles, silicon nanoparticles, fluorescent quantum dots (ZnS: Mn^{2+}), and others (Lu *et al.*, 2006; Bauer *et al.*, 2004; Chan *et al.*, 2002; Kim *et al.*, 2002).

In fact, presence of nanoscale particles in nature is well documented. For example, *Lowenstam* (1962) was the first to discover naturally occurring biogenic magnetite (Fe_3O_4), as a capping material in the radula teeth of chitons (Lowenstam, 1962). Later *Blakemore* (1975) discovered magnetotactic bacteria (Fig. 1), which now represent the most extensively studied biomagnetic system. Magnetotactic bacteria are perhaps the first living organisms to orient themselves with the earth's magnetic field (Blakemore and Frankel, 1981). These bacteria are known to contain aligned chains of magnetite or greigite (Fe_3S_4) particles of various shapes, which are covered with an intracellular phospholipid membrane vacuole, forming structures called 'magnetosomes' (Fig. 1) (Schuler and Frankel, 1999).

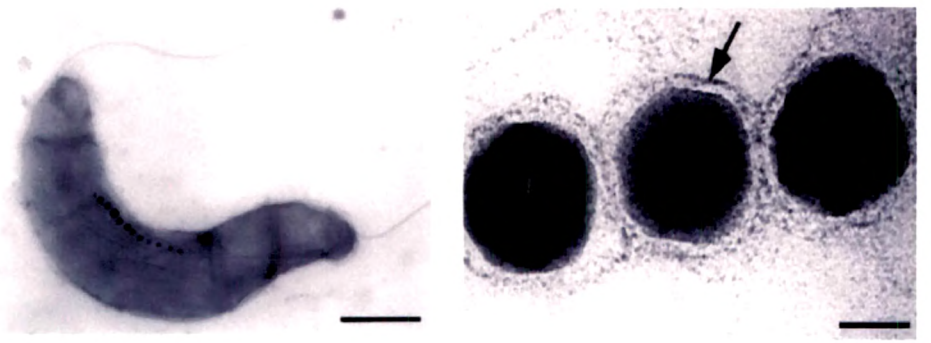

Figure 1.

(Left image) Electron micrograph of a *Magnetospirillum gryphiswaldense,* harboring up to 60 intracellular magnetite particles in magnetosomes which are arranged in a chain. The bar is equivalent to 0.5 μm. (Right image) Magnetosome particles isolated from *M. gryphiswaldense*. The magnetite crystals are typically 42 nm in diameter and are surrounded by the magnetosome membrane (arrow). The bar is equivalent to 25 nm. (Source: Schuler and Frankel, 1999)

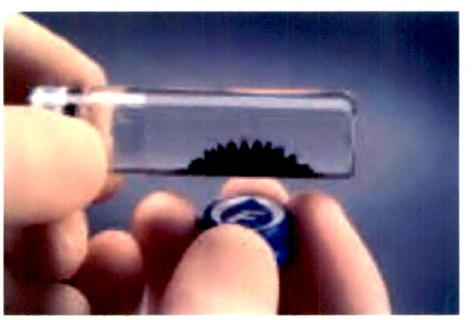

Figure 2.

Magnetic fluid being influenced by the presence of external magnet

The presence of these magnetic nanoparticles has also been reported in a large variety of organisms ranging from bacteria to humans (Frankel and Bazylinski, 1994). Existence of this nanoparticle in biological system indicates their biocompatibility and thereby provides a possibility to exploit them for various bioapplications. Thus, the current study stems from the finding of magnetic iron oxide based nanoparticles namely magnetite (Fe_3O_4). In addition to their natural occurrence, these magnetic nanoparticles can be synthesized chemically in the laboratory in the form of magnetic fluids (or ferrofluids).

Magnetic fluids as they are often called mainly consist of nano-sized iron oxide particles (Fe_3O_4 or γ-Fe_2O_3), which are often suspended in an organic carrier liquid. Although often referred to as magnetic, many of the particles currently used are **superparamagnetic**; i.e. these particles can be easily magnetized with an external magnetic field (Fig. 2) and demagnetized immediately once the magnet is removed (Ramchand *et al.*, 2001).

Magnetic fluids were first developed in 1965 at NASA (National Aeronautics and Space Administration), for the elaboration of a propulsion system in microgravity. The applications of the magnetic fluids are generally in hi-tech fields, which include aerospace machineries, chemical industry, sensors, and computer technology. Magnetic fluids are advanced materials that can solve complex engineering problems — **for example sealing rotary shafts and computer disk drives, to avoid deposition of harmful dust particles and**

other impurities. One of the most important military applications developed using magnetic fluid in the late 1980s **was for radar-repulsion purposes.** Painted with a ferrofluid or non-magnetic paints, airplanes are able to absorb radar waves and remain invisible to radar when they pass overhead (www.wisegeek.com/what-is-ferrofluid.htm). Additionally, magnetic fluids are suited for applications in extreme temperatures, ranging from **-55°C to 200°C** (-67°F to 392°F), which makes them ideal for use in any location on earth and particularly in space conditions.

The application of magnetic fluids in the field of biology was of limited use up to the 1970s. Typically in late 70s, use of magnetic fluids for preparation of magnetic polymers as supports for immobilizing molecules (proteins, enzymes or drugs) created general interest among the scientists. Such immobilization procedures for proteins, enzymes or drugs had a major impact in areas of medicine and biology. The immobilized molecules were used as **magnetic-affinity matrix**, or as component of **immunoassay** systems. Furthermore, the property of magnetic fluid to possess **high specific absorption rate** (SAR); led to the use of magnetic fluid as hyperthermia causing agents to treat cancerous tissue. The first use of magnetic fluids for causing hyperthermia to treat mammary carcinoma in rats was reported in 1979 (Gordon *et al.,* 1979). Subsequently, during 1980s several studies were done, where biocompatible ferromagnetic particles were investigated as **potential drug carriers** and as **hyperthermia causing agent** to treat cancer cells. The concept of

magnetoliposomes came up at the same time. Magnetoliposomes are magnetic derivatives of liposomes and can be prepared by entrapment of magnetic fluids within the core of liposomes. Several groups then investigated the uses of **magnetoliposomes for site-specific drug targeting, cell sorting and as magnetic resonance contrast enhancing agents** (Reviewed by Ito *et al.*, 2005; Babincova *et al.*, 2002; Margolis *et al.*, 1983; Bulte *et al.*, 1999).

The **Human Genome Project** (HGP) begun in 1990 by the coordinated efforts of the U.S. Department of Energy and the National Institutes of Health. This required an unprecedented increase in DNA purification and sequencing capabilities. In order to reach the goal, a need for high-throughput genome isolation procedure for the HGP was realized. In view of the above, an automated sequencing system was developed based on solid phase reversible immobilization (SPRI) chemistry. **This procedure utilizes carboxylate-coated paramagnetic beads that exhibit a reversible affinity to precipitated DNA** (Hawkins *et al.*, 1994; 1997). This greatly facilitated automation by eliminating centrifugation/filtration steps associated with traditional template purification protocols. **SPRI purification technology was used to sequence over a third of the human genome.**

During recent years, magnetic separation techniques using **magnetizable solid-phase supports** (MSPS) have been increasingly applied to a number of biotechnological applications (Safarik and Safarikova, 1997; Safarikova and

Safarik, 2001). Here, magnetic separation is used sometimes in combination with traditional separation or identification methods, to purify cells, cell organelles and biologically active molecules especially proteins and nucleic acids (DNA, or mRNA) directly from cell lysate. With its growing popularity, large collections of biocompatible magnetic particles/beads are commercially available from different companies for a wide variety of applications. Some of these products include magnetic bead immobilized antibody for the isolation of antigen expressing specific cell type (e.g. Miltenyi Biotec, Germany) or silica coated magnetic bead for rapid isolation of genomic DNA (Tecan Inc., Switzerland) from blood.

As mentioned earlier, magnetic nanoparticles suited for biological applications usually exist in nature. For example, bacterial magnetite nanoparticles obtained from magnetotactic bacteria have been used for variety of bioapplications. Due to the presence of lipid layer the particles are biocompatible (Fig. 1), their suspensions are very stable and the particles can be easily modified (Matsunaga and Sakaguchi, 2000; Matsunaga and Takeyama, 1998).

1.1 TYPES OF MAGNETIC PARTICLES

The commercially available formats of magnetic particles are broadly classified into three classes:

a. **Unmodified or naked particles,**

b. Chemically derivatized particles with **general specificity ligands**

c. Chemically derivatized particles with **specific recognition groups.**

General specificity particles are largely produced as substrates to attach a variety of affinity ligands; examples of such ligands are oligo-(dT) (used for isolation of poly(A)$^+$mRNA), streptavidin (used for separation of biotinylated molecules), or protein A (used to purify antibodies). On the other hand the specific recognition groups include particles coated with polyclonal or monoclonal antibodies; for example anti *CD34+* tagged magnetic particles have been used for selective separation of stem cells (Kato and Radbruch, 1993).

The use of magnetic technique offers an advantage in terms of subjecting the analyte to very little mechanical stress compared to other methods. Secondly, these methods are non-laborious, cheap and often highly scalable. Moreover, techniques employing magnetism are more amenable to automation and miniaturization. The following section discusses the selected advancement made in the field of molecular biology, cell biology and medicine using magnetically driven separation techniques.

1.2 MOLECULAR BIOLOGY APPLICATIONS

1.2.1 DNA isolation

Isolation of DNA is a prerequisite step for many molecular biology techniques. The separation of DNA from the complex mixtures in which they are often

found is necessary before being subjected to other uses like sequencing, amplification, hybridization, detection etc. The presence of large amounts of cellular or other contaminating material like proteins and RNA, in such complex mixtures often impedes many of the reactions and techniques used in molecular biology (Ahern, 1995). The conventional protocol for extracting DNA involves cell lysis followed by removal of contaminating cellular components such as proteins, lipids and carbohydrates; and finally isolating DNA using a series of precipitation and centrifugation steps, which are difficult to automate. Improvement in methods for isolating DNA has been made and more recently, methods that rely on the use of solid phase have been proposed. Adsorbents that provide fast, efficient DNA purification are important for making this procedure amenable to automation. The discovery in late 80's that silica can be used as adsorbent for DNA isolation (Vogelstein and Gillespie, 1979), became the basis for most of the DNA isolation kits currently available. Some of these kits involve isolation of DNA using silica coated magnetic particles (Tecan Inc., Switzerland; Dynal, Oslo, Norway). A high throughput genome isolation protocol has been developed, which is based on **SPRI** chemistry (Hawkins *et al.*, 1994; 1997). The SPRI protocol is based on DNA binding to the surface of carboxyl coated paramagnetic particles under the condition of high salt and PEG. The procedure has been developed and optimized for single stranded DNA isolation, such as M13 phage and double stranded plasmid DNA. The SPRI protocol has allowed the development of an automated procedure in a microplate format with a throughput of about

200,000 DNA preparations per day, thus becoming a fastest microplate based DNA purification system that was used for human genome sequencing. Furthermore, a novel method was reported for the preparation of superparamagnetic carboxyl-modified nanobeads and this were checked for isolation of genomic DNA from human blood (Xie *et al.*, 2004).

Recently, magnetic silica microspheres were used as adsorbent for isolation of genomic DNA from *Saccharomyces cerevisiae* and maize kernels, respectively (Zhang *et al.*, 2006). Using silica-coated magnetic bead purification technology, an automated workstation was developed and validated for forensic DNA analyses (Nagy *et al.*, 2005). The workstation has been used for analysis of more than 20,000 routine lab samples. Also, Chiang and coworkers (2005) have reported the use of polyethylenimine (PEI) modified magnetic nanobeads for purification of supercoiled plasmid DNA from bacterial cells. The following table 1 list selected examples of magnetic affinity separations of nucleic acids.

Table 1. Selected examples of magnetic separation of DNA

Nucleic acid	Magnetic system used	Typical examples
DNA	Dynabeads DNA direct universal method	DNA
	Biotinylated cloned genomic DNA immobilized on Dynabeads Streptavidin	cDNA
	Dynabeads M-280 streptavidin with immobilized biotinylated oligonucleotide complementary to the lacZ region	M13 single-stranded DNA
	Magnetic particles with immobilized pyrimidine oligonucleotide	Double-stranded target DNA (triple helix formation)
	-COOH terminated magnetic beads (in presence of high-salt and polyethylene glycol)	Double-stranded DNA, PCR products, M13 single-stranded DNA
	Magnetic silica beads (Tecan Inc., Switzerland)	DNA

(Compiled from Rudi *et al*, 1997, Deggerdal and Larsen, 1997, Hawkins *et al*, 1994; Safarik and Safarikova, 2000)

1.2.2 Magnetic separation of poly(A) mRNA

A number of methods have been reported for isolation of total RNA from a variety of cells or tissues. Chirgwin *et al.* (1979) developed a method for efficient isolation of total RNA by homogenization in a 4M solution of guanidium thiocyanate containing 0.1M 2-mercaptoethanol. Homogenization is followed by extraction of RNA by ethanol or by ultra-centrifugation through cesium chloride. This method was further modified by Chomczynski and Sachi (1987) to devise a rapid single step isolation procedure for RNA. It involves extraction of RNA using a mixture of guanidium thiocyanate and phenol-chloroform. Many RNA isolation kits are available based on the above two protocols. All these methods isolate RNA on the basis of its biochemical properties. In contrast, biomagnetic separation of mRNA is based on specific complementary hybridization between polyA sequence of isolated mRNA and oligo $(dT)_{25}$ sequence covalently linked to the surface of paramagnetic particles. In this method oligo $(dT)_{25}$ coated magnetic beads are added to crude cell or tissue lysate. During incubation polyA mRNA from the lysate binds to oligo $(dT)_{25}$ coated magnetic beads. The beads/mRNA complex is then washed magnetically. The mRNA thus isolated is either eluted or directly applied for many downstream applications, which includes cDNA library construction, Substractive hybridization, Northern hybridization, RT-PCR and *in vitro* translation (Mrazek and Petrek, 1999).

More recently, an automated procedure for viral RNA extraction from plasma was reported based on magnetic bead technology using chemagic RNA isolation kit (Pichl *et al.*, 2005). Table 2 provides list of commercial magnetic beads designed to specifically isolate nucleic acids (such as DNA, plasmid, or RNA) based on magnetic separation technology.

Table 2. Selection of few commercially available magnetic and superparamagnetic particles used (or suitable) for the isolation and purification of nucleic acids

Manufacturer/supplier	Product name	Surface modification	Applications
Advanced Biotechnology, Epsom, UK	Magnacil	Silica	DNA and RNA isolation
PerSeptive Biosystems, Farmingham, MA, USA	Biomag	-COOH and $-NH_2$ terminated superparamagnetic beads	PCR clean-up before DNA sequencing
Bangs Labs, Fishers, IN, USA	Magnetic beads	-COOH terminated polystyrene beads	Purification of M13 single stranded sequencing templates
CPG, Lincoln Park, NJ, USA	MPG	Streptavidin	RNA capture system based on binding of biotinylated oligo(dT) to MPG streptavidin
Promega Corporation, Madison, WI, USA	MagneSil paramagnetic particles	Silica	Purification of nucleic acids including genomic DNA, plasmid, PCR cleanup
Chemagen AG, Baesweiler, Germany	Magnetic beads	Polyvinyl alcohol	DNA isolation

Ref Compiled from Safarik and Safarikova (2000), chemagen Inc Baeswailer, Germany - www chemagen de

1.2.3 Isolation of nucleic acid binding biomolecules

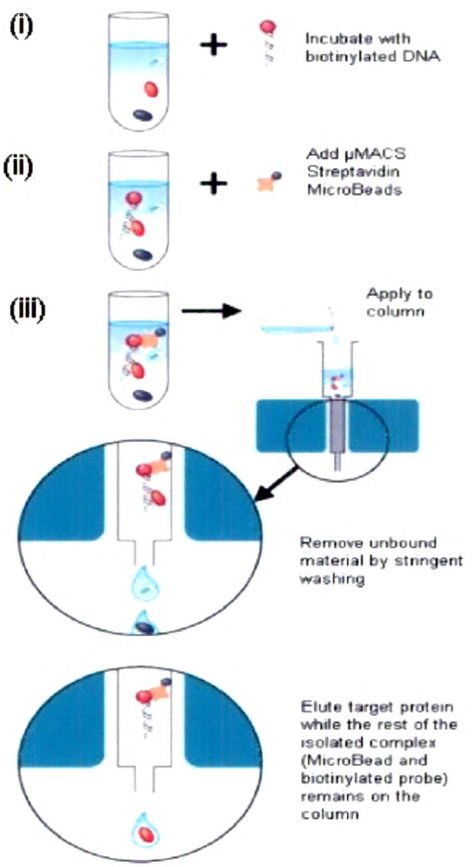

Figure 3.

Isolation of nucleic acid binding molecules. Biotinylated DNA is added to the cell lysate or a protein mixture that contains protein that interacts with DNA (i). Streptavidin magnetic beads are added that binds to the complex (biotin-DNA protein) based on its affinity for biotin (ii). The complex is separated magnetically from the mixture (iii). (Source: Miltenyi Biotec, GmbH, Germany).

The isolation of specific molecules based on its interaction with complementary binding partner is emerging as important technologies in many field of research. The isolation and characterization of specific transcripts or proteins can be employed to monitor the progression of disease. Several kits are available in the market that works on the principle of magnetic labeling and direct isolation of biotinylated molecules such as DNA, RNA or proteins onto streptavidin coated magnetic beads (μMACS streptavidin Microbeads from Miltenyi Biotec, Germany). These biotinylated molecules can then be used for indirect isolation of non-biotinylated target molecules that may interact with them (Albig, 2001). The procedure involves complex formation between the biotinylated probe (DNA, RNA or proteins) and the target molecule (i.e. interacting biomolecules DNA, RNA or protein). The probe-target complex is then separated from rest of the component by addition of streptavidin coated magnetic beads. The complex is magnetically isolated and washed to remove non-specifically bound molecules. The non-biotinylated target molecules can be either eluted off from the complex with high purity, whereas the magnetically labeled biotinylated probe remains bound to the column (Fig. 3). This technique has potential for rapid and efficient screening of transcriptional and translational regulatory proteins. For example, target DNA-conjugated magnetic beads have been used for rapid screening of DNA-binding peptide ligands from solid phase combinatorial library (Alam *et al.*, 2000). More recently, Mojsin and coworker (2006) have confirmed the

binding of the human recombinant USF1 protein to its putative binding site (E-box) employing magnetic separation technique.

1.2.4 Magnetocapture protein interaction assays

Magnetic particles are now increasingly used as carriers for binding proteins, enzymes and drugs. Magnetic support for immobilization purposes can be either prepared by co-polymerization of magnetic particles along with the synthesis of the supporting polymer (Horisberger, 1976; Robinson *et al.*, 1973) or magnetic particles can itself be coated with common support materials such as dextran or agarose (Rusetski and Ruuge, 1990). On this basis, Ni-NTA (nitriloacetic acid) tagged magnetic agarose beads have been used for versatile magnetocapture assays using 6xHis-tagged proteins (Sinclair, 2000). The procedure involves use of metal chelating nitriloacetic acid (NTA) groups covalently bound to the surface of agarose beads, which contain strong magnetic particles. The beads are pre-charged with nickel, which is ready to capture 6xHis-tagged proteins for sensitive interaction assays or microscale purification of 6xHis tagged proteins (Fig. 4). Thus, this technique bridges the gap between purification-scale procedures using Ni-NTA metal chelate affinity chromatography resins and microplate-based assays (Qiagen, GmbH, Germany). The immobilized biomolecules can be used directly for a bioassay or as affinity ligands to capture or modify target molecules or cells (Zimmerman and Heberg, 1998).

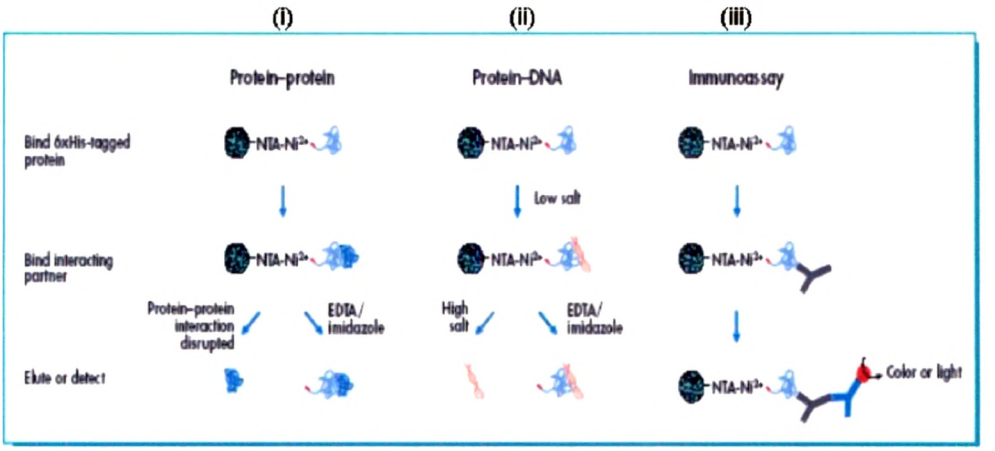

Figure 4.

Magnetocapture protein interaction assays. Ni-NTA magnetic agarose beads pre-charged with nickel captures 6xHistagged proteins, which can be used for studying protein-protein (i) and DNA-protein interactions (ii), and immunoassay procedures using antibodies specific for antigens present on the captured 6xHis-tagged biomolecule or antigens present on interaction partners bound to the captured 6xHis-tagged biomolecule (iii). (Source: Qiagen, GmbH, Germany)

In addition, studies have shown that proteins and enzymes can be bound covalently to naked magnetic particles in the presence of carbodiimide (Koneracka *et al.*, 2002). Such immobilization procedures for proteins, enzymes or drugs will have a major impact in various areas of medicine and biotechnology. This direct binding of protein to magnetic particles is possible, as the particle have a chemisorbed hydroxyl (-OH) group, in a lattice of Fe_3O_4 at a pH between 6 and 10 (Bacri *et al.*, 1990). Additionally, the presence of carbodiimide (CDI) modifies the carboxyl group of the protein at a slightly acidic pH, which increases the percentage binding of the protein (Packer *et al.*, 1979). Immobilization of bovine serum albumin, glucose oxidase, streptokinase chymotrypsin, cholesterol oxidase and dispase on naked (uncoated) magnetic particles using carbodiimide as coupling agent has already been reported (Koneracka *et al.*, 2002; Kouassi *et al.*, 2005a; 2005b). Following tables (3 and 4) discusses selected examples for isolation of protein and biologically active compounds using magnetic separation technique.

Table 3: Selected examples of magnetic separation of proteins

Isolated protein	Magnetic support used	Typical examples
Enzymes	Sub-micron ferrite particles with immobilized soybean trypsin inhibitor	Trypsin
	Ferrofluid-modified 5-AMP-Sepharose 4B	Alcohol dehydrogenase
	Magnetic agarose beads with immobilized dye	Lactate dehydrogenase
	Magnetic chitin	Lysozyme
	Dynabeads with immobilized polyclonal antibodies	Angiotensin-converting enzyme
	Imidoacetic acid coupled to magnetic agarose and charged with Zn^{+2}	Angiol-TEM-β-lactamase
	Alginate-magnetite beads	Pectinase
	Magnetic affinity aqueous two-phase system	Hexokinase
Antibodies	Magnetic particles with immobilized antibodies against human IgG	Human IgG antibodies
	Magnetic particles with immobilized protein A or protein G	Antibodies
	Human serum albumin immobilized onto ferromagnetic Dacron	Antibodies against human serum albumin
Lectins	Magnetic cross-linked chitosan	*Solanum tuberosum* lectin
Receptors	Dynabeads M-450 sheep anti-mouse IgG, with immobilized monoclonal antibody	Human transferrin receptor

	Magnetic particles with immobilized oligonucleotide containing EcdR binding sequence	Ecdysteroid receptor (EcdR) from *Drosophila melanogaster*
Other proteins	Magnetic particles with immobilized DNA/RNA fragment containing the specific binding sequence	DNA/RNA binding proteins
	Magnetic particles with immobilized m-aminophenylboronic acid	Glycated haemoglobin
	Organomercurial-agarose magnetic beads	Transcriptionally active chromatin restriction fragments with accessible histone H3 thiols
	Ni-NTA Magnetic agarose beads (Qiagen Inc., GmBH, Germany)	6xHis-tagged proteins

(Compiled from Sinclair, 2000; Mosbach and Andersson, 1977; Safarik and Safarikova, 2000)

Table 4: Selected examples of magnetic separation of low-molecular weight biologically active compounds and organic xenobiotics

Type of compound	Magnetic system used	Typical examples
Biologically active compounds	Magnetic particles with immobilized aldosterone antiserum	Aldosterone
	Molecular imprinted polymer containing magnetic iron oxide	(S)-propanolol
	Magnetic charcoal	Separation of free antigens in radioimmunoassays
Organic xenobiotics	Magnetic particles with immobilized Cu-phthalocyanine	Polyaromatic hydrocarbons, triphenylmethane dyes
	Magnetic polyethyleneimine microcapsules	Carcinogens
	Magnetic particles with immobilized specific antibodies	Pesticides, polyaromatic hydrocarbons, TNT, PCBs
	Bacterial cells adsorbed to magnetite	Chlorinated hydrocarbons and pesticides
	Magnetic charcoal	Water soluble dyes, pesticides

(Adopted from Safarik and Safarikova, 2000a)

1.3 CELL BIOLOGY APPLICATIONS

1.3.1 Immunomagnetic cell isolation and separation

Immunomagnetic cell separation methods have become increasingly popular among the cell biologist. The general approach involves use of paramagnetic particles coated with antibodies against the target specific cell surface molecules. The method employs two schemes for isolating the target cell, direct method that involves coupling of affinity ligand (antibody) onto the magnetic particles, which are then directly added to the sample containing the target cells. During the incubation the magnetic particles bind the target cells that can then be recovered using a magnet. Whereas in case of indirect method, a free affinity ligand (an appropriate antibody) is first added to the cell suspension. After incubation, excess unbound affinity ligand is removed by washing, and the antibody-target cell complex is then captured by magnetic particles bearing an affinity ligand (secondary antibody) with the affinity for the primary label. Both positive and negative selection can be performed with immunomagnetic separation. In case of positive selection target cellular subsets are magnetically labeled and subsequently separated. On the other hand negative selection involves target purification by removing all other contaminating cells (Safarik and Safarikova, 1999; Safarik and Safarikova, 2000b).

Using this technology large number of cell types has been isolated to date. Some of the selected examples, where immunomagnetic separation technology has been applied, are listed below in table 5.

Table 5 Selected examples of antibody tagged magnetic particles for isolation and purification of different cell types

REFERENCE	DETAILS
Tsoumakidou *et al.*, 2006	Isolation of myeloid and plasmacytoid dendritic cells from human bronchi-alveolar lavage fluid
Corona-Barrera *et al.*, 2004	Separation of intestinal spirochaetes from porcine faeces
Matsunami *et al.*, 2003	Detecting micro-metastasis in the bone marrow of gastric cancer patients
Bilkenroth *et al.*, 2001	Detection and removal of circulating tumor cells from peripheral blood
Favrin *et al.*, 2001	Detection of *Salmonella enterica* in broth
Luxembourg *et al.*, 1998	Isolation of antigen specific CD 8+ T cells
Kato and Radbruch, 1993	Selective separation of CD34+ cells
Shoge *et al.*, 1999	Isolation and enrichment of retinal ganglion cells (RGCs) for culturing
Cotter *et al.*, 2001	Novel method for isolation of neutrophils from murine blood using negative selection immunomagnetic separation

In addition to whole cell isolation, even cell organelles can be selectively separated using magnetic particles. The lysosome fraction was isolated from the amoeba ***Dictyostelium discoideum*** after feeding with dextran-based nanoparticles and subsequent homogenization (Temesvari *et al.*, 1994). Additionally, immunomagnetic separation has been used extensively for detection of pathogenic bacteria in clinical, food and environmental samples (Safarik and Safarikova, 1995; 1999; 2002; 2000b). Likewise a variety of cell types can be isolated using automated systems and different surface coated magnetic particles that are available from different companies (Table 6).

Table 6 List of few commercially available magnetic and superparamagnetic particles used (or suitable) for the isolation of cells

Manufacturer/supplier	**Product name**	**Surface modification**	**Immobilized compounds**	**End groups**
PerSeptive Biosystems, Framingham, MA, USA	BioMag ($\phi \sim 1$ μm)	Silanization of iron oxides	Protein A, ProteinG, Streptavidin, Biotin, Secondary Abs, Anti-CD Abs, Anti-fluorescein Ab	-COOH, $-NH_2$
Dynal, Oslo, Norway	Dynabeads M-280 (ϕ 2.8 μm) Dynabeads M-450 (ϕ 4.5 μm) Dynabeads M-500 (ϕ 5.0 μm)	Polystyrene	Streptavidin, Oligo (dT), Secondary Abs, Anti-CD Abs, Abs against microbes such as *E. coli*, *Salmonella* etc.	Tosyl-activated
Scigen, Sittingbourne, UK	M-100, M-104, M-108 (ϕ 1- 10 μm)	Cellulose	-	-OH
Cotex Biochem, San Leandro, CA, USA	Magabeads (ϕ - 3.2 μm)	Polystyrene	Streptavidin, Protein A, Protein G, Secondary Abs	-COOH, $-NH_2$ -epoxy
ProZyme, Madison, WI, USA	Magnetic beads (ϕ - 0.8 μm)	Latex	Streptavidin, Protein A, Protein G	-

CPG, Lincoln Park, NJ, USA	MPG (ϕ - 5 μm)	Porous glass	Streptavidin, Avidin	$-NH_2$, hydrazide, glyceryl
Seradyn, Indianopolis, USA	Sera-Mag (ϕ - 1 μm)	Polysytrene	Streptavidin	-COOH
chemagen AG, Baesweiler, Germany	Magnetic beads (Polydisperse size)	Polyvinyl alcohol (PVA)	Streptavidin, $Oligo(dT)_{30}$	-COOH, $-NH_2$ -CHO,-epoxy

(Ref Compiled from Safarik and Safarikova, 1999, chemagen Inc. [www chemagen de])

1.4 DIAGNOSTICS APPLICATIONS

1.4.1 Immunomagnetic assays

Immunoassays are becoming an important tool in clinical diagnostics and in fundamental investigations, because of its sensitivity, specificity and general applicability. Magnetic particles have been applied increasingly for various immunoassays that include fluoroimmunoassays, enzyme immunoassays or radioimmunoassays. Magnetic particles bound with primary or secondary antibodies are used for separation and quantification of antigens. Use of magnetically bound antibodies allows quicker separation thus reducing assay time and simplifying operation, thereby increasing the efficiency and accuracy of the assay. Nakamura and co-workers (1993) have developed a novel fluoroimmunoassay method, where a fluorescein isothiocyanate (FITC) conjugated monoclonal anti-*Escherichia coli* antibody was immobilized onto bacterial magnetic particles (BMPs) for detection and removal of *E. coli*. The same group has also developed a chemiluminescence enzyme immunoassay with BMPs using IgG as a model antigen (Matsunaga *et al.*, 1996). Likewise a magnetic bead based ELISA has been developed for detection of *Staphylococcus species* (Yazdankhah *et al.*, 1998).

An immunomagnetic bead immuno-liposome fluorescence assay has been developed for detection of *E.coli* O157:H7 in aqueous samples (DeCory *et al.*, 2005). More recently, a comparative study of a monoclonal antibody-based

capture/enrichment sandwich enzyme linked immunosorbent assay with immunomagnetic bead separation for detection of attachment effacement *E.coli* O26 strains from cattle faeces was reported (Finlay *et al.*, 2006). Additionally, a novel method was developed for the simultaneous detection of *E.coli* O157:H7, Salmonella, and *Listeria monocytogenes* using an immunomagnetic bead sandwich assay (Chen and Durst, 2006). Also, a rapid method based on immunomagnetic capture-fluorescent PCR assay has been developed for detection of *Campylobacter jejuni* in foods and water (Liu *et al.*, 2006).

Now a days, several instruments are available from different companies that couples separation of biomolecules with its detection in terms of its quantification or its interactions with other biomolecules. These instruments either use directly ferromagnetic particle as label (magneto assay) or couples magnetic particles with other detection methods such as fluorescence or chemiluminescence. It might be possible in near future that magnetic particles would be used as detection probes for a variety of assays, replacing labeling techniques such as fluorescence, chemiluminescence and radioactivity.

1.5 BIOMEDICAL APPLICATIONS

1.5.1 Drug targeting using magnetic targeted carriers (MTCs)

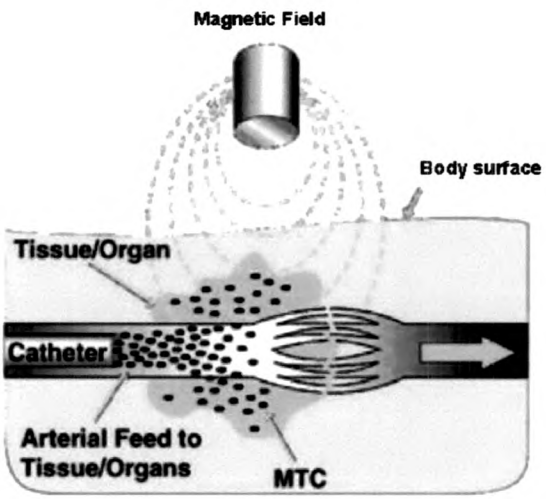

Figure 5.

Drug targeting using MTCs. MTCs are composite of elemental iron and activated carbon with anticancer drug adsorbed onto it. MTCs are delivered intra-arterially via a catheter to the desired site by externally applied magnetic field. The magnetic field aids in particle localization and retention at targeted site by extravasation of the particles into the surrounding tissue. (Source: FeRx Inc., San Diego, CA).

Magnetically guided drug targeting has been attempted in order to increase the efficacy and reduce the unpleasant side effects associated with chemotherapy (Reviewed by Ito *et al.*, 2005). This method of drug delivery involves immobilization of drug or radionuclide in biocompatible magnetic nano- or microspheres (Pankhurst, 2003). It aims to make chemotherapy more effective by increasing the drug concentration at the tumor site, while limiting the systemic drug concentration. FeRx Inc. of San Diego have designed Magnetic Targeted Carriers termed MTCs for site-specific targeting, tissue retention, and sustained release of drugs. These MTCs are composed of elemental iron particles and activated carbon. MTCs (1–2 μm in size) can adsorb and desorb pharmaceutical agents such as doxorubicin (DOX). MTCs are mixed with an anticancer drug already in solution; the mixture is then introduced into the catheter. The drug delivery using MTCs includes insertion of catheter into an arterial feed to the tumor, followed by application of powerful magnetic field to cause the MTC-DOX to extravasate through the capillary bed into the targeted tissue (Fig. 5). The field is left in place for another 15 minutes; after removal of the magnet, the particles remain trapped in the tumor, where the drug is then released (Fricker, 2001). FeRx has already initiated phase III clinical trial for MTC-DOX formulation. Like wise, Schutt and co-workers (1999) have carried out pre-clinical studies in collaboration with FeRx for treatment of liver and brain tumors using MTCs labeled with γ-emitters such as Rhenium-188 and Yttrium-90. Although the main research concern of

MTC-drug therapy is cancer treatment, the technology is not limited to this; other drugs that are deliverable in this manner include antibiotics, thrombolytics, anti-inflammatories, peptides and steroids (Sinclair, 1998).

Magnetic drug targeting employing magnetic nanoparticles (coated with starch derivatives having phosphate group) bound to mitoxantrone has been attempted for treatment of VX2 squamous cell carcinoma in rabbits (Alexiou *et al.*, 2003; Alexiou *et al.,* 2006). Recently, magnetite polymer (polylactic acid, PLA) nanospheres loaded with indomethacin (IND) were prepared and characterized; this magnetite-PLA-IND nanospheres have been proposed for anti-inflammatory treatment (Timko *et al.*, 2006).

1.5.2 Magnetic fluid hyperthermia

Magnetic fluids have been investigated as potential hyperthermia causing agents due to their high specific absorption rate (SAR). Hyperthermia is a promising approach for cancer treatment that uses AC magnetic fields to heat target areas (cancer tissue) containing magnetic fluids (Ramachandran and Mazuruk, 2004). In late 1970s, Gordan *et al.* (1979) first proposed the concept of intracellular hyperthermia using dextran magnetite nanoparticles to treat mammary carcinoma in rats. Since then, various types of biocompatible magnetic particles (Brusentsov *et al.,* 2001; Gonzales *et al.,* 2005; Jordan *et al.,* 2001), cationic magnetoliposomes (Yanase *et al.,* 1998), or affinity magnetoliposomes (Le *et al.,* 2001) have been used for hyperthermia treatment (Jordan *et al.,* 1999). To study the biological effects of AC magnetic field

excited ferrofluids, both *in vitro and in vivo* studies have been carried out in cancer cell lines and spontaneously induced tumors in animal models. The results of these studies have shown that magnetic fluid hyperthermia is able to reduce the viability of cancer cells, thereby indicating the potential of this therapy (Jordan *et al.*, 2006; 1997). During recent years, Johannsen and coworkers (2006, 2005, 2004) have successfully demonstrated the reduction in prostate tumor size due to the effect of magnetic fluid hyperthermia (alone) and in combination with external radiation in a rat tumor model.

1.5.3 Magnetoliposomes as drug delivery vehicles

As mentioned earlier, magnetoliposomes are magnetic derivatives of liposomes and can be prepared by entrapment of ferrofluids within the core of liposomes (De Cuyper and Joniau, 1988). The concept of magnetoliposomes for various biological and medical applications is in existence since 1980s. Table 7 provides few selected applications where magnetic liposomes (MLs) have been used.

Table 7: Use of magnetoliposomes for application in *in vitro* and *in vivo* studies

Authors Name/Year	Details
Margolis *et al.*, 1983a; Margolis *et al.*, 1983b	Antibodies associated with MLs to selectively concentrate target cells.
De Cuyper and Joniau, 1990	Study on non-protein mediated transfer and exchange of phospholipids between sonicated phospholipids dispersion and magnetoliposomes.
De Cuyper and Joniau, 1992	Immobilization of cytochrome-C oxidase as a model bound membrane enzyme into the phospholipids envelope
Shinkai *et al*, 1995	Antibody conjugated MLs for targeting cancer cells for hyperthermia*
Masuko *et al*, 1995	Demonstrated a possibility of thermosensitive MLs as a new agent for electromagnetic induced hyperthermia*
Viroonchatapan *et al.*, 1996	Reported magnetic targeting of thermosensitive MLs to mouse liver in an in situ on-line perfusion system.
Matsunaga *et al.*, 1997	Magnetoliposomes containing cis-diamminedichloroplatinum (II) (CDDP) prepared using bacterial magnetic particles (BMPs) were evaluated for targeting and controlled release of drugs at tumor site#
Babincova and Machova, 1998	Proposed a method where MLs coupled with HIV receptor protein might be used for elimination of HIV from infected individual.
Viroonchatapan *et al.*, 1998	*In vivo* study that provided evidence for the electromagnetic field-induced thermosensitive release of 5-fluorouracil from thermosensitive MLs in a tumor with the use of microdialysis.
Bulte *et al*, 1999	Used MLs as a contrast agent for magnetic resonance (MR) imaging of bone marrow
Babincova, 1999	Studied leakage of encapsulated drug from MLs when the liposome-enwrapped magnetite was heated with

	microwave (*in vitro* study)
Kubo *et al.*, 2000; 2001	Results of study indicate magnetic liposomes encapsulated with adriamycin can effectively deliver ADR to osteosarcoma in hamster #
Babincova *et al.*, 2000	Demonstrated site-specific *in vivo* targeting of MLs encapsulated with human serum albumin labeled with technetium-99m.
Domingo *et al.*, 2001	Immunomagnetoliposome were used to select CD34+ cells
Shinkai *et al.*, 2001	Targeted antibody conjugated MLs against MN antigen present on mouse renal cell carcinoma for hyperthermic treatment*
Babincova *et al.*, 2002	Release of doxorubicin from MLs in presence of AC-magnetic field with a frequency of 3.5 MHz (*in-vitro* study)
Luciani *et al.*, 2004	Demonstrated significant improvement in tumor targeting of paramagnetic agent for MR imaging using PEGylated niosomes.
Kullberg *et al.*, 2005	Used Epidermal Growth Factor (EGF) conjugated magnetoliposomes for targeting to tumor cells over expressing EGF receptors.
Fortin-Ripoche *et al.*, 2006	Investigated the feasibility of targeting sterically stabilized MLs after intravenous injection in mice.

* Use of magnetic liposomes to cause hyperthermia; # *in vivo* drug targeting attempted.

There also exists the possibility of combination therapy, which would include hyperthermia treatment followed by chemotherapy or gene therapy. The approach involves use of magnetic nano- or microspheres or magnetoliposomes containing a drug to cause hyperthermia using the standard procedure, followed by the release of encapsulated drug that will act on the injured cells. It is anticipated that the combined treatment might be very efficient for treating solid tumor (Safarik and Safarikova, 2002). Although, active targeting of anticancer drugs using magnetically responsive carriers (either magnetic fluids or magnetoliposomes) offers very attractive treatment approach for solid tumors. However, to date successful results are limited.

As discussed in the foregoing sections, many different types of magnetic micro- and nanoparticles and molecular magnetic labels have been used for a great number of applications in various areas of biosciences and medicine and it has been extensively reviewed (Ito *et al.*, 2005; Saiyed *et al.*, 2003; Pankhurst *et al.*, 2003; Safarik and Safarikova, 2002; 2004). The majority of review papers consider magnetic micro- and nanoparticles to be equally important. Indeed, in many areas magnetic microparticles with diameters above 1 μm are used (e.g. immunomagnetic separation of pathogenic microorganisms in food and clinical microbiology), whereas for other applications nano-sized particles necessitates as well.

Thus, from the review of literature it is obvious that magnetic particles have been used in various biological studies. Its application indicates ease in handling and quicker separation of biomolecules. In addition, one can clearly understand that most of the previous studies with magnetic particles have been done with particles **coated with biopolymers or synthetic polymers.** In those cases, only the magnetic property of the particles has been exploited to achieve faster separation. Moreover, magnetic bead based separation **kits available commercially are very expensive** to be used for routine purposes. Some of these commercially available beads can be prepared in the laboratory, but **most of the times the coating procedure is tedious** and requires sophisticated instruments. In addition, the **magnetic beads/particles endures chemical instability** (with respect to oxidation) and especially larger magnetic particles (typically ≥500 nm) retain a remnant magnetic moment after having subjected to the magnetic field, thus **leading to magnetic bead clustering** (Gijs, 2004).

Similar to coated magnetic particle, as mentioned earlier, a naked magnetic particle also possesses chemically reactive functional group (-OH) on the surface, which may help in direct binding of the biomolecule on the surface of the nanoparticles. There are several inherent advantages of such system where molecules are directly linked to magnetic support. Due to the absence of polymer coating the particle size is small (≤100 nm), which **provides higher surface area (on a weight basis) for the binding of the**

biomolecules. Additionally, this allows a greater response of particles to the applied magnetic field.

The results of previous studies on the use of magnetic particles for cell isolation have suggested that larger the particle size used for separation, **the higher the extent of non-specific entrapment in the larger aggregates of the magnetic particles.** Thus, nano-sized magnetic particles hold the promise of greater specificity. Moreover, **magnetic nanoparticles can exist as stable colloidal suspension that will not aggregate, allowing for uniform distribution in a reaction mixture.**

There are reports, where researchers have attempted using naked magnetic nanoparticles for biological applications instead of magnetic microparticles/beads to overcome some of the limitations of coated particles. Use of naked magnetite particles for isolation of plasmid and genomic DNA has been attempted (Davies *et al.*, 1998; Taylor *et al.*, 2000). In spite of this, the uses of naked (uncoated) magnetic nanoparticles for various biological applications have **not been exploited to its full potential.**

In view of the above information, the current work was mainly focused on the use of naked (uncoated) magnetic nanoparticles for molecular biology and drug targeting applications. Naked magnetic particles have the property of adsorbing genomic DNA on its surface under specific conditions, but in previous studies it has not been utilized for direct

isolation of DNA from samples such as blood, cultured cells, tissue homogenate, etc. Therefore, in the present study **the adsorption properties of magnetic particles were studied.** An effort was made to develop a universal high-throughput genome isolation system using magnetic nanoparticles as a solid-phase support. The other aspect dealt with, include immobilization of proteins and enzymes to naked magnetic particles using covalent linking. Such **immobilized proteins have several applications, such as solid phase reactant, for purification of proteins and peptides (as affinity matrix or in diagnostics), for terminating reactions and allowing repeated use of enzymes.** The advantage of using these magnetic particles for immobilization is the ease in recovery, speed and extreme specificity by which a protein could be isolated from a mixture. With the growing interest in nanoparticles as targeted drug delivery agents, the present work was also focused to evaluate the use of **magnetoliposomes for targeting drugs to solid tumors.**

1.6 OBJECTIVES OF THE PRESENT STUDY

Three of the applications were evaluated using the properties of naked magnetic particles:

- To develop a universal DNA extraction procedure using magnetic nanoparticles as solid-phase adsorbent.
- Immobilize alkaline phosphatase and streptavidin by direct binding procedure onto naked magnetic particle using coupling agent and check the applications of immobilized proteins in molecular biology and biotechnology experiments.
- To develop and optimize the magnetoliposomal formulation and check its *in vivo* efficacy as targeted drug delivery agent.