

Synopsis of the thesis on



**Application of magnetic nanoparticles
in the field of molecular biology and
drug targeting**

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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.

Magnetic fluids or ferrofluids as they are often called mainly consist of nano-sized iron oxide particles (Fe_3O_4 or $\gamma\text{-Fe}_2\text{O}_3$) which are suspended in carrier liquid. Although often referred to as magnetic, many of the particles currently used are **superparamagnetic**, these particles can be easily magnetized with an external magnetic field and redispersed immediately once the magnet is removed. Magnetic fluid research was initiated more than three decades ago and today has received a great interest due to its wide range of scientific applications and numerous technological advances. NASA (National Aeronautics and Space Administration) first developed magnetic fluids in 1965, for the elaboration of a propulsion system in microgravity. The applications of the magnetic fluids are generally in hi-tech fields, which include aerospace techniques, chemical industry, medical field, sensors, and computer technology. Magnetic fluids, are advanced materials that can solve complex engineering problems — **for example sealing rotary shafts**. High-speed **computer disk drives** use magnetic fluid exclusion seals to contain harmful dust particles and other impurities. One of the most important military applications that was developed using ferrofluid in the late 1980s was **for radar-repulsion purposes**. Another convenience of ferrofluid is their capability to operate in extreme temperatures, ranging from **-55°C to 200°C** (-67°F to 392°F), making them ideal for use in any location on Earth and particularly suited to space conditions.

The application of magnetic techniques in the field of biosciences was of limited use up to the 1970s. 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 various areas of medicine and biology. The immobilized molecules were used as **magnetic-affinity matrix** systems, or as components of **immunoassay** systems. One of the important properties of magnetic fluid is their good power absorption capability; this 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. 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 in 1980s. 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.**

The **Human Genome Project (HGP)** started 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. 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.** In recent years, magnetic separation techniques using **magnetisable solid-phase supports (MSPS)** has become increasingly applied to a number of biotechnological applications. 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.

Magnetic particles having connections to biological systems and bioapplications usually exist or can be prepared in the form of either single domain or superparamagnetic magnetite, greigite, various types of ferrites, iron, nickel *etc.* Currently available formats of particles are broadly classified into three classes: unmodified or naked particles, chemically derivatized particles with general specificity ligands and chemically derivatized particles with specific recognition groups. Magnetic separation technique works due to the presence of an affinity group or biomolecule on the surface of the magnetic particle. The 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.

In most of the previous studies, **magnetic particles coated with biopolymers or synthetic polymers were used for various applications**. Isolation of a specific cell, protein, or nucleic acid using magnetic beads (e.g. Dynabeads from Dynal, Norway or dextran-based magnetic particles from Miltenyi Biotec, Germany), utilizes only the magnetic property of the particles. There exist several kits based on magnetic support for purification and isolation of biologically active molecule. **These kits are very expensive** to be used for routine purposes. However, some of the coated beads available can be made in the laboratory, but **most of the times the coating procedure is tedious** and requires sophisticated instruments. Additionally, the **magnetic beads/particles suffers from being chemically unstable** (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**. There are reports, where researchers have attempted using naked magnetic particles for biological applications instead of magnetic microparticles/beads to overcome some of the above-mentioned limitations.

A naked magnetic particle possesses chemically reactive functional group on the surface, which may help in direct adsorption/linking of the biomolecule. . There are several inherent advantages that are envisaged for the use of such naked particles, where molecules are directly linked to a magnetic material/support. The absence of the polymer coat results in smaller particles (≤ 100 nm), **thus providing more surface area (on a**

weight basis) for adsorption/linking. Also, it allows a greater response to any magnetic field; studies on the use of magnetic particles for cell isolation have revealed that larger the particle size used for separation, **the higher the extent of non-specific entrapment in the larger aggregates of 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.** However, the use of naked (uncoated) magnetic nanoparticles for various biological applications **has not been exploited to its full potential.** In view of the above information, the present work is 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 complex samples such as blood, cultured cells, etc. Therefore, in the present study **the adsorption properties of magnetic particles were studied.** So an effort is made to develop a universal high-throughput genome isolation system using magnetic nanoparticles as a solid support. The other aspect that was studied includes, immobilizing 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 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 drug delivery agents, the present work was also focused to evaluate the use of **magnetoliposomes for targeting drugs to solid tumors.**

◆ **OBJECTIVES OF THE STUDY:**

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

- **To develop a universal high throughput genomic 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 for targeting drugs to the desired site.**

SUMMARY OF THE WORK DONE:

◆ **Review of literature:**

This involves a general overview about applications of magnetic microspheres, magnetic nanospheres and ferrofluids in various areas of biosciences. The “Introduction” describes the use of magnetic separation techniques for isolation and purification of biomolecules such as DNA, mRNA and proteins. Also, details of immunomagnetic cell separation and uses of magnetic particle immobilized biomolecules (especially protein and/or enzyme) as an affinity matrix and for diagnostics are given. In addition biomedical applications, which involve magnetically guided drug delivery using ferrofluids and magnetic liposomes for cancer drug targeting is described.

◆ **Preparation and characterization of magnetic nanoparticles for nucleic acid purification:**

The procedure for preparation of magnetic nanoparticles for biological applications was optimized. The particles were prepared by co-precipitating ferric and ferrous salts in an alkaline medium. Yield of precipitated magnetic particle was calculated by removing known aliquots of the suspension and drying to constant mass in an oven at 60°C. The particles were stored in appropriate solvent (deionized water or buffer) at a suspension concentration of 30 mg/ml. The particles were examined by transmission electron microscopy (TEM) and Fourier transform infrared spectrophotometry (FT-IR). It was found to be in a size range of 28-70 nm. The IR spectrum of magnetic particle was highly consistent with magnetite (Fe_3O_4) (bands at 583 and 632 cm^{-1}).

◆ **Development of universal genomic DNA isolation methodology using magnetic nanoparticles as solid phase adsorbent:**

DNA isolation utilizing superparamagnetic particles as a solid phase adsorbent is developed. The protocol was initially developed and optimized for genomic DNA isolation from human whole blood, since it is a readily available source of genomic DNA from humans or other vertebrates. Further this protocol was extended for DNA extraction from PBMCs (peripheral blood mononuclear cells), buffy coat (leukocyte rich layer), cultured cells (HCT116 cell lines) and tissues homogenates (rat liver and brain). The present protocol was able to successfully isolate DNA from the above biological specimen. In the initial experiments, performance comparison between the use of naked magnetic particle and silica coated particles for genomic DNA isolation was done. The yield of DNA isolated using naked magnetic particles was equivalent or slightly better

than obtained with silica magnetic particles. But the DNA yield using both silica coated and naked magnetic particles was better than traditional phenol extraction method.

In order to check the universality of the isolation procedure, it was applied for DNA isolation from bacterial species, which include *S. flaviscleroticus* (gram-positive), *E.coli* and *S. typhi* (gram-negative). Also, isolation of DNA from *S. cereviceae* (yeast), and *D. discoideum* (slime mould) was attempted. Depending on the sample types, optimization of sample amounts and lysis conditions was carried out. Additionally, isolation of plasmid DNA (pBR322) from *E.coli* (DH5 α) and elution of DNA from agarose gel using naked magnetic particles was performed successfully. A performance comparison of developed magnetic particle based DNA extraction method with Qiagen extraction kit was done (Qiagen QIAamp DNA Blood Mini kit for blood and QIAamp DNA Mini kit for cultured cells). The magnetic based DNA extraction method was tested for its efficiency of DNA extraction, ease of use and quality of DNA extracted. The genomic DNA extractions from both blood and cultured cells (HCT 116 cell lines) using magnetic particles were successful. The procedure gave consistently good results with both sets of samples. The yield of DNA isolated using magnetic particle was on an average 1.3 fold more than Qiagen method. The whole procedure using magnetic method takes less than 20 minutes, while Qiagen method takes on an average 40 minutes for isolation of DNA from blood and cultured cells.

◆ **To investigate the compatibility of isolated DNA in further applications such as polymerase chain reaction amplification and restriction endonuclease digestion:**

In order to compare the magnetic method with conventional procedures, the isolated DNA from all sources was analyzed by agarose gel electrophoresis and checked for its

performance in restriction digestion and PCR (Polymerase Chain Reaction) amplification. In all extractions the isolated genomic DNA band migrated to an equivalent distance to the 23kb of λ phage/Hind III digested molecular mass marker. Further to this the isolated DNA from blood and cultured cells were successfully amplified for a 226bp fragment of glyceraldehyde-3-phosphate dehydrogenase gene. Also, the isolated DNA from all sources functioned satisfactorily in restriction endonuclease digestion. A high throughput genomic DNA isolation system is also devised for simultaneous isolations from hundred of samples. This system originates from the growing demand in the field of molecular phylogeny, population and evolutionary genetics, for extraction of PCR ready genomic DNA from large population or a number of cellular sources. The present method using naked magnetic particle has advantages over conventional method and silica based supports (silica coated non-magnetic/magnetic beads). The method is simple, rapid, cost effective and reproducible. Further it can be performed in any laboratory without the need of any sophisticated instrument. Also, it doesn't require use of centrifuge or hazardous time-consuming and laborious purification steps with organic solvents like phenol/chloroform. There is scope for robotic manipulation system for high throughput DNA extraction.

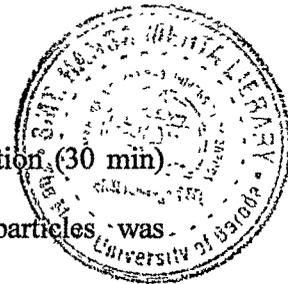
◆ **Direct immobilization of alkaline phosphatase and streptavidin onto naked magnetic particles:**

Immobilized drugs or proteins onto magnetic particles have potential applications as affinity matrix, in diagnostics and for clinical use. In earlier reports, several enzymes and proteins such as bovine serum albumin, lactate dehydrogenase, glucose oxidase, streptokinase, etc., have been immobilized onto magnetic particles using carbodiimide as

a coupling agent. In the present study, proteins that include alkaline phosphatase (ALP) and streptavidin were immobilized directly onto magnetic nanoparticles without the aid of primary coating. Immobilization of alkaline phosphatase using the direct binding procedure was optimized using different ratios of magnetic particles: carbodiimide: protein. Also, two different methods were used for immobilization: 30 minutes sonication and 24 hours shaking method. In all cases, 80-100% of the added enzyme (ALP) was bound to magnetic particles whereas up to 43% of the enzyme activity was retained compared to original activity. The immobilized ALP was stable for at least 16 weeks and interestingly there was an increase in the activity of immobilized ALP, which peaked at 3rd week post-immobilization, followed by decrease and finally the activity following just below the basal activity at 16th week.

Magnetic particle immobilized ALP was also checked for its efficiency in molecular cloning applications for dephosphorylation of plasmid DNA. Recircularization of vector DNA (plasmid) can be minimized by removing the 5'-phosphate residue from both termini of the linear, double stranded plasmid DNA with ALP. In traditional dephosphorylation procedure, once the ALP treatment is completed the enzyme reaction is terminated by incubation at 65°C for 30 minutes. But use of magnetic particle immobilized ALP facilitates the termination step, because after the dephosphorylation reaction the magnetic particle-ALP is removed by application of external magnetic field. This reduces the procedure time and also avoids exposure of reaction mixture to high temperature.

Immobilization of streptavidin using direct coupling procedure was also optimized using different ratios of magnetic particle: carbodiimide: protein. Similar to previous



experiments, two different methods were used for immobilization: sonication (30 min) and shaking (24 hours). The binding of streptavidin to magnetic particles was investigated by carrying out its interaction with biotinylated ALP. ALP activity was used as an indirect measure for determining the functionality and amount of immobilized streptavidin. From the Biotin-ALP activity it was observed that streptavidin immobilized using sonication procedure (219 pmole/mg particles) had higher biotin-binding ability than shaking procedure (157 pmole/mg particles). This higher activity by sonication method can be attributed due to higher amounts of immobilized streptavidin or increasing accessibility of immobilized streptavidin. The stability study indicates that immobilized streptavidin was stable over a period of at least 12 weeks. Also, the presence of protein (ALP and streptavidin) onto magnetic particles was confirmed by FTIR spectra of protein and protein coated particles. The characteristic bands of protein occur at 1647 cm^{-1} and 1542 cm^{-1} .

◆ **Magnetically guided drug delivery using magnetoliposomes: Formulation preparation and optimization, characterization, *in-vitro* stability and flow characteristics, and *in-vivo* efficacy studies**

This section discusses the results of evaluating magnetoliposome as drug targeting agents. The existing reported methods were used as a base to devise a new method for synthesis of magnetoliposomes. As characterized using transmission electron microscopy, the magnetoliposomes exhibited spherical shape with a diameter range of 200–500 nm. The phospholipid: magnetite ratio was determined to be 0.32 mmol/gm. Doxorubicin (DOX) was loaded into the magnetoliposome in response to the pH gradient. The drug encapsulation efficiency was found to be higher than 90-95 %. The

circulation stability of DOX encapsulated magnetoliposome was checked in an *in vitro* closed circulatory system using fluid having viscosity equivalent to whole blood. The magnetoliposomal formulation was found to be stable and no leakage of the drug was detected due to the resistance exerted by the fluid.

Magnetoliposomal drug release *in vitro* was characterized over 24 hours at 37°C. In the absence of serum, more than 90% of encapsulated doxorubicin remained liposome associated. This served as a control for spontaneous drug release, illustrating that the phospholipid-cholesterol bilayer remains tightly packed at 37°C, maintaining the pH gradient and preventing drug transport across the bilayer. In the presence of 50% serum, however 15-25 % of drug was released. The release of drug therefore appears to be at least partially dependent on protein-lipid interactions. These results illustrate the potential for these systems to sustain release at the disease site following intravenous administration. Our study also shows that this formulation is stable for at least 2 months at 5°C.

Also, regression studies in tumor bearing mice model have shown reduction in the size of tumor after treatment with magnetoliposomal doxorubicin compared to liposomal doxorubicin alone. A small magnet was implanted subcutaneously in the vicinity of the tumor on the day when first dose was given. Our results suggest that this treatment approach, which involves a combination of magnet implantation at the diseased site and intravenous administration of magnetic liposomes, can improve the treatment option for cancerous tissue.