6 CONCLUSIONS

As mentioned in the previous chapters, magnetic separation techniques have been applied in diverse field of biology. It offers comparatively a quick and easy method for sensitive and reliable capture of specific proteins, genetic material and other biomolecules. In this post genomic era, technologies based on magnetic separation are becoming an integral part of today's biology laboratory. As already discussed in the **introduction** that most of the currently used magnetic beads are coated with biopolymers or synthetic polymers with the magnetite (magnetic material) entrapped with the core of the polymer matrix. To this polymers general specificity ligands or specific recognition biomolecules such as monoclonal or polyclonal antibodies, receptors or enzymes are linked for different applications. Thus, in most of the earlier studies only the magnetic property of the particles has been exploited to achieve faster separation.

In 1983, Bacri *et al.* reported that the magnetite particles prepared by co-precipitation method possesses a chemisorbed hydroxyl (-OH) group in an aqueous medium, which is amphoteric in nature. Mehta *et al.* (1997) made use of this information (presence of –OH group) to directly link protein (BSA – Bovine serum albumin) on the magnetite particles in the presence of carbodiimide. This naked magnetite particle also possesses the property of adsorption, which was later found and exploited by Davies *et al.* (1998), where the researcher reported isolation of plasmid DNA using magnetite as solid-

phase adsorbent. To date barring the above few studies, uses of naked (uncoated) magnetic particles for various biological applications have **not been exploited to its full potential.**

Therefore, in the present study naked magnetite particles were used for molecular biology experimental applications and drug delivery purposes. As mentioned above, naked magnetic particles have the property of adsorbing DNA on its surface, using this property an effort was made to develop a universal high-throughput DNA isolation system using magnetite as solid support. The other aspect studied include, covalent coupling of biologically important proteins which in the present case were alkaline phosphatase and streptavidin. These immobilized protein preparations were tested for their application in biotechnology experiments. During recent years, there is a growing interest in nanoparticles as targeted drug delivery agents, the current work was also focused on developing a magnetoliposomal formulation with entrapped anticancer drug for targeting to specific areas in tumor bearing animal models.

For the development of a method for DNA isolation using naked magnetite; one can say that although it was not a totally new concept but was more or less neglected to date. The earlier attempts to use magnetite for adsorption of DNA were reported by Davies *et al.* (1998) and Taylor *et al* (2000), where isolation of plasmid and genomic DNA from a cleared cell lysate was peformed from bacteria and plant tissue, respectively. The authors reported that magnetite was

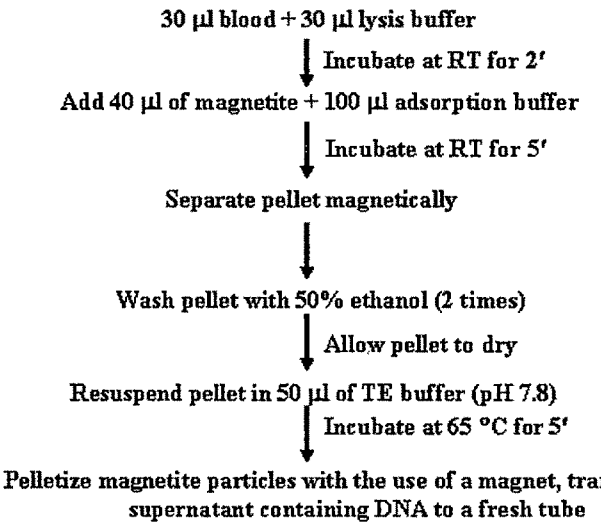
able to efficiently adsorb DNA, however, the yield was found to be low due to incomplete recovery of DNA from the magnetite support during elution (Taylor *et al.*, 2000). Additionally, it was suggested that magnetite particles with larger size (150 nm) prepared by the Sugimoto's method (1980) were more easier to work with, due to their greater magnetic susceptibility.

In the present study however, it was possible to prepare and successfully use the magnetite particles of nano-size (40 nm) for DNA isolation from different sources. The particles prepared were characterized using FTIR spectroscopy and were found to be consistently magnetite. The particles were also highly susceptible to the external magnetic field (0.2 Tesla). The binding capacity of this magnetite was determined with the use of standard human genomic DNA (Bangalore Genei, Bangalore, India) and was found to be in the range of 12-14 μg DNA per mg of magnetite as against ~10 μg reported by Davies *et al.* (1998). The higher binding capacity in the present case is possibly due to the nano-size of the magnetite particles, which might provide greater surface area for binding of DNA. In addition, use of adsorption buffer with optimized concentration of components (1.25M sodium chloride and 10% PEG 6000) could aid in the increased binding efficiency. Furthermore, at least 85-90% of the adsorbed DNA was recovered by elution in TE buffer or sterile water within 10 min as compared to 80% reported by Davies *et al.* (1998). The higher recovery of DNA with present method is possibly attributed to the optimized elution step, which involved mechanically breaking the magnetite-

DNA pellet mechanically by 25-50 pipetting strokes followed by incubation at 65°C for 5 min in the elution buffer.

The DNA isolation procedure was initially optimized using whole blood as a sample. Once the isolation procedure for blood genomic DNA was standardized, further optimization for DNA isolation was carried out for bacteria, yeast, *Dictyostelium*, plant tissue, and variety of mammalian cells. Additionally, the protocol was also successfully used for the extraction of plasmid DNA from bacterial cells and also recovering DNA from agarose gels.

Protocol for genomic DNA isolation using magnetite support



The above flow-chart describes standardized procedure to extract DNA using magnetite particles from blood sample.

Additionally, comparison of the present method with conventional procedures, coated magnetic beads and commercially available kit (Qiagen

kit) was also performed. Some of the obvious advantage of the present procedure includes

- The present method requires small quantities of starting materials to isolate DNA, for example the procedure yields enough DNA for 30 PCR from a small quantity (30 µl) of blood in less than 20 min. In contrast conventional procedure and Qiagen method requires higher amounts of starting materials (for e.g. 200 µl of blood)
- The yield of DNA isolated with the present method was comparable to that obtained with silica magnetite particles. Although the method was not compared with silica magnetic bead kit available from Dynabeads, nonetheless the results obtained with laboratory prepared silica magnetic particles were comparable in terms of its efficiency to extract DNA from blood. However, the yield was 1.3-fold higher than Qiagen method.
- Time taken to extract DNA using present method is less than 30 min as against several hours with conventional procedure. Whereas the Qiagen procedure takes an hour.
- The present method requires no tube transfers or centrifugation steps or organic solvent treatment.
- The method requires only a magnet and a heating block to achieve quicker separation.

- The present method doesn't require treatment of the sample with RNase or proteinase K (especially for blood, buffy coat, PBMCs and cultured cells), whereas conventional and Qiagen procedure requires treatment with both.
- The present procedure is cost effective. For example the cost per sample with Qiagen kit is about Rs 180/-, whereas with present method it is less than Rs. 5/- (single isolation from 30 µl whole blood).
- The procedure can be performed in any laboratory without sophisticated instruments.
- The procedure is amenable to automation and suitable for high throughput isolation of DNA.

To make the present method universal for extraction of DNA from different samples; modifications were made in the standardized protocol especially at the lysis step. In addition to its applicability for different cell types, the present procedure was also successful in extracting and recovering DNA from agarose gel. The use of naked magnetite particles for this application (extraction of DNA from agarose) has been reported for the first time from the present work. The results showed that more than 80% of DNA could be recovered from agarose gels, which is comparable to reported by Hawkins *et al.* (1994) using carboxyl coated magnetic particles.

In all the samples tested the isolated DNA was of comparable or higher yield than conventional procedures. Furthermore, isolated DNA from all the sources

functioned satisfactorily in restriction endonuclease digestion. Also, PCR amplification of GAPDH gene was successfully performed using the isolated DNA from blood and cultured cells. This gave an indication that the isolated DNA with the present method is suitable for downstream application such as genotyping, sequencing, cloning, hybridization, etc. The same processing (PCR amplification and restriction digestion) can also be performed without eluting the DNA from magnetic particles. However, it is important to mention that the magnetic particles concentration should not exceed 50 μg per 50 μl of the reaction medium.

The ultimate goal of the present study was to develop a high throughput-genomic DNA isolation system using magnetite as a solid-support, so as to allow DNA isolation from 100 to 1000 samples simultaneously. Therefore, an attempt was made to devise a prototype of the system that can isolate DNA from several samples simultaneously. However, the final format required tiny ceramic electromagnet that were to be impregnated in the lid of the 96 – 384 well plates to aid magnetic separation. But the availability and procurement of this electromagnet was not possible so the final format of this system was difficult to fabricate. Nonetheless, the method worked successfully in the prototype format, which hints that the procedure would function with high throughput system if the technology were to be taken by a commercial organization.

Thus, the developed method is simple, quick, reproducible, and cheap compared to conventional and commercially available kits.

After the use of the adsorption property of magnetite, the next section discusses the use of magnetite particles as a solid-support to covalently immobilize proteins and enzymes. The advantage of using these magnetite particles for immobilization is the speed and ease in recovery of the immobilized protein. Furthermore, the nano-size of the particles provides greater surface area for binding of the proteins as compared to the coated particles. This allows higher loading of protein on the surface of magnetite particles, which makes it possible to use lesser quantity of magnetite particles per assay. This helps in minimizing the loss of expensive proteins.

In the present study, immobilization of ALP and streptavidin was attempted using using two different methods viz shaking and sonication method, respectively. The reason for comparing two immobilization methods stem from the studies conducted by Koneracka *et al.* (2002), where shaking method has been described for immobilizing proteins on magnetic particles, whereas Kouassi *et al* (2005a, 2005b), have used sonication method for the immobilization enzyme onto magnetic particles.

The results from these studies indicated that sonication method for immobilization was quick and retained higher amount of functional activity compared to shaking method in both the cases. This higher loss of activity with shaking method could probably be attributed to the exposure of enzyme

at 37°C for extended period of time (24 h), whereas with sonication method the enzyme was exposed to sonic wave for only 30 min at 4°C. The activity of immobilized ALP with sonication method was 30-43%, whereas it was 24-38% with shaking method. The loss of functional activity is a common observation for enzymes immobilized by covalent linking; when ALP was immobilized on chitosan microparticles with glutaraldehyde cross-linking, the activity yield was also in the range of 38-49% (Zubriene *et al.*, 2003). In most of the previous reports where enzymes have been covalently linked, the % activity retained varied from as low as 6% to as high as 90% (table 21 – section 4.1.2). These differences are very large, however it was summarized that the retention of the functional activity for an immobilized enzyme depends on its nature, size and type of the support matrix. Additionally, there are lots of factors that contribute to the loss of enzyme activity upon covalent linking. For example covalent linking of the protein may result in a change in conformation of the enzyme where the active site is partly or totally obscured by the immobilization matrix and thus not accessible for binding the substrate (Goldman *et al.*, 1968).

The immobilized ALP with both the above methods were found to be stable for at least 16 weeks when stored at 4°C. In the case of storage stability, the ALP immobilized with shaking method was found to retain higher amounts of activity (especially 3:1:1 ratio of magnetite:CDI:ALP) compared to sonication method. The extended stability of the immobilized ALP compared to free

(unbound) ALP indicates that, covalent coupling with carbodiimide might cause inter and/or intra cross-linking of the enzyme providing a better stability to its quaternary structure (Simons *et al.*, 2002). Thus to conclude, sonication method is the choice, if the immobilized ALP is to be used only for short-term purposes. Whereas shaking method is preferable for long-term stability. Additionally, it is advisable to use higher amounts of ALP for coating onto magnetic particles (i.e. ratio of 3:1:1) with both shaking and sonication methods. Furthermore, in the present study, successful plasmid dephosphorylation was demonstrated with immobilized ALP, thus, providing the evidence for its use in molecular biology experiments.

As mentioned earlier, streptavidin was also immobilized with two different methods. In the present study, the biotin-binding capacity of the immobilized streptavidin was determined with the use of biotinylated-ALP. The biotin binding capacity of present streptavidin magnetic particles was at least 4-5 times higher (157 and 219 pmole/mg magnetite with shaking and sonication method, respectively) than commercially available streptavidin coated magnetic beads (30-50 pmole/mg beads; chemagen AG, Baesweiler, Germany).

However, when comparing two immobilization strategies, the biotin binding capacity of streptavidin magnetite particles coupled with shaking method was found to be significantly lower than sonication method, though the amount of protein bound was same. As mentioned earlier, this is possibly due to the

denaturation of streptavidin either during coupling reaction or due to extended exposure at 37°C for 24 h.

Their also exist a possibility that the biotin-binding capacity reported in the present study could be an under-estimate, since it was determined using larger size probe i.e. biotin-ALP. Such a possibility is already reported in case of magnetic streptavidin particles available from Bangs laboratories; the biotin-binding capacity of the same particles when measured by Seradyne Inc. using BF (Biotin Fluorescein) probe was 15-fold higher than reported by the Bangs Lab (100 pmoles) (Source: Technote, Sera-Mag Magnetic Streptavidin microparticles from Seradyne Inc. Indianapolis, USA). The probes used for determination of biotin-binding capacity have inverse relation to the binding capacity, that is higher the molecular weight of the probe lower is the binding capacity. From the literature reported, the binding capacity determined using free biotin or biotinylated-oligonucleotide is expected to be at least 5 fold higher than reported with biotinylated-protein probe. Therefore, according to this statement the free biotin binding capacity of magnetic streptavidin nanoparticles of the present study is expected to be in the range of 800-1000 pmoles. This value is higher or comparable than reported by commercially available particles from different companies, which includes Spherotech, Dynal Biotec, Scipac and Cortex Biochem. The higher biotin binding capacity of present streptavidin magnetic particles indicates that lesser quantity of particle is required per assay.

Furthermore, the immobilized streptavidin with both the methods was also found to be stable for at least 12 weeks. Thus, the covalent linking method described here can be used for preparation of streptavidin magnetic nanoparticles, which could be used for various bioapplications where biotinylated tagged biomolecules can be selectively isolated by binding to streptavidin magnetic particles. One example where this techniques has already been applied involves mRNA isolation with biotinylated oligo(dT) probe, which simplifies the purification of mRNA by addition of streptavidin magnetic beads (Sinclair, 1998). In addition to the direct isolation of biotinylated molecules such as DNA, RNA or proteins onto streptavidin coated magnetic beads; this strategy can also be exploited for indirect isolation of non-biotinylated target molecules that may interact with them (e.g. transcriptional or translational factors). Using this technique mRNA binding protein was purified; in that case the probe mRNA sequence was biotinylated and mixed with the cell extract containing the proteins that bind to this sequence (Albig, 2001). Based on the interaction of biotin-streptavidin, the probe-target complex was then separated and the non-biotinylated molecule was eluted thereof. Thus it can be said that the streptavidin magnetic particles prepared in the present study could also be applied in different areas such as nucleic acid separation, cell separation and isolation and many more.

The direct coupling method for protein immobilization is simple, quick, inexpensive and it can be performed in any laboratory without the requirement

of sophisticated equipments. The present procedure could have potential applications in molecular biology, where expensive enzymes such as restriction endonucleases and other DNA/RNA modifying enzymes (ligases, kinase, nucleases and polymerases) can be immobilized in a similar manner. **Such immobilized preparations could simplify the operation, reduce procedural time and allow repeated use of enzymes and proteins.**

With the increasing interest in nano-sized particles for drug delivery, the present work was also focused on developing a stable magnetoliposomal formulation for targeting drug to specific site in the body. As described in **introduction** section drug delivery using ferrofluids reversibly bound to anticancer drug has already been attempted (Alexiou *et al.*, 2003; Alexiou *et al.*, 2006). However, the problem with this approach is non-specific release of bound drug once the formulation is brought into the circulation. Additionally, there also exist possibilities of particle aggregation while in circulation, which could result in blockage of vital capillaries and may be fatal. To overcome some of these problems the use of magnetoliposomes for treatment of cancerous tissue has also been reported (Shinkai *et al.*, 1995; Viroonchatapan *et al.*, 1998; Shinkai *et al.*, 2001; Babincova *et al.*, 2002). Most of these studies have used magnetoliposomes as a hyperthemia-causing agent to kill cancer cells. However, there are few *in vitro* and *in vivo* studies where magnetoliposomes have been used as drug delivery agents (Kubo *et al.*, 2000; 2001; Kullberg *et al.*, 2005). Although active targeting of drugs using

magnetoliposomes offers very attractive treatment approach, yet successful results are limited. This gives a scope to further try alterations in this approach of drug delivery and investigate its efficacy as a drug delivery carrier.

In the present study preparation procedure for magnetoliposomes was standardized. The method of preparation of magnetoliposome reported earlier was used as a base to devise a modified and an improved method (Sangregorio *et al.*, 1999; Babincova *et al.*, 2002). In the present method direct encapsulation of the magnetic particles within the liposome core was attempted. The prepared magnetoliposomes were characterized and found to exhibit spherical shape with a diameter range of 200-500 nm.. The phospholipid: magnetite ratio was determined to be 0.32 mmol/gm. Drug loading efficiency was determined using encapsulation of doxorubicin (DOX); which was loaded into the magnetoliposomes in response to a pH gradient. The drug encapsulation efficiency was found to be consistently high (90-95 %). The circulation stability of DOX encapsulated magnetoliposomes 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. Additionally, dilution induced drug leakage, and storage stability of the DOX entrapped magnetoliposomes was also performed. The results showed that more than 90% of encapsulated doxorubicin remained liposome associated. Thereby 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. After the preparation and characterization was accomplished the next step was to check the *in vivo* efficacy, pharmacokinetics and toxicity of the prepared formulation.

An *in vivo* study of the present formulation was performed to check the tissue distribution of these magnetoliposomes in rats. However, due to lack of facilities and problem associated with extraction of doxorubicin from blood, it was rather difficult to account the amount of doxorubicin injected and recovered. From the results obtained, it probably hints at the RES uptake of liposomes and this could be minimized by use of Pegylated phospholipids. As mentioned earlier, it is important to mention that the idea of present magneting targeting approach is to concentrate magnetoliposomes using a strong magnet at the site of the tumor. Nonetheless, PEgylation may help to increase the circulation time of magnetoliposomes that are less susceptible to the applied magnetic field.

A preliminary *in vivo* efficacy study of this formulation was done in fibrosarcoma tumour bearing BALBc mice. On zero day of the treatment, a

small magnet with strength of 0.2 Tesla was subcutaneously implanted near the site of the tumour. Actually a strong magnet with a magnetic field strength equal or greater than 0.45 T could have been a better option, however the availability of small button shaped magnet with such high field strength was not possible. The present magnetoliposome-DOX injected animals were compared with liposome-DOX group. The results indicated a faster tumour regression in magnetoliposomal groups compared to liposome group, which might be attributed to the rapid accumulation of ML in tumours due to the influence of external magnetic field. However, confirmation on these lines was difficult to obtain due to the lack of proper imaging techniques that can provide the actual concentration of particles at the site of tumour. Nonetheless, the preliminary findings suggest this targeted approach with magnetoliposomes may be better than normal liposomes. However, much remains to be done before the magnetoliposomal approach for drug delivery is validated. In spite of its potential as targeted delivery agents, the present approach is currently limited to its applicability to peripheral tumours only. However, there are some successful reports of drug delivery using doxorubicin coated magnetic microparticles in patients with hepatic carcinoma (FeRx Inc., San Diego, CA). Therefore, it can be said that in the near future the present approach could also be applied to internal tumors.

Thus, the prepared magnetoliposome in the present case were found to be consistent in terms of its characteristics and *in vitro* stability. The primitive *in*

vivo efficacy study has given exciting results, indicating this as one of the potential treatment approach for solid tumors.

As a part of future prospect one can plan to carry out required modification in the magnetoliposomal composition (especially pegylation) and use a combined treatment approach of hyperthermia (using liposome entrapped magnetite) and drug delivery with magnetic liposomes.

To conclude, it can be said 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 of solid tumors.

These nanoparticles have a versatile application in biological fields and opens a new avenue for developing bio-tools and applications, which are specific, efficient, rapid and economical.