2. MATERIALS AND METHODS

2.1 Preparation of magnetic nanoparticles (Fe_3O_4)

In order to achieve the objectives, the procedural condition for preparation of magnetic particles was standardized. Initially, using small quantities of Fe^{+3} and Fe^{+2} salt solutions the method described by Mehta *et al.* (1997) was used as the base to optimize conditions in the laboratory. Once the consistency in preparation was obtained, everytime a known quantity of Fe^{+3} and Fe^{+2} solutions was mixed and added to an alkaline solution. The detailed protocol is described in the following section.

Magnetic nanoparticles (Fe_3O_4) were prepared by co-precipitating di and trivalent iron ions by alkaline solution and treating under hydrothermal conditions as described by Mehta *et al.* (1997)

$$2FeCl_3 + FeCl_2 + 8\ NH_3 + 4H_2O \text{ ------- } > Fe_3O_4 + 8NH_4Cl$$

27.8 g of Iron (II) sulphate hepta hydrate ($FeSO_4.7H_2O$) in 100 ml double distilled water and 32.2 g of Iron (III) chloride ($FeCl_3$) in 100 ml were thoroughly mixed and dissolved. This solution mixture was then added drop wise over a period of 30 min under constant stirring to 1M sodium hydroxide that was kept at 80°C as against 3M sodium hydroxide used by Huang *et al.* (2003). This resulted in precipitation of black magnetite particles.

In case of the magnetic particles that were used for protein immobilization studies, 1M sodium hydroxide was replaced with 8M ammonium hydroxide. This is because reports from previous studies have demonstrated that immobilization of proteins and enzymes is more efficient with magnetic particles prepared using NH_4OH rather than with NaOH (Mehta *et al.*, 1997; Koneracka *et al.*, 1999; Koneracka *et al.*, 2002; Kouassi *et al.*, 2005).

The solution containing precipitates of magnetite particles was kept on a magnet to allow the particles to settle and then the supernatant was discarded. The magnetic particles thus formed were thoroughly washed with hot distilled water to remove impurity of ions such as chlorides and sulphates. Finally, the magnetic particles were suspended in slightly alkaline medium (pH 8.0–8.5) and stored at the suspension concentration of 30 mg/ml. The yield of precipitated magnetite was determined by removing known aliquots of the suspension and drying to constant mass in an oven at 60°C. This preparation procedure routinely yield 28 ± 2 g of magnetite.

The magnetic nanoparticles thus prepared were characterized for size and consistency using transmission electron microscopy (TEM) and Fourier-transform infrared spectroscopy (FTIR), respectively.

2.2 Characterization of magnetic nanoparticles

2.2.1 Transmission electron microscopic (TEM) examination of magnetic nanoparticles for size determination

The size of the magnetite nanoparticles was determined using negative staining technique of **transmission electron microscopy.** This work was carried out at Department of Zoology, School of Life Sciences, Gujarat University, Ahmedabad.

Principle: The basic principle is the same as light transmission microscopes; the electron beam passes through the sample and is affected by its interaction with it that means the electron beam gets deflected by the surface of the sample and hence forms the image. The use of high-energy electron beams allows magnification leading practically to atomic observation. If electrons are accelerated under 100 kV potential, the associated wavelength is only 0.037 Å. Hence, magnification up to 500000 is possible. However, the sample thickness should not exceed 10nm approximately.

Materials: Sample (magnetic particles), formvar-coated grids

Dye used: 0.5% uranyl acetate in 50% methanol

Procedure:

1. Magnetite sample was placed on a formvar-coated grid
2. It was allowed to dry and then stained with uranyl acetate dye.

3. After allowing the stain to dry, the samples were examined under TEM with various magnifications and the size of magnetic particles determined.

2.2.2 Determination of composition of magnetic nanoparticles (Fe_3O_4) using Fourier transform infrared spectroscopy.

This part of the work was carried out at Department of Chemistry, The M.S. University of Baroda, Vadodara.

Principle: Photon energies associated with infrared (from 1-15 kcal/mole) are not large enough to excite electrons, but may induce vibrational excitation of covalently bonded atoms and groups. The covalent bonds in molecules are not rigid sticks or rods, such as found in molecular model kits, but are more like stiff springs that can be stretched and bent. In addition to the facile rotation of groups around single bonds, molecules experience a wide variety of vibrational motions, characteristic of their component atoms. Consequently, virtually all compounds will absorb infrared radiation that corresponds in energy to these vibrations. Infrared spectrophotometer provides absorption spectra of compounds that are a unique reflection of their molecular structure.

Materials: sample (magnetite, Fe_3O_4), KBr crystals

Procedure: The sample was ground with KBr powder in a mortar and pestle. The mixture was placed in-between the tablet punch and the assembly was put under hydraulic press and the sample was compressed to

form a pellet. The pellet was then put gently in a disc holder of the infrared spectrophotometer and then the spectrum was recorded in the wave number range of 400-4000 cm^{-1}.

2.3 Preparation of silica magnetite beads

Silica magnetic beads were prepared by coating the core magnetite with siliceous oxide layer according to the procedure described by Pryor *et al.* (2001). Superparamagnetic iron oxide particles were prepared according to the protocol described above in section 2.1. The prepared particles were then washed thoroughly with water and finally resuspended in distilled water. From the precipitated magnetite 75 g of particles were then treated under sonication with 600 ml of 3 % hydrogen peroxide (H_2O_2). The reaction mixture was maintained in this condition for 60 min and then the magnetite was separated from the solution by application of an external magnet. The magnetite particles were then washed thrice with 2 liters of distilled water. However, treatment with H_2O_2 resulted in considerable loss of magnetite particles especially at washing steps. The recovered particles were only 60-70% (~50 g) to that added initially. This is possibly attributed to extensive sonication, which may break micron or nano-meter sized clumps of magnetite particles. Thus, resulting in colloidal suspension of particles with some having size below 10 nm and thereby less susceptible to the applied magnetic field. In order to get 75 g of wet particles after treatment with H_2O_2, a higher amount of wet magnetite particles (~110 g)

were treated from the beginning. After treatment with H_2O_2, 75 g of wet magnetite were again resuspended in 75 ml of distilled water. The particle preparation was then heated to about 90°C and further subjected to agitation in a sonicator bath. An aqueous sodium silicate solution (10.7 % SiO_2 and 3.24 % Na_2O) was added to the particles slowly under constant sonication. A total of 315 ml of sodium silicate was added over a period of 2 hours with the addition rate not exceeding 5 ml per min. The pH of the resulting suspension after silicate addition was about 10. The pH was then brought down to 7.5 by addition of 1N HCl. The siliceous oxide coated particles were then allowed to age under agitation for at least 1 hour at 90°C. After that the particles were washed twice with 2 liters of distilled water, followed by two washes with 3% NH_4Cl solution for about 30 min. Finally, the particles were washed again with distilled water and resuspended at a concentration of 40 mg/ml. The particles thus prepared were reported to have mean particle size of 5 μm (Pryor *et al.* 2001)

2.4 Genomic DNA isolation using magnetite as solid-phase support

2.4.1 Determination of DNA binding capacity of the magnetite particles

Prior to the development of genomic DNA isolation procedure, it was found necessary to check whether the prepared magnetite was capable of adsorbing and subsequently releasing, DNA The binding capacity of DNA

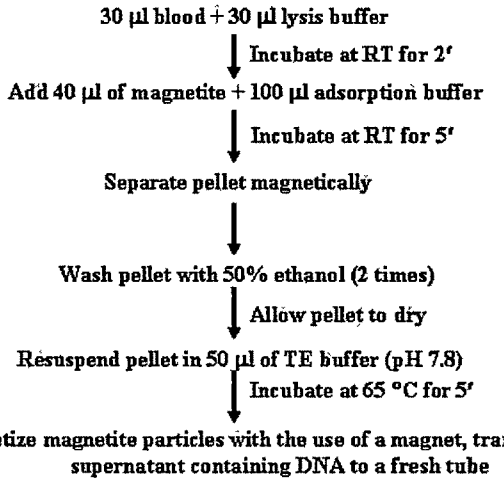
to magnetite particles was determined using standard human genomic DNA (Bangalore Genei, Bangalore, India). Standard human genomic DNA (stock concentration – 40 ng/μl) was adsorbed and eluted from the magnetite particles using the following procedure: 50 μl of magnetite particles (concentration - 10 mg/ml) suspended in TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 7.8) was pipetted in a 0.5 ml microfuge tube. Varying concentration of standard human genomic DNA (0-20 μg) was added to the magnetite followed by addition of 50 μl of adsorption buffer (1.25M NaCl and 10% polyethylene glycol 6000; PEG 6000) (the optimization of adsorption buffer is described in following section). The suspension was incubated for different time intervals (2-10 min) at room temperature to allow DNA to adsorb onto magnetite; however 2 min incubation was found to be sufficient. The magnetite bound to DNA was then immobilized using an external magnet. The supernatant was removed, and a further 100 μl of 50% ethanol was added to wash the magnetite by gentle resuspension. The magnetite particles were again immobilized using an external magnet and the wash supernatant was removed. Finally, the magnetite-DNA complex was resuspended completely in 50 μl of sterile water by repeated pipetting strokes (25-50 times) and then incubated at 65°C for different time intervals. The results obtained indicate that, 5 min incubation at 65°C was enough to elute most of the bound DNA. The magnetite particles were then pelleted by application of an external magnet

and supernatant containing the eluted DNA was analyzed by agarose gel electrophoresis and the DNA binding capacity of the magnetite was judged by the intensity of DNA bands in the gel after ethidium bromide staining and UV transillumination using Bioimaging system (UVP Systems, Cambridge, UK) and densitometry software.

2.4.2 Optimization of procedure for genomic DNA extraction from whole blood

In the present study, method for isolation of genomic DNA was initially optimized using whole blood and then investigated for its suitability in cultured cells (HCT116) and tissue (rat liver and brain) homogenates. The sample preparation for DNA extraction involved collection of whole blood in a tube containing EDTA as anticoagulant.

Protocol for genomic DNA isolation using magnetite support



The flow chart describe the steps involved in extracting DNA from whole blood

In the process of optimizing the protocol, initially the lysis step was standardized. Different concentrations of Triton-X 100 and sodium dodecyl sulphate (SDS) were examined so as to check efficient cell lysis in a shorter duration. SDS at a final concentration of 0.5% was found to cause complete cell lysis of blood leucocytes in less than 2 min. The composition of adsorption buffer was also optimized so as to allow efficient binding of DNA on magnetite, whereas minimize the contamination with RNA and protein. The components that were considered in the adsorption buffer include isopropanol, absolute ethanol, sodium iodide (alone) and in combination with high PEG, and sodium chloride (alone) and in combination with high PEG. Furthermore, PEG of different molecular weight (6000, 8000 and 10,000) and varying concentration (2.5 – 10%) were investigated to optimize the conditions for efficient DNA binding on magnetite. Finally, the optimized buffer components include 1.25M sodium chloride and 10% PEG 6000. This combination of adsorption buffer resulted in high yield of isolated DNA.

The elution of DNA from magnetite was also optimized, since one of the problems using magnetite as adsorbent was already reported by Taylor *et al.* (2000); that it is difficult to elute magnetite bound DNA, which results in lower yields of extracted DNA. To solve this problem, initially elution was attempted by incubation of DNA-magnetite pellet at different temperatures (65°, 75° and 85°C) and time intervals (5, 10, 15 and 20 min).

It was found that incubation at 65°C for 5 min was sufficient to elute about 50% of the magnetite bound DNA. However, in cases where magnetite-DNA pellet was disrupted (complete resuspension) by repeated pipetting strokes (25-50 times) before incubation at 65°C, the yield of recovered DNA increased to about 85%. Therefore, the optimized elution step involved resuspending the magnetic-DNA pellet by repeating pipetting, followed by incubation at 65°C for 5 min with slight agitation. This resulted in recovery of 85-90% of bound DNA; further increasing the incubation time at 65°C doesn't considerably increases the recovery. Nonetheless, the 90% recovery of DNA is still higher than reported earlier by Davies *et al.* (1998) to be about 80% with pure plasmid. The purity of the recovered DNA with the present method as judged with UV260/280 ratio was 1.75-1.85. Additionally, no RNase or proteinase K treatment of the sample was required with the present method. The optimized blood genomic DNA extraction procedure is described below.

2.4.3 Standardized procedure for DNA extraction from whole blood using magnetite as support

In a typical extraction, a 30 µl of sample (whole blood), 30 µl of 1% (w/v) sodium dodecyl sulphate (SDS) solution was added. The tube was mixed by gentle inversion for two or three times and incubated at room temperature for a minute. After incubation, to the cell lysate 10 µl of

magnetic nanoparticles (20 mg/ml) were added; followed by addition of 75 µl of adsorption buffer (1.25 M sodium chloride and 10% PEG 6000). The suspension was mixed by inversion and allowed to stand at room temperature for at least 3 min. The magnetic pellet was immobilized by application of an external magnet and supernatant removed. The magnetic pellet was washed with 50 % ethanol and dried. The pellet was then completely resuspended in 50 µl of TE buffer (pH 8.0) by repeated pipetting strokes and magnetic particle bound DNA was eluted by incubation at 65 °C for 5 min with continuous agitation. The magnetite particles were then immobilized with a magnet and the clear supernatant containing DNA was transferred to a fresh tube.

The above procedure was also found to be applicable for genomic DNA isolation from buffy coat and peripheral blood mononuclear cells (PBMCs). However, in those cases only 10 µl of starting material was taken instead of 30 µl used for blood. This is due to the fact that buffy coat and PBMCs contains only nucleated cells.

2.4.4 Optimized procedure of genomic DNA extraction from cultured cells and tissue homogenate

Cultured cells used in this study were of colon carcinoma cell lines (HCT116). For experiment purpose, the cells were trypsinized and adjusted to a cell density of 7 x 10^6 cells per ml with PBS (phosphate buffered saline, pH 7.4). For tissue DNA extraction a 10% homogenate of rat liver and brain was prepared in 0.32 M buffered sucrose (pH 7.5) using Teflon homogenizer. The same protocol that was standardized with blood was found to be suitable for DNA isolation from cultured cells without any further modifications. However, for tissue DNA extraction in addition to the standard procedure, 10 µl of proteinase K (20 mg/ml) and 2.5 µl of RNase (1 mg/ml) treatment was found to be necessary to improve the yield and purity of DNA. Once the cell lysis step in the presence of proteinase K was accomplished, the released DNA was extracted by binding to magnetite particles in the presence of adsorption buffer as described in standard procedure.

2.5 Isolation of genomic DNA from prokaryotic cells

After the successful isolation of DNA from mammalian cells, the applicability of this procedure to isolate genomic DNA from bacterial cells was investigated. Due to the presence of cell wall in bacterial cells, the

same protocol cannot be directly used to isolate DNA. Appropriate modifications were made and the procedure was optimized.

2.5.1 Optimized procedure for genomic DNA extraction from gram positive bacteria (*S. flavisclerotícus*)

S. flavisclerotícus cells were grown in Yeast Extract Malt Extract (YEME) media to a cell density of 10^8 to 10^9 cells per ml. 1.5 ml of cells were pelleted in a microcentrifuge at 12000 g for 30 sec. The supernatant was discarded. The pellet was resuspended in 500 µl of SET buffer (50mM Tris; 1mM EDTA; 150mM NaCl, pH 7.2) + 10 µl of lysozyme (2 mg/ml) was added and the tubes were incubated at 37°C. After 10 min, 10 µl proteinase K (20 mg/ml) was added along with 60 µl of lysis buffer (10% SDS) (Pospiech and Neumann, 1995). The cell lysis was performed at 65°C for 10-min. 100 µl of magnetic nanoparticles (10 mg/ml) was added to the cell lysate followed by addition of adsorption buffer (700 µl). After incubation for 5 min, an external magnet was applied to pelletize the magnetic particles bound with DNA. The magnetic pellet was washed twice with 50 % ethanol and dried. Finally, the pellet was completely resuspended in 50 µl of TE buffer as described in earlier experiment and magnetic particle bound DNA eluted by incubation at 65°C for 5 min with continuous agitation.

2.5.2 Optimized procedure of genomic DNA extraction from gram negative bacteria (*E. coli*)

E.coli (DH5α) was grown overnight at 37°C in Luria broth (LB). Late log phase bacteria (10^7 to 10^8 cells) were pelleted in a microcentrifuge at 12000 g for 30 seconds. The pellet was resuspended completely in 100 µl TE buffer (pH 7.5); 10 µl of lysozyme (2 mg/ml) was also added and the tubes incubated for 2 min at room temperature To the cell suspension, 12.5 µl of lysis buffer (10% SDS) plus 3 µl of proteinase K (20 mg/ml) and 2.5 µl of RNase A (1 mg/ml) was added to the cell suspension (Sambrook *et al.*, 1989). The tube was mixed by gentle inversion for 5-6 times and incubated for 10 min at 55°C. The cell lysate was now centrifuged at 2000 g for 5 min at 4°C. The supernatant obtained was transferred to a fresh tube; the amount of volume recovered everytime from the cell lysate was between 110-120 µl. To the cell lysate, 50 µl of magnetic particle (20 mg/ml) was added; followed by addition of adsorption buffer (200 µl). Tubes were kept for 5 min at room temperature. After incubation, external magnet was applied to pelletize the magnetic particles bound with DNA. The magnetic pellet was washed twice with 50 % ethanol and dried. Finally, the pellet was completely resuspended in 50 µl of TE buffer by repeated pipetting strokes and magnetic particle bound DNA eluted by incubation at 65 °C for 5 min as mentioned earlier. The magnetite particles

were then immobilized with a magnet and the clear supernatant containing DNA was transferred to a fresh tube.

The same procedure was employed for isolation of genomic DNA from *Salmonella typhii.*

2.5.3 Optimized procedure for genomic DNA extraction from yeast (*S. cereviceae*)

S. cereviceae were grown in yeast potato dextran (YPD) medium to a cell density of 10^8 cells per ml. Cells were pipetted in a 1.5 ml microcentrifuge tubes and pelleted by spinning at 12000 g for 30 seconds. The supernatant was discarded and the pellet was completely resuspended in a 100 µl sorbitol buffer containing zymolase (200U/ml), followed by incubation at 37°C for 10 min (Sambrook *et al.*, 1989). The cell suspension was then treated with 12.5 µl of lysis buffer (10 % SDS) plus 10 µl of proteinase K (20 mg/ml) and incubated at 65°C for 10 min. The tubes were then centrifuged at 8000 g for 5 min. The supernatant was transferred to a fresh tube; the amount of volume recovered from the cell lysate was about 120µl. To the cell lysate, 75 µl of magnetic nanoparticles (10 mg/ml) were added followed by 200 µl of adsorption buffer. After incubation at room temperature for 5 min, the magnetic pellet was washed twice with 50% ethanol and allowed to get dry. Finally, the pellet was resuspended in 50 µl of TE buffer and DNA eluted from magnetic particles as mentioned earlier.

2.5.4 Optimized procedure for genomic DNA extraction from *Dictyostelium discoideum*

D. discoideum cells were grown in HL-5 medium to a density of 2 x 10^6 cells per ml. 1.5 ml of cells were pipetted in a microfuge tube. The cells were pelleted by centrifuging at 5000 g for 5 min. The supernatant was discarded and pellet was resuspended in 100 µl of GTE buffer (15% (w/v) Glucose; 25 mM Tris; 10 mM EDTA, pH 8.0) (Nellen *et al.*, 1987). To the cell suspension, 100 µl of lysis buffer (0.4 % SDS) was added. The incubation time for lysis was optimized and found 10 min to be sufficient to bring about complete cell lysis. To the cell lysate, 50 µl of magnetic nanoparticles (10 mg/ml) and 250 µl of adsorption buffer were added. After allowing the DNA to bind the magnetic nanoparticles, the particles were separated by application of an external magnetic field. The magnetic pellet bound with DNA was washed twice with 50% ethanol and the pellet was allowed to dry. As mentioned in earlier experiments, the pellet was finally suspended in 50 µl of TE buffer for to eluted the bound DNA.

2.5.5 Optimized procedure for isolation of plasmid DNA from bacterial cells

E. coli strain (DH5α) harboring plasmid pBR322 was grown overnight at 37°C in Luria broth. The boiling lysate method described by Sambrook *et al.* (1989) was used as a backbone for devising a plasmid DNA isolation

method using magnetic nanoparticles. 1.5 ml of bacterial cells was pelleted in an eppendorf tube. 420 µl of lysis buffer (STET - 8% sucrose; 5% Triton X-100; 50 mM Tris-HCl, pH 8.0; 50 mM Na_2EDTA, pH 8.5) and 15 µl of lysozyme solution (10 mg/ml) were added to the pellet. The pellet was resuspended thoroughly by vortexing. Tubes were incubated on ice for 7 min. The tubes were then kept in the boiling water bath for 60 sec. After boiling, tubes were centrifuged at 10000 g for 10 min in a microcentrifuge at 4°C. Pellet was pulled out with the help of a toothpick. 50 µl of magnetic nanoparticles (20 mg/ml) and 450 µl of binding buffer were added to the supernatant. The tubes were incubated at room temperature for 4 min. Magnetic pellet/DNA complex was then separated by applying external magnetic field. The pellet was washed twice with 200 µl of 50% ethanol and then dried to allow alcohol to evaporate. Pellet was resuspend in the 50 µl of TE buffer and plasmid DNA eluted by pipetting up and down for at least 20-25 times, followed by incubation at room temperature at 25°C for 5 min. The supernatant containing the plasmid was transferred to a fresh tube.

2.5.6 Isolation of genomic DNA from plant tissue

Genomic DNA isolation from maize leaf was attempted using magnetite as solid-support. The method reported by Sharma *et al.* (2003) was used as a base to devise a procedure to extract plant genomic DNA without the use

of liquid nitrogen. 1 gm of leaf tissue was submerged in 5 ml of alcohol. The alcohol was allowed to evaporate; the tissue was then ground in a mortar and pestle with the addition of 2 ml of TE buffer. 50 µl of the crushed homogenate was taken in a 1.5 ml microfuge tube. A 7.5-µl volume of 10% (w/v) SDS, 10 µl of proteinase K (20 mg/ml) and 7.5 µl of RNase A (1 mg/ml) was added; the sample was vortexed and incubated at 60°C for at least 30 min. After incubation, the samples were centrifuged and the pellet discarded. The supernatant (about 70 µl) was transferred to a fresh tube, to this supernatant; 75 µl of magnetite particles (10 mg/ml) along with 145 µl of adsorption buffer was added. The tubes were incubated at room temperature for 5 min. After that a magnet was applied and the magnetite-DNA pellet was separated. The magnetite-DNA pellet was washed twice with 50% ethanol and the pellet was dried. Finally the pellet was completely resuspended in 50 µl of TE buffer. The DNA was then eluted as previously described by breaking the pellet and incubating at 65°C for 5 min.

2.5.7 Extraction of DNA from agarose gel using magnetite as solid-phase support

In an optimized procedure, DNA samples (23-kb) were electrophoresed on a 0.8 % agarose gel. The separated DNA was visualized on a UV transilluminator and the band of interest was excised with a sterile blade

and transferred to a microcentrifuge tube. 4 volumes of SSC (0.75 M sodium chloride, 0.0075 M sodium citrate, pH 7.0) buffer was added to the agarose plug and the tubes were incubated at 80°C for 5 min to allow agarose to melt. After incubation, immediately 20 µl of magnetic nanoparticles (10 mg/ml) were added from the stock, followed by addition of 200 µl of binding buffer (1.25 M sodium chloride and 10% PEG 6000). The suspension was mixed by inversion and allowed to stand at room temperature for 5 minutes. The magnetic pellet was immobilized by application of an external magnetic field and supernatant was discarded. The magnetic pellet was then washed twice with 50 % ethanol and dried. The pellet was then resuspended in 30 µl of TE buffer and magnetic particle bound DNA was eluted by incubation at 65 °C with continuous agitation. Finally, the particles were separated magnetically and supernatant was transferred to a fresh tube as described in earlier experiments.

2.5.8 PCR amplification of genomic DNA from mammalian cells

All PCRs were performed in a 50 µl reaction volume; 25 µl of PCR 2X master mix (Genetix, USA) was added. Five picomoles each of primers GAPDH-forward (5-ACAGTCCATGCCATCACTGCC-3) and GAPDH-reverse (5-GCCTGCTTCACCACCTTCTTG-3) were added per reaction for amplification of an amplicon in the glyceraldehyde 3-phosphate

dehydrogenase (GAPDH) gene. 100 ng of template DNA was used in the reaction mixture; PCR was performed on Techne thermal cycler PCR system (Roche, USA). PCR conditions were 4 min at 94°C; 34 cycles of 30 seconds at 94°C, 30 seconds at 61°C and 1 min at 72°C; and 10 min at 72°C. The PCR products were analyzed on 1.5% agarose gel stained with ethidium bromide.

2.5.9 Restriction endonuclease digestion of extracted DNA

A 10-µl volume of the eluted DNA solution was mixed with the manufacturer's reaction (Bangalore Genei, Bangalore, India) buffer (1 µl), sterile water (quantity sufficient) and incubated with the restriction endonuclease Eco R1/Hind III (1 µl, 10 units) at 37°C for 4 hours. The digestion mixture was analyzed directly after electrophoresis on 0.8% agarose gel.

2.5.10 Agarose gel electrophoresis of isolated DNA

Isolated genomic DNA from all the experiments were visualized on ethidium bromide stained 0.8% agarose gel. Electrophoresis was performed in 1X TAE and the resulted were documented with UVP BioImaging gel documentation system (UVP Cambridge, UK).

2.6 Covalent immobilization of alkaline phosphatase and streptavidin onto naked magnetic particles

As mentioned earlier (Section 2.1), the preparation of magnetic particles for protein immobilization required different conditions. In the present case 8M ammonium hydroxide instead of 1M sodium hydroxide was used to provide the alkaline conditions. The rest of the preparation protocol was same as described earlier.

In order to immobilize proteins onto the naked magnetic particles, a mixture of magnetic particles, carbodiimide-HCl (1-ethyl-3-3-dimethyl-aminopropyl) and protein (Calf alkaline phosphatase or Streptavidin) dissolved in phosphate buffer pH 6.3 was taken in the desired ratios. The immobilization was performed with two different methodologies:

1. Shaking method was adopted from Mehta *et. al.* 1997.
2. Sonication method adopted and modified from Kouassi *et. al.* 2005a.

In shaking method the mixture was kept on a shaker for 24 hours at 37°C in a shaking water bath at 50-100 rpm speed. In sonication method, the mixture was sonicated in an ultra sonic bath for 30 minutes at 4°C. After completion of this step, the magnetic particles bound with protein were attracted by application of an external magnetic field. The supernatant was removed in another tube to

remove all the unbound protein. The pellet was washed thrice with phosphate buffer pH 6.3 to remove non-specifically bound proteins. The washes were also collected and preserved for further analysis. Finally the pellet was resuspended in appropriate volume of phosphate buffer pH 6.3 and stored between 0-4°C until further use.

2.6.1 Determination of ALP activity

Free and immobilized ALP activity was assayed according to the procedure of Walter and Schutt (1974).

Principle: The substrate, p-nitrophenyl phosphate (PNPP) is hydrolyzed by ALP to p-nitrophenol and phosphoric acid. PNPP is colorless in acid or alkaline medium, whereas PNP is yellow in color in the alkaline medium and colorless in the acid medium.

The reaction is terminated by addition of NaOH. The intensity of yellow colored was estimated at 405 nm. The amount of p-nitrophenol liberated per unit time is a measure of the phosphatase activity.

Requirements:

- Stock substrate of PNPP (4 mg/ml)
- Glycine buffer (50 mM glycine, 1 mM $MgSO_4$, 0.1 mM $ZnSO_4$, pH 10)

- Glycine buffered substrate: Prepared freshly by mixing equal volumes of glycine buffer and stock substrate of PNPP.
- 0.05 M sodium hydroxide
- Enzyme: ALP (pure) and magnetic ALP particles

Protocol:

The sample tubes were read against blank to which the enzyme was added after NaOH.

Incubation volume: 0.525 ml; final volume: 5.525 ml

Sr. No.	**Buffered Substrate (ml)**	**ALP (ml)**	Incubate at 37° for 30 min	**0.05 N NaOH (ml)**	Read at 405 nm
Sample tube	0 5 ml	0 025 ml		5 0	

Activity expressed as µmol of PNP formed/min/ml of enzyme.

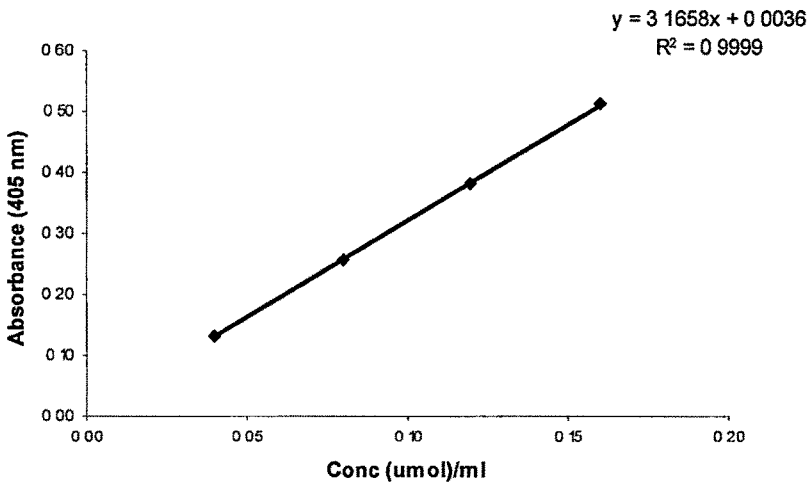


Figure 6 PNP calibration curve [Range 0 04-0 16 µmol/ml]

2.6.2 Procedure for performing streptavidin-biotin interaction

Biotin-binding capacity of magnetic streptavidin particles was determined by studying its interaction with biotinylated-alkaline phosphatase (B-ALP). The protocol for streptavidin-biotin interaction was followed as given in streptavidin biotinylated ALP complex kit (Bangalore Genei, Bangalore, India).

According to the procedure: 75 µl each of magnetic streptavidin particles and B-ALP (1:50 times diluted) were taken in a microfuge tube. 850 µl of reaction buffer (provided in the kit) was then added to the tube and incubated for 1 hour at 37°C. After the incubation step, the magnetic particles were separated by application of an external magnet. The supernatant was collected in another tube. The pellet was washed thrice with constant shaking in phosphate buffer (pH 7.5). The washes were also collected separately. Finally the magnetic pellet was resuspended in an appropriate volume of the phosphate buffer (pH 7.5). Biotin binding was confirmed by performing ALP activity tagged to biotin.

2.6.3 Procedure for stability study of immobilized preparation

In order to check the storage stabilities, immobilization of ALP and streptavidin was performed according to the method described in section 2.6 (materials and methods). Samples were then aliquoted in different vials with proper labels corresponding to the dates on which the activity is to be

performed and then stored at 0-4°C in a refrigerator. At defined time intervals, sample vials were thawed and the enzyme activity was checked. The results obtained were compared to the basal level (zero day) activity. In case of magnetic ALP particles, directly the ALP activity was measured as discussed in section 2.6.1, whereas magnetic streptavidin particles were first interacted with B-ALP; then ALP activity (tagged to biotin) bound to streptavidin was estimated.

2.6.4 Application of magnetic ALP particles for plasmid dephosphorylation

To perform plasmid dephosphorylation with magnetic ALP particles, pcDNA3 (size 5.4 kb) was first linearized by digestion with Hind III. The linear plasmid DNA obtained was then dephosphorylated (hydrolysis of 5'-phosphate residues of DNA) with ALP (pure) and magnetic ALP particles (immobilized). To check whether dephosphorylation was successful, the dephosphorylated DNA was then ligated with DNA ligase. The absence of self-ligation indicates that dephosphorylation was successfully accomplished. The protocol employed for restriction digestion and plasmid dephosphorylation are described in the following section.

2.6.5 Restriction digestion of plasmid DNA (pcDNA3) with Hind III restriction enzyme

Restriction digestion was performed according to the procedure described in Sambrook *et al.* (1989). A 15-µl volume of the plasmid DNA (2-3 µg) was mixed with the manufacturer's reaction (Bangalore Genei, Bangalore, India) buffer (2 µl), sterile water (2 µl) and incubated with the restriction endonuclease Hind III (1 µl, 10 units) at 37°C for 3-4 hours. The digestion reaction was monitored by electrophoresing the digested plasmid on agarose gel.

2.6.6 Protocol for dephosphorylation of 5'-termini of DNA

For this experiment, ALP immobilized on magnetite particles was procured from Fermentas Life Sciences (Lithuania, Lietuva). Dephosphorylation of 5'-termini of DNA was performed according to the protocol described by the ALP manufacturer. Plasmid DNA dissolved (1-20 picomoles of DNA termini) in 10-40µl of deionized water was mixed with 10x reaction buffer (5 µl), sterile water (q.s) and alkaline phosphatase (1U). The reaction mixture was incubated at 37°C for 30 min. After incubation was complete, the tube in which the dephosphorylation was performed using magnetic ALP particles; the reaction was terminated by application of a magnetic separator and the supernatant was transferred to a fresh tube. Whereas in case of pure ALP, reaction was stopped by heating at 85°C for 15 min,

followed by DNA extraction with phenol-chloroform method. The dephosphorylated plasmid was then checked for self-ligation using DNA ligase according to the protocol described by the authors (Sambrook and Russell, 2001). All the plasmid DNA was visualized on 1% agarose gel stained with ethidium bromide.

2.6.7 Bradford dye binding method for protein estimation

The binding of ALP to magnetic particles was judged by estimation of protein in unreacted and residual fractions. Bradford dye binding method was used to estimate the amount of protein (Bradford, 1976).

Priniciple: Bradford dye (Commassie Blue G-250) at acidic pH (< 1) has red brown color. However, when it binds to protein the blue color is restored, which is attributed to the shift in pKa of the bound dye. So, a solution containing protein when mixed with dye gives blue color that is read at 595 nm.

Reagent:

- Bradford dye: 600 mg of Commassie Blue G-250 was dissolved in 1 litre of 2% perchloric acid and filtered to remove undissolved dye. The solution once prepared is stable indefinitely.

Procedure: To 1.5 ml of sample containing up to 50 μg protein 1.5 ml Bradford dye was added. The blue color formed was read at 595 nm after 2 min and before 30 min of the addition of Bradford dye.

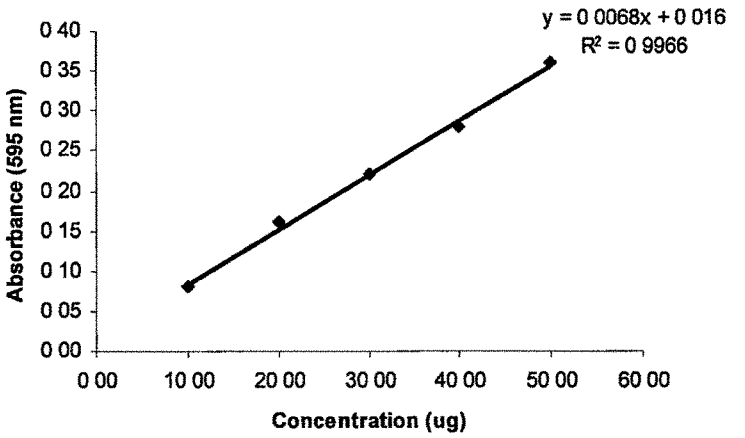


Figure 7. Standard curve of bovine serum albumin (BSA) by Bradford dye binding method [Range 10-50 µg]

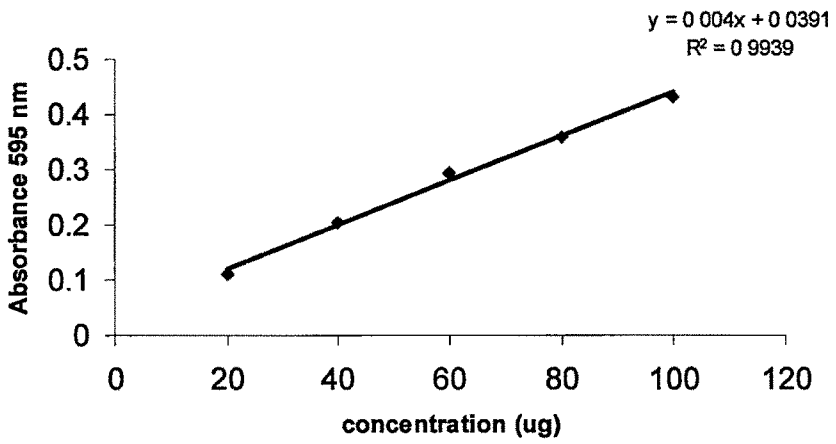


Figure 8. Standard curve of ALP by Bradford dye binding method [Range 20-100 µg]

2.7. Magnetoliposomes as drug delivery carrier

2.7.1 Preparation of magnetoliposomes

Magnetoliposomes consist of small, magnetizable iron oxide cores that are wrapped by a phospholipid bilayer. In the present study, use of magnetoliposomes for site-specific drug targeting was evaluated.

Materials: Soyabean phosphatidyl choline (Kind gift from Nabros Pharma, Ahmedabad, India), Cholesterol (Sigma, USA), Di hexa decyl phosphate (DHDP) (Sigma, USA), chloroform, isopropyl alcohol, Doxorubicin (Kind gift from Sun Pharmaceuticals Ltd., Vadodara, India) and magnetic nanoparticles (prepared as described above in section 2.1).

Apparatus: Round bottom flask, rotary evaporator, vortex machine, bath sonicator.

Protocol

The method described below was modified and optimized using the basic framework of protocol reported by Sangregorio *et al.*, (1999) and Babincova *et al.*, (2002). Phospholipid, cholesterol and DHDP were dissolved in chloroform and mixed in a ratio of 5:4:1. Magnetic particles prepared with either 8M NH_4OH or 1M NaOH were found to be suitable for this part of the work. Magnetic nanoparticles were stored in methanol and just before the preparation of magnetoliposomes, they were dispersed in chloroform. These magnetic particles were added to the lipid mixture to

a final concentration of 1.5 mg per ml of lipid solution. The solvent was evaporated in a rotary evaporator under nitrogen atmosphere. The dry lipid film obtained was then reconstituted with 1.25 ml of isopropyl alcohol. The lipid solution now in isopropyl alcohol was then injected in 300 mM citrate solution (pH 4.0) under sonication. This resulted in formation of magnetoliposomes, while the extra vesicular iron was removed by application of weak magnets. The magnetoliposomes prepared were stored at 4°C for further use.

2.7.2 Characterization of magnetoliposomes

Transmission electron microscopy was used to determine the size of magnetoliposomes. The magnetoliposomes were characterized in terms of phospholipid/Fe_3O_4 ratio. The phosphorus estimation was performed by Fiske Subbarow method (Fiske and subbarao, 1925) and iron estimation was performed using 2,2' dipyridyl method (Ramsay, 1957).

2.7.3 Drug encapsulation in magnetoliposomes

Doxorubicin (DOX) was encapsulated in the core of the liposomes in response to the transmembrane pH gradient (Mayer *et al.,* 1990). Magnetoliposomal suspension was taken and the exterior pH was adjusted to 7.5 with 400 mM sodium carbonate. The suspension was diluted with buffer to achieve lipid concentration of 10 mg/ml. Doxorubicin was then added from a stock solution and incubated with the magnetoliposomal

suspension at 60°C for 10 min; so as to allow complete entrapment of doxorubicin inside the core of magnetoliposomes. The drug to lipid ratio was kept at 0.2%.

Free and encapsulated doxorubicin in magnetoliposomal system was determined spectrophotometrically. This procedure utilizes the pronounced change in absorbance (absorbance maxima 480 nm, pH 7.5; 550 and 592nm pH 10.5) observed on increasing the pH of doxorubicin solution from 7.5 to 10.5. This is also followed by a change in color from red-orange to blue. The liposomal/magnetoliposomal doxorubicin sample was diluted to achieve the drug concentration between 50 and 100 μM. The measurement were done according to the following sequence:

1. The absorbance at 600 nm of the diluted magnetoliposomal suspension was adjusted to zero.
2. The pH of the sample was increased to 10.5 with 1.0 M NaOH (0.02 ml per 1.0 ml of sample) and the absorbance at 600 nm was recorded with in 2 min.
3. The spectrophotometer was then zeroed against 0.2% Triton X-100 solution
4. The absorbance was then determined at 600 nm of the magnetoliposomal doxorubicin sample to which Triton X-100 (0.01 ml of 20% Triton per ml of sample) had been added.

5. Free/total doxorubicin ratios were calculated as the absorbance at 600 nm upon NaOH addition divided by the absorbance after Triton X-100 addition.

2.7.4 Storage stability of magnetoliposomal formulation

For this purpose, magnetoliposomes were prepared by the method described in section 2.7.1 (Materials and methods). The magnetoliposomal and liposomal suspension were then aliquoted in a vial labeled appropriately. These vials were then placed in a refrigerator at 0-4°C. At different time interval, the appropriate vials were removed and thawed to room temperature and the amount of free and total DOX was determined by spectrophotometric method.

2.7.5 *In vitro* study to evaluate drug leaching from liposomes/magnetoliposomes

In order to check the dilution induced release and effect of serum components on integrity of drug-loaded liposomes and ML following experiments were carried out:

2.7.6 Dilution induced drug leakage

For this study, liposomal/magnetoliposomal formulations were prepared as outlined in section 2.7.1. As mentioned earlier, DOX was encapsulated in response to the transmembrane pH gradient. The resulting drug-loaded liposomes/MLs were transferred into 25 mm diameter dialysis tubing (10,000-12,000 molecular weight cut-off) and the samples (3 ml) were

dialyzed against 1 liter of phosphate buffered saline at 37°C for 48 h. At the indicated time points, 150-µl of samples were withdrawn from the dialysis bag and assayed for DOX as described above.

2.7.7 Effect of serum component on drug loaded liposomes/magnetoliposomes

Liposomal/magnetoliposomal formulation (DOX encapsulated at a concentration of 1mg/ml) and fetal calf serum (Sigma Chemicals, USA) were mixed in 1:1 proportion (final concentration of serum 50%) and incubated at 37°C for a period of 24 hours with intermittent shaking. At different time intervals, samples were thawed and free and total DOX was determined spectrophotometrically.

2.7.8 Study of flow characteristics of liposomal / magnetoliposomal formulation

In order to study the flow properties of the liposomal formulation; an *in vitro* closed circulatory model system was devised using the HPLC peristaltic pump. This was operated in a purge mode with the flow rate of 10 ml per min. The basic scheme of this model is as shown in **figure below**

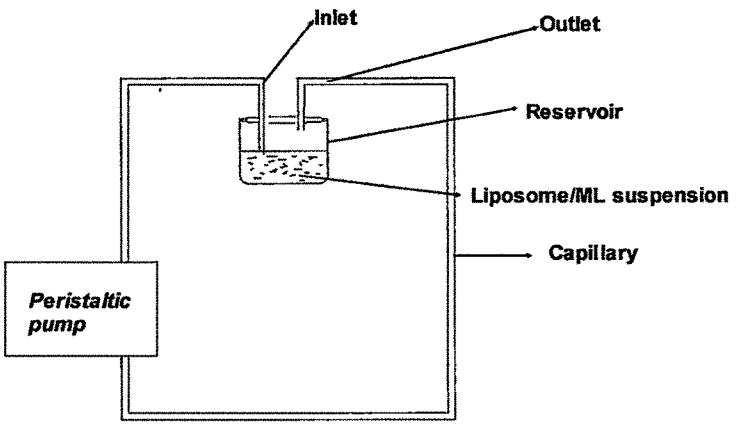


Figure 9. *In vitro* closed circulatory model system

(a) Reservoir contains liposomes or magnetoliposomes encapsulated with doxorubicin and suspended in appropriate carrier liquid (normal saline or 5% dextran T500).

(b) The **inlet** capillary from the reservoir was connected to the peristaltic pump.

(c) The **peristaltic pump** drives the fluid into another capillary of defined diameter (0.3mm) at a desirable flow rate (10ml/min).

(d) The **outlet** capillary through which the fluid was circulated back to the reservoir (to make a closed circulatory system). At different time intervals samples were collected from the outlet capillary. The total length of the capillary was 1 meter and diameter 0.3mm.

Liposome/ ML formulation encapsulating 1mg/ml dox was diluted with saline/ dextran T 500 (5%) in 3.5: 6.5 ratio and then passed in the circulatory model system.

2.7.9 *In vivo* biodistribution of DOX loaded liposomes / magnetoliposomes

Healthy female adult Charles Foster strain of rats weighing 150-200 g obtained from department animal house were used for *in vivo* animal experimentation. Animals were divided into three groups: (1) Free DOX (2) Liposomal DOX (3) Magnetoliposomal DOX. Formulations were injected intravenously through caudal vein with concentration of doxorubicin 5-mg/kg body weights in all sets. After 24 hours animal was anaesthetized, blood was collected from the orbital sinus and cardiac puncture and then the animal was sacrificed by cervical dislocation. Liver, lungs, heart, kidney and spleen were removed and washed thoroughly in saline. A 10% homogenate was made in acid alcohol (0.3 N HCl in 70% absolute ethanol) and incubated at 4°C for extraction of doxorubicin. After 24 hours supernatant was collected in a fresh tube and doxorubicin was estimated spectrophotofluorimetrically. The fluorimetric estimation of doxorubicin was performed according to the method described by Gabizon *et al.* (1989). The excitation and emission wavelength were 490 and 570 nm, respectively.

2.7.10 Determination of DOX encapsulated liposome / magnetoliposome concentration in plasma

In addition to the tissue, DOX was extracted and estimated from the plasma with the protocol described by Mayer *et al.* (1997).

Plasma was separated from the whole blood by centrifuging at 1500 g for 10 min. 800 µl of plasma was mixed with 100 µl of 10% SDS. The volume was made to 1.0 ml with double distilled water. An equal amount of chloroform isopropyl alcohol (1:1) was added. The tubes were mixed thoroughly by constant vortexing to extract doxorubicin. The tubes were then incubated in -20°C overnight. Next day tubes were thawed and again mixed by vortexing. The tubes were allowed to stand at room temperature till the phase separation occurs. Finally, the organic phase was collected and examined using spectrofluorimeter with excitation wavelength of 470 nm and emission wavelength of 590 nm.

2.7.11 *In vivo* efficacy of DOX loaded liposomes / magnetoliposomes in tumor regression

Sarcoma cell lines (WEHI 164) from liquid nitrogen were grown in a flask for at-least 24 h to allow the cells to adapt to the condition. Once the monolayer was formed, the spent media was decanted; cells were washed twice with PBS to remove traces of media. Adherent cells were trypsinized to detach them from the support and then resuspended in minimum amount of sterile DMEM medium. Cell clumps were broken with the use of a

syringe and finally single cell suspension was prepared. Viability of cells was checked in a haemocytometer after staining with Trypan blue. 5 x 10^5 cells were injected intradermally in each mouse on the dorsal side. After the tumor became palpable (usually after 6-7 days), diameter in three perpendicular planes were measured on alternate days with vernier calipers. After two weeks the two animals were sacrificed and tumor was excised, chopped with a sterile scalpel to small pieces and then injected again in fresh mice to establish tumor bearing animal model. Animals with necrotic and irregular shaped tumor were excluded from the study. The day on which the experiment started was considered as "Zero day". Mice were injected with a dose of 9-mg/kg body weight of DOX. Since it was a preliminary study, the formulation was injected every alternate day. The efficacy of the formulation was examined by measuring the reduction in the size of the tumor.