4.1 Covalent immobilization of calf alkaline phosphatase on magnetic nanoparticles

Immobilization of macromolecules on various inert support matrices has been known since long (Nelson and Griffin, 1916; Silman and Katchalski, 1966; Vandamme, 1983; D'souza, 1999). However, during recent years magnetic support has been increasingly used for immobilization of enzymes and proteins. The first use of magnetic polymers as supports for biomolecules was reported in late 1970s (Chaplin and Kennedy, 1976; Horisberger, 1976; Munro *et al.*, 1977).

Magnetic supports for immobilization purposes can be either prepared by co-polymerization of magnetic particles along with the synthesis of the supporting matrix (Horisberger, 1976; Robinson *et al.*, 1973) or the magnetic particles can itself be coated with common support materials such as dextran or agarose, and the biomolecule covalently linked to their surfaces (Rusetski and Ruuge, 1990)

In most of the earlier studies, coated magnetic particles have been used as support matrix. Direct immobilization by covalent linking on naked (uncoated) magnetic particles has also been attempted for proteins such as bovine serum albumin, glucose oxidase, streptokinase, chymotrypsin, dispase, neutral protease and cholesterol oxidase using carbodiimide as a coupling agent (Mehta *et al.*, 1997; Koneracka *et al.*, 2002; 1999; Kouassi *et al.*, 2005a; 2005b). As already mentioned in chapter 2, there are several

advantages envisaged for the use of such naked particles. In the present study, an attempt was made to immobilize alkaline phosphatase (ALP) and streptavidin on magnetic nanoparticles using 1-[3-(dimethylamino)propyl]-3-ethylcarbodiimide hydrochloride (carbodiimide, CDI) as a coupling agent.

ALP was selected for immobilization since it was easier to monitor the activity using p-nitrophenyl phosphate as substrate at 405 nm (Walter and Schutt, 1974). Additionally, magnetic ALP particles were also checked for application in molecular biology experiment for dephosphorylation of plasmid DNA. Dephosphorylation of 5'-phosphate residue from both termini of the linear, double stranded plasmid DNA with ALP minimizes the recircularization (self-ligation) of plasmid. In conventional dephosphorylation procedure, once the ALP treatment is completed, the enzyme reaction is terminated by incubation at 65°C for 30 min or by protease treatment followed by phenol chloroform extraction of dephosphorylated DNA. 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 an external magnetic field. Moreover, immobilized ALP can be reused for another cycle of reaction.

Streptavidin-biotin interaction has been exploited for various applications such as diagnostic assays, biomedical research, cell separation and nucleic

acids isolation. Streptavidin coated magnetic beads (or microparticles) are available commercially from several companies, however they are expensive to be used for routine purposes. Therefore, in the present study direct binding of streptavidin was attempted on naked magnetic nanoparticles. The idea was to achieve high streptavidin surface loading with greater biotin-binding ability, so that less quantity of particles is required per assay. Additionally, the present method for protein immobilization can be performed in any laboratory without the requirement of sophisticated instruments and is cost-effective.

4.1.1 Preparation of magnetic particles

As mentioned earlier, the magnetic particles used for protein immobilization were prepared with 8M NH_4OH according to the protocol described by Mehta *et al.* (1997). The preparation procedure of these particles differed from that applied for DNA isolation in the use of ammonium hydroxide instead of sodium hydroxide for creating alkaline conditions. The mechanism for direct coupling of protein on magnetite particles is attributed to the presence of chemisorbed hydroxyl (-OH) group on the surface of magnetite at a pH between 6-10 (Koneracka *et al.*, 2006; Mehta *et al.*, 1997; Bacri *et al.*, 1980). Recently, it has also been suggested that the magnetite particles prepared by co-precipitation of Fe^{+2} and Fe^{+3} using concentrated ammonia solution displays an amino functional group that might be involved in direct coupling of protein using carbodiimide

(Kouassi *et al.*, 2005a; Huang *et al.*, 2003; Liao and Chen, 2002). The possible mechanisms of protein binding to naked magnetite are shown in figure 34. However, the particles prepared using NaOH requires further modification for example, thiophene acetylation was performed when glucose oxidase (GOD) was covalently linked on such magnetic particles. In that study bound enzyme efficiencies achieved was only 66-72% as against 94-100% for amino-functionalized particles (Kouassi *et al.*, 2005). Also, the authors reported that the GOD immobilized on magnetite particles prepared with sodium hydroxide via thiophene acetylation (GOD-Fe_3O_4-I) had lower stability at higher temperature and pH compared to GOD immobilized on particles prepared with NH_4OH (GOD-Fe_3O_4-II). Furthermore, the storage stability of GOD-Fe_3O_4-I was also lower than GOD-Fe_3O_4-II when stored at 25°C (Kouassi *et al.*, 2005a). Therefore, in the present study only magnetite particles prepared with NH_4OH were used to immobilize ALP and streptavidin

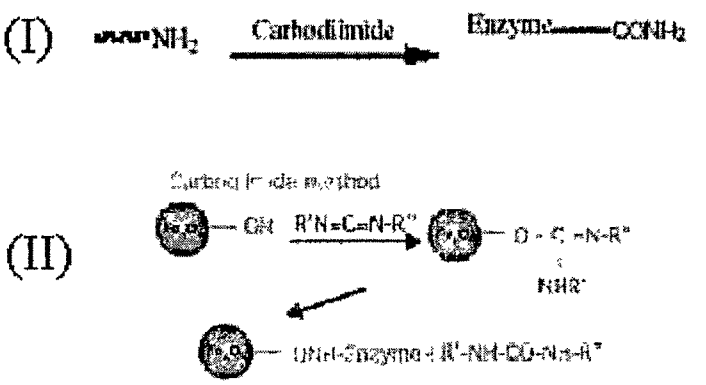

Figure 34. Mechanism of direct linking of protein on naked magnetite (I) Binding of protein via amino group on magnetite particles (II) Binding of protein via hydroxyl group on magnetite particles

The particles prepared for the present work were characterized using transmission electron microscopy and were found to have spherical shape with the particle size range of 10-50 nm. Fourier Transform infrared (FTIR) spectroscopy was also performed to check the consistency of the prepared magnetite (presence of vibrational bands at 632 and 583 cm^{-1}). These magnetite particles were used for immobilization of both ALP and streptavidin.

4.1.2 Immobilization of ALP onto magnetic particles

As already described in materials and methods, ALP was immobilized onto magnetic particles using two different strategies viz., shaking and sonication methods. The binding of ALP is affected by the concentration of carbodiimide as well as the amount of magnetic particle. To determine the

optimum proportion of all the three, reaction mixtures containing magnetic particles (x), CDI (y) and ALP (z) in phosphate buffer (3 mM, pH 6.3) were prepared using different weight fractions of x:y:z. In all cases, magnetic particles were separated by placing the flasks on a rectangular permanent magnet having surface magnetization equal to 4500 (0.45 T) gauss. The binding of ALP was judged from the estimation of protein in the unreacted fraction by the dye binding method (Bradford, 1976). Initially, the concentrations of magnetic particles and carbodiimide were standardized. When the ratio of magnetite:CDI:ALP were 2:2:1 and 3:2:1, the ALP binding efficiency was less than 25%. Additionally, there was substantial loss of enzyme specific activity. However, when the ratio of magnetite:CDI:ALP was 3:1:1, the efficiency of ALP binding was in the range of 80 - 90% with both shaking and sonication method, respectively. The following table discusses the ALP binding and activity retained on magnetite at a ratio of 3:1:1.

Table 18. Percentage ALP bound and activity retained onto magnetic nanoparticles with two methods (3.1:1)

x:y:z (mass ratio)	Method 1*		Method 2#	
(n=3) in duplicate	% Protein bound	% Activity retained	% Protein bound	% Activity retained
3:1:1	94	24.53	84	30.02

Where x = magnetic nanoparticles, y = CDI and z = ALP

* Shaking method, # sonication method

Although a higher amount of protein binding occurs with both the methods at a ratio of 3:1:1, there was substantial loss of enzyme's specific activity (Table 18). The activity yield was found to be about 24% with shaking method and 30% for sonication method. This is a common observation that is reported for enzymes immobilized by covalent bonding. Similar loss of activity (> 70%) has been reported when glucose oxidase was covalently linked on magnetic particles in the presence of carbodiimide (Koneracka *et al.*, 2002). Likewise, when invertase was immobilised on silica particles with glutaraldehyde linking, the activity yield was only 24% (Bergamasco *et al.*, 2000). Moreover, when papain was covalently coupled onto the surface of a vinyl alcohol/vinyl butyral copolymer (PVB) membrane the activity yield was only 6% (Zhuang and Butterfield, 1992).

Since with the sonication method the % ALP bound was lesser than shaking method, the sonication time was optimised so as to allow more coating of ALP onto magnetic particles with the ratio of 3:1:1. Two different time points i.e. 30 and 60 min were tried and the results are summarized below.

Table 19 Effect of different sonication time on the % ALP bound and activity recovered (3 1 1)

Samples	**Set A (30 min)***		**Set B (60 min)***	
n=3 (in duplicate)	% Protein bound	Activity (%)	% Protein bound	Activity (%)
ALP bound on magnetic particles	82	32	84	29
Leftover ALP (unbound + residual)	18	58	16	59

* Magnetite: CDI:ALP mass ratio of 3:1:1

From the results obtained (table 19), it is evident that increasing the sonication time does not have any influence on the amount of protein getting immobilized. So, it was inferred that once a particular amount of ALP is coated onto the magnetic particles, further increase in sonication time has no effect. Hence for all further experiment the standardized 30 min sonication time was used.

In next set of experiments, the amount of alkaline phosphatase was reduced, whereas the concentration of magnetic particles and carbodiimide kept constant. The ratio of magnetite:CDI:ALP of 3:1:0.4 and 3:1:0.2 were attempted. The results of these ratios are given below:

Table 20: Percentage ALP bound and activity retained onto magnetic nanoparticles with two methods (3 1 0 4 and 3 1 0 2)

x:y:z (mass ratio)	**Method 1***		**Method 2#**	
n=3 (in duplicate)	% Protein bound	% Activity retained	% Protein bound	% Activity retained
3:1:0.4	100	38	99	43.5
3:1:0.2	100	20.7	100	30.4

With both these methods, 100 % of the added ALP was bound to the magnetic particles at a ratio of magnetite:CDI:ALP of 3:1:0.4 and 3:1:0.2 at room temperature and pH 6.3, respectively (Table 20).

However, the % enzyme activity retained with the ratio of 3:1:0.2 was similar to that obtained with 3:1:1. Of all the above ratios checked, the highest % activity retention was found with the ratio of 3:1:0.4 and it was about 38% and 43% with shaking and sonication method, respectively. In all these experiments, the activity loss with shaking method was higher than sonication method. This could possibly be attributed to the exposure

of enzyme at 37°C for extended period of time (for e.g. 24 h), whereas with sonication method the enzyme was exposed to ultrasonic wave for only 30 min at 4°C. Thus, the optimum conditions for the binding of ALP to the magnetic particles were observed at a ratio of magnetite:CDI:ALP of 3:1:0.4 with both shaking and sonication method

Table 21: Selected examples where enzymes have been covalently linked to solid matrices and comparison with present work

Inert support	**Immobilized protein**	**% Activity retained**		**Reference**
Magnetic particles (Fe_3O_4) Reactant ratio: Magnetite:CDI:protein	Alkaline phosphatase	Method 1*	Method 2#	Current study
a) 3:1:1		24.5%	30%	
b) 3:1:0.4		38%	43.5%	
c) 3:1:0.2		20.6%	30.4%	
Chitosan microparticles (different molecular weight)	Alkaline phosphatase β-galactosidase Maltogenase Pullulanase	49.5% ($7.5x10^5$ – chitosan M_w) 89.9% ($7.0x10^4$ – chitosan M_w) 61.6% ($7.5x10^5$ – chitosan M_w) 79.1% ($7.5x10^5$ – chitosan M_w)		Zubriene *et al.*, 2003
Magnetic particles	Glucose oxidase	30%		Koneracka *et al.*, 2002
Silica particles	Invertase	24%		Bergamasco *et al.*, 2000
Magnetic poly(styrene) particles	Glucoamylase	70%		Bahar and Celebi, 1999
Magnetic particles (Fe_3O_4)	Dispase Chymotrypsin Streptokinase	80% 50% 55%		Koneracka *et al.*, 1999

Polystyrene microspheres	β-lactamase	20.5%	Wu *et al.*, 1998
Vinyl alcohol/vinyl butyral copolymer (PVB) membrane	Papain	6%	Zhuang and Butterfield, 1992
Tresyl activated silica	Lipase	26-34% (Direct coupling) 56-67% (bound via polyethylene glycol spacer arm	Stark and Holmberg, 1989
Cyanogen bromide-activated agarose	Metapyrocatechase	30 %	Iwaki and Nozaki, 1982
Magnetic sepharose beads	Trypsin	30%	Mosbach and Andersson, 1977

* Shaking method; # Sonication method

As described in table 21, the enzyme activity retained in the present study is comparable to that reported earlier with other proteins. However, important points to be noted from the comparison are: the activity of immobilized protein depends on its type, property of the support matrix and the orientation of the bound protein with respect to the solid-support (the microenvironment). From the selected examples of enzyme immobilization; it indicates a very high loss of functional activity by covalent linking to solid-support. The % activity retained ranges from as low as 6% to as high as 90%. The differences are very large, however the retention of functional activity for an immobilized enzyme depends on its nature and the size of the support matrix. For example, when Zubriene *et al.* (2003) attempted immobilization of several types of hydrolases on chitosan microparticles of different molecular weight; the activity yields were higher with chitosan molecular weight of 7.5×10^5 and drastically reduced with molecular weight of 2×10^6.

Therefore, in the present study the apparent loss of enzyme activity with both the methods (i.e. shaking and sonication) in all the experiments described above could be due to the denaturation of the protein during the covalent coupling reaction. Additionally, the 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). Yet another possibility is that covalent bond formation could take place through residues, which though not

actually present at the active site may be essential for maintaining the active conformation of the active site (Koneracka *et al.*, 2002). One or more of these factors could have contributed to the loss of enzyme activity.

4.1.3 Confirmation of ALP binding with FTIR spectroscopy

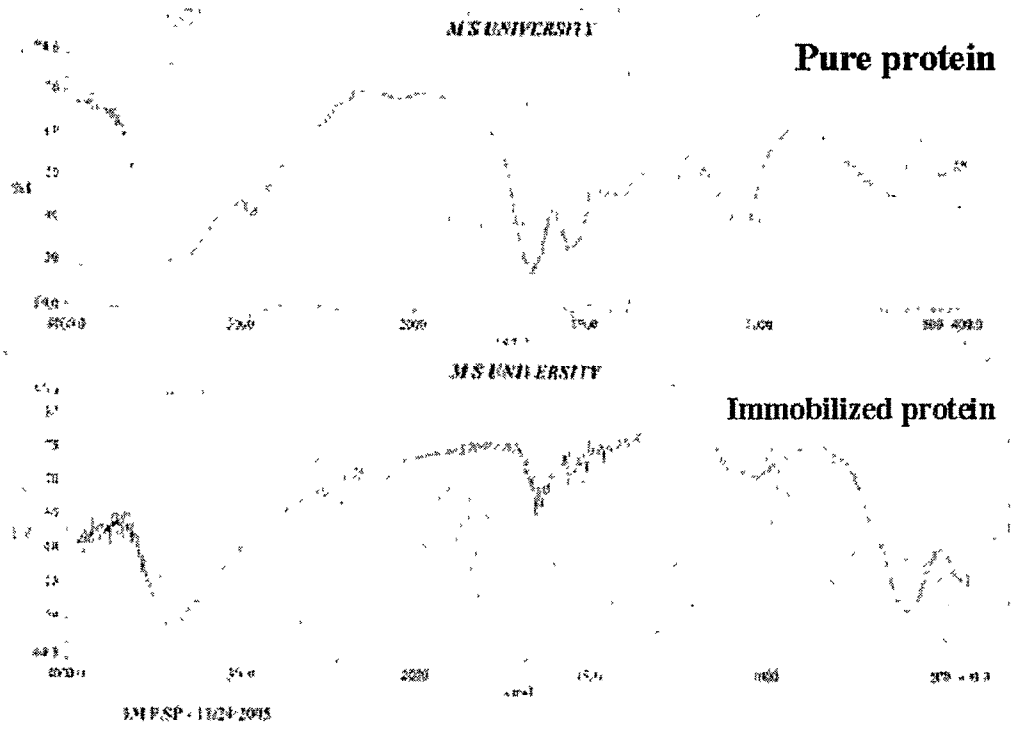

Figure 35. FTIR spectra of ALP alone (top) and ALP bound to magnetic particles (bottom)

Fig. 35 shows the FTIR spectral characteristics of ALP bound to magnetic particles. The characteristic bands of protein at 1647 and 1542 cm^{-1} assigned to amide I and amide II, respectively are visible in the spectra of pure ALP and ALP-Fe_3O_4. These peaks are associated with two additional peaks in the region 1420-1300 cm^{-1}, typical of carboxylate groups, from the enzyme (Kouassi *et al.*, 2005a). The characteristic peaks of pure protein in the spectra of ALP-Fe_3O_4 indicates presence of enzyme on the particles.

4.1.4 Stability of immobilized ALP

Loss of enzyme activity on storage is a major concern for long-term stability of immobilized enzyme preparations. Ideally, once the batch of immobilized protein is prepared, it is important that the functional activity of the protein is maintained, so as to use it for long-term applications. The storage stability of the immobilized ALP was examined for 16 weeks. Figure 36 and 37 shows the storage stabilities of free (unbound) ALP and immobilized ALP with shaking and sonication method respectively. Each data point is the result of three independent observations done in duplicates. In the case of shaking method, with ratios of 3:1:1 there was an increase in the activity of ALP, which was quite high compared to the basal activity (zero day). This showed peak activity at 3 weeks post-immobilization, followed by decrease in activity. However, the activity still remained higher than the basal level even at 16 weeks. While with 3:1:0.4 and 3:1:0.2 concentrations, the rise in activity was not significant,

showing peak activity at 2 weeks after immobilization followed by a gradual decrease and finally the activity falling below the basal level.

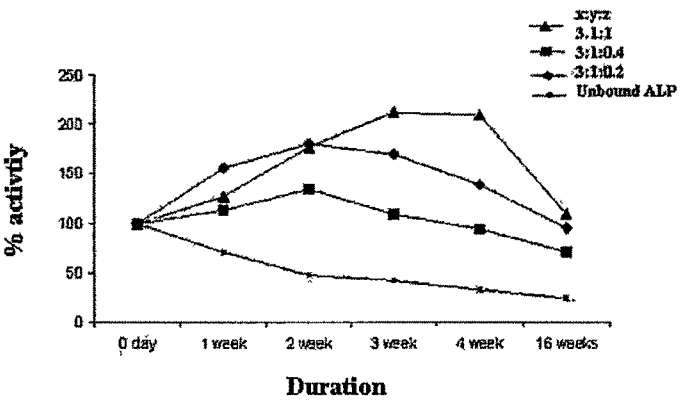

Figure 36. Storage stability of free and immobilized ALP covalently linked to magnetite with the shaking method x = magnetite, y = CDI and z = ALP

However, the free (unbound) ALP activity constantly decreased in both the sets (i.e. shaking and sonication) during the storage period and finally only about 20% of the activity was retained at 16^{th} week (Fig. 36 and 37) This indicates that the stability of the enzyme was found to improve upon binding to magnetic nanoparticles but the most significant improvement in stability was observed for the ratio of 3:1:1 in both the sets.

Immobilization using sonication method with ratio of 3:1:1, showed a similar trend as seen in shaking method with ALP activity showing peak at 3 week post-immobilization, and then declining at 16 week and remaining just below the basal level. With ratio 3:1:0.4 the enzyme activity declined after 1 week,

after which it remained almost constant up to 4 weeks at 60% with a slight decrease at 16 weeks. Similar pattern was observed with ratio of 3:1:0.2 with decrease in enzyme activity every week and finally retaining about 40% activity even at 16 weeks.

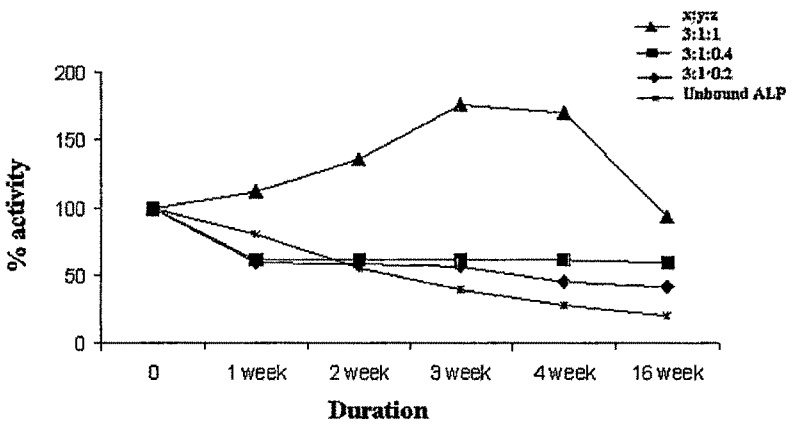

Figure 37. Storage stability of free and immobilized ALP covalently linked to magnetite with the sonication method x = magnetite, y = CDI and z = ALP

As seen from the results, the stability study indicates an interesting pattern of increase in activity above the basal level in both shaking and sonication method, and this was more consistent with sets in which higher amount of protein (3:1:1) was used from immobilization. It is a well-studied fact that, the tertiary structure of an enzyme is maintained primarily by a large number of weak non-covalent bonds. In practical terms, an enzyme molecule is a very delicate and fragile structure. If the molecule absorbs too much energy, the tertiary structure will disrupt and the enzyme will be denatured, that is it will

lose the catalytic activity (Segel, 2004). The low basal activity on zero day could be attributed to the stressful condition the protein encounters during the coupling reaction. These conditions could lead to disruption of vital bonds (such as hydrogen bonds, hydrophobic interactions), which maintains the optimal conformation of the enzyme. The plausible explanation for the increase in activity after 1 week could be due to the reformation of those bonds or interactions that were broken earlier, this might allow the enzyme to achieve the optimal orientation for binding the substrate and hence increase in activity was seen. The decrease in enzyme activity observed thereafter (i.e. 4 weeks) and in other sets (3:1:0.4 and 3:1:0.2) could be due to the natural course of protein denaturation. The extended stability of the immobilized ALP against 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) and thereby providing higher stability to the enzyme. Such improvement in stability upon immobilization as compared to free enzyme has also been reported by Kouassi *et al.* (2005a), where the authors attempted covalent linking of glucose oxidase on magnetic nanoparticles. Additionally, Grabarek *et al.* (1990), suggested that the efficiency of protein coupling via carbodiimide activation could be attributed to the potential of carbodiimide to activate the carboxylic acid side chains partially buried at the surface or in active sites of the enzyme, favoring the formation and the stability of the amide bond.

From the present comparative study, it is concluded that for long-term storage it is favorable to use higher amounts of protein (i.e. 3:1:1 ratio of magnetite, carbodiimide and ALP) for immobilization with both shaking & sonication method. This is in agreement with the study where decreased stability of the enzyme preparations was noticed when low enzyme loading was used (Wehtje *et al.*, 2004). Moreover, in sets with ratio of 3:1:0.4 and 3:1:0.2 there was a visible clumping of magnetic particles observed at the 16^{th} week. Thus, results indicate that shaking method appeared to be better than sonication method and use of higher amounts of protein for coupling could be recommended for long-term stability of immobilized preparation.

4.1.5 Use of immobilized ALP for plasmid dephosphorylation (molecular biology application)

Magnetic particle immobilized ALP was also checked for its efficiency in molecular cloning applications for dephosphorylation of plasmid DNA. Use of magnetic ALP particles reduces procedure time, also avoids exposure of reaction mixture to high temperature. Additionally, it also prevents the need to extract DNA once dephosphorylation reaction is accomplished; this allows direct use of the reaction mixture for ligation step. Also, immobilized ALP can be reused for another cycle of reaction.

In the present study plasmid (pcDNA3, size 5.4 kb) was restriction digested (Fig 38) and then dephosphorylated using immobilized ALP. The dephosphorylated plasmid DNA was further kept for ligation using DNA

ligase. If the plasmid DNA has been completely dephosphorylated then it cannot be re-ligated using DNA ligase, because for re-ligation the plasmid requires 3'-OH and 5'-P group to form the phosphodiester bond. After the ligation reaction, plasmid DNA was analyzed directly on agarose gel electrophoresis.

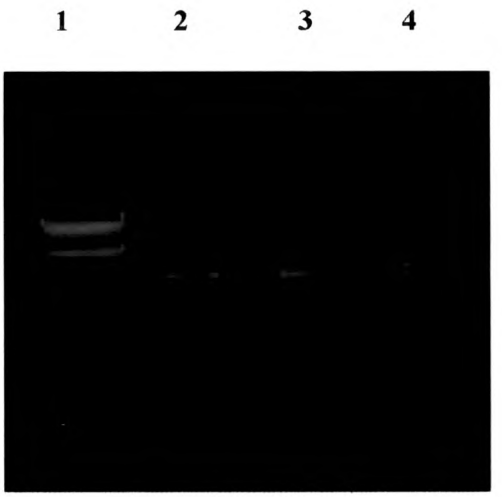

Figure 38. Restriction digestion of pcDNA 3. Lane 1: λ phage DNA/HindIII digest; Lanes 2-4:Linear band of pcDNA3 after digestion with Bam H1

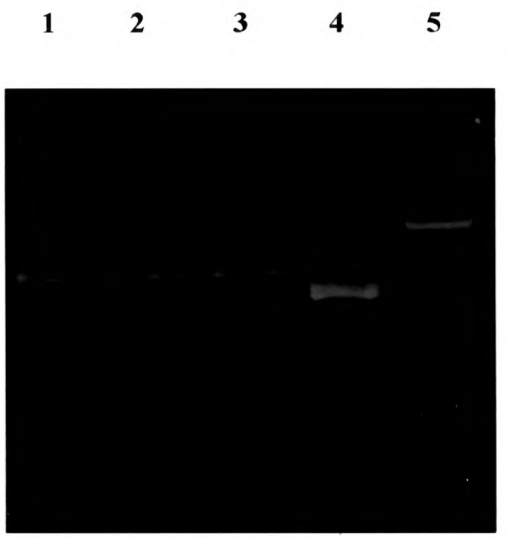

Figure 39. Agarose gel electrophoresis of plasmid DNA (pcDNA 3 – 5.4 kb) following ligation reaction. Lanes: 1-2 = Linear band of non-ligated plasmid (treated with immobilized ALP); 3 = Linear band of non-ligated plasmid (treated with pure ALP); 4 = Ligated plasmid DNA (non-dephosphorylated positive control); 5 =. λ phage DNA/Hind III digest.

From the gel pictures (Figure 39) it was confirmed that dephosphorylation of the plasmid DNA (pcDNA3, 5.4kb) was successfully performed by the immobilized ALP. Only a single band (corresponding to the linear form of plasmid) was observed with the DNA that was dephosphorylated with the immobilized ALP as comparable to that obtained with pure ALP. A positive control included a non-dephosphorylated but digested DNA that showed successful ligation using DNA ligase.

4.2 Covalent immobilization of streptavidin on magnetic nanoparticles

Streptavidin was also immobilized onto magnetic nanoparticles by covalent coupling in the presence of carbodiimide. As mentioned earlier, the strong non-covalent interaction, between avidin-biotin ($K_d=10^{-15}M$) has been exploited for many separation techniques in immunology, cell biology and molecular biology, (TechNote 205). In the past, achieving high activity and stable binding of solid phase ligands has been a major problem. Biomolecules that are difficult to attach to microparticle surfaces by conventional means may be amenable to biotinylation. Due to the avidin-biotin interaction, binding biotinylated compounds to streptavidin coated magnetic nanoparticles may improve specific activity.

Some of the benefits of direct coupling of streptavidin to magnetic nanoparticles include colloidal stability, which means monodisperse particle suspension that settles slowly in absence of magnetic field. Additionally, coating of streptavidin onto magnetic nanoparticles may allow higher activity of surface bound ligands, so that there is decreased particle quantity required per assay.

In the present study streptavidin was covalently linked to magnetic particles using two different strategies i.e. shaking and sonication method, respectively. The amount of streptavidin bound to the particles was determined by measuring the left over protein in the unreacted fractions. The biotin-binding

ability of magnetic streptavidin particles was determined using biotinylated-alkaline phosphatase according to the manufacturer's procedure (Bangalore Genei, Bangalore, India). ALP activity was estimated using p-nitrophenyl phosphate as substrate at 405 nm.

4.2.1 Immobilization of streptavidin on magnetic particle

The coupling of streptavidin to magnetic particles was carried out to determine the optimum conditions for immobilization, i.e. change of pH of the reaction mixture and proportion of magnetic particles to streptavidin. The pH was varied using sodium and potassium phosphate buffers (3 mM). The streptavidin and the CDI, were dissolved in buffer of the desired pH.

The first set of experiment involved determination of optimum concentration of streptavidin that can bind per milligram of magnetite. The magnetite:CDI mass ratio was kept constant at 3:1, whereas the concentration of streptavidin was varied from 10 – 80 μg. The binding of streptavidin was determined by estimating the amount of protein in the unreacted and residual fraction. The protein estimation was performed by Bradford's dye binding method and UV spectrophotometry at 280 nm. Immobilization was performed with both shaking and sonication methods described in materials and methods. From the results obtained in Fig. 40, it indicates that with both the immobilization methods, increasing the concentration of added streptavidin; the maximum binding achieved was on an average 20 μg streptavidin per milligram of

magnetite. Therefore, the optimum binding concentration of streptavidin to magnetite was considered to be 20 μg per milligram magnetite.

In order to check whether pH influences the coupling reaction, the binding was performed at different pH values viz., 6.3, 7.0, 7.5 and 8.0. The ratio of magnetite:CDI:streptavidin of 3:1:0.1 was used for this experiment. In all cases, the binding of streptavidin to magnetic particles didn't exceed 20 ± 2 μg per milligram magnetite. Thus, it was inferred that variations in pH of the coupling buffer do not affect the linking efficiency. Additionally, the biotin-binding ability of magnetic streptavidin particles immobilized at different pH value showed no differences in the activity of BALP (Fig. 41). Hence forth, all the experiments were performed with magnetite:CDI:streptavidin ratio of 3:1:0.06 (i.e. 20 μg streptavidin per milligram of magnetite).

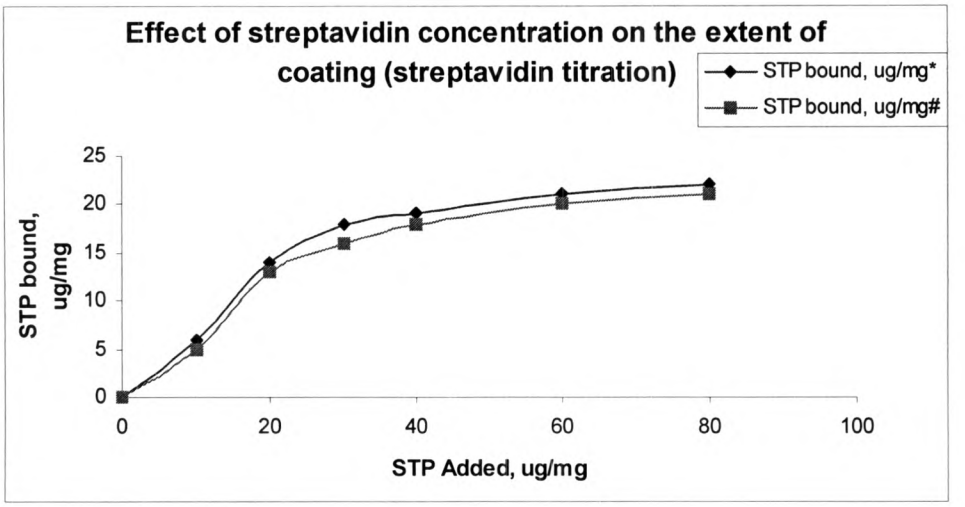

Figure 40: Streptavidin (SA) binding to magnetic particles. *shaking method, #sonication method.

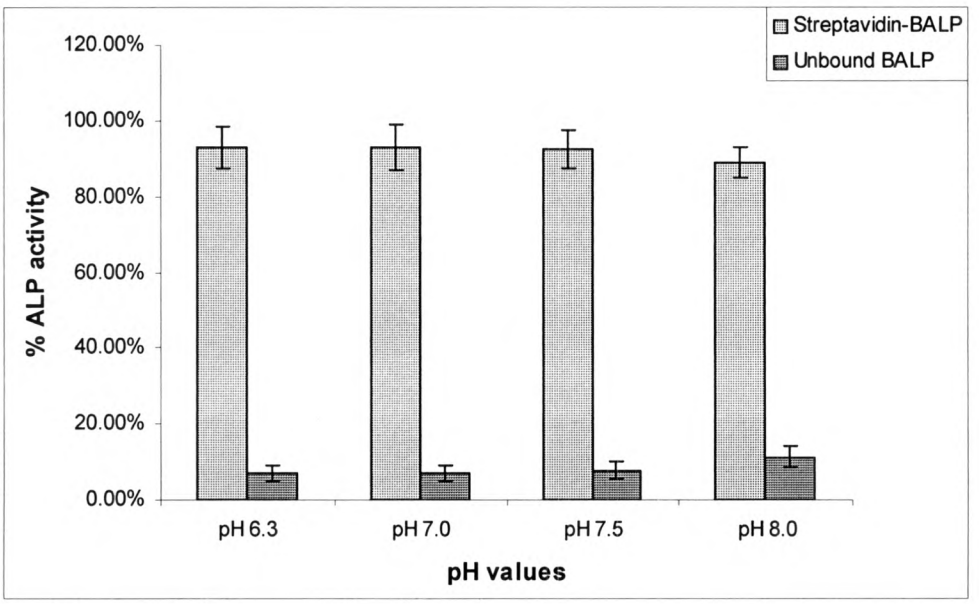

Figure 41: Biotin-ALP activity of magnetic streptavidin nanoparticles immobilized at different pH.

4.2.2 Biotin binding capacity of magnetic streptavidin nanoparticles

Determining the amount of streptavidin bound to the magnetic nanoparticles and their capacity to bind biotin are important analytical steps. Biotin-binding capacity is usually determined by two methods, biotinylated probes or biotinylated enzymes. In the present study, due to lack of funds and instrument facilities it was difficult to use biotinylated probes (especially Biotin-Fluorescein); therefore the biotin-binding capacity was determined using biotinylated alkaline phosphatase. Some of the inherent problems with the use of biotinylated enzymes were known from the beginning. This is because enzymes may vary considerably in specific activity depending on pH, buffer and temperature. This makes standardization difficult and limits precision.

Table 22: Comparative study of streptavidin binding to magnetic particles and its capacity to bind biotin (as judged from streptavidin bound biotin-alkaline phosphatase activity)

Samples	**Method A[1] (%)**	**Method B[2] (%)**
Streptavidin bound to MP[3]	**100**	**100**
MP-streptavidin bound BALP activity	**62.8**	**88.15**
Unbound/Residual biotin-ALP activity	**37.2**	**12.1**

MP, magnetic particles; BALP, biotinylated alkaline phosphatase; [1]shaking method; [2]sonication method; [3]calculated based on the estimation of protein in unreacted fraction.

Table 22 shows the % biotinylated-ALP activity in the unbound fraction and that associated with magnetite-streptavidin fraction. It was observed that the ALP activity associated with magnetic-streptavidin fraction is greater than unbound fraction. However, when comparing the two methods, the result indicates that the biotin-ALP activity of magnetic streptavidin particles immobilized with sonication method is higher compared to shaking method. Though, the amount of protein bound was similar with both the methods, the apparent loss of biotin-binding ability of immobilized streptavidin with shaking method (37°C for 24 hours) can be due to either the denaturation or inactivation of the protein because of extended (24 hours) exposure at 37°C in presence of carbodiimide.

Additionally, covalent linking of streptavidin may result in a change in conformation of the protein where the biotin-binding site is partly or totally obscured by the immobilization matrix and thus not accessible for binding the ligand. Alternatively, covalent coupling may lead to inter and/or intra molecular cross-linking between free protein or immobilized protein, thus resulting in loss of one or more binding sites per tetramer of streptavidin that is between the surface of the particle and the streptavidin molecule (Koneracka *et al.*, 2002; Technote Sera-Mag Magnetic Streptavidin microparticles from Seradyne Inc. Indianapolis, USA).

The biotin-binding capacity (in pmoles per mg particles) was calculated from the BALP activity associated with magnetite-streptavidin. From the ALP

activity, the amount of ALP present on magnetic streptavidin particle was determined. The biotinylated-alkaline phosphatase used in the present study had the ratio of Biotin:ALP of 1.5:1. So, once the amount of ALP was determined, the biotin bound to magnetic streptavidin was calculated and the binding capacity estimated. In all cases, a control set that includes base nanoparticles free of streptavidin was included, so that any non-specific binding of BALP can be taken care.

Table 23. Biotin-binding capacity of magnetic streptavidin particles (given per mg particles)

Magnetic streptavidin particles	**Biotin binding (pmole per mg particles)**
Shaking (present work)	$157^{a} \pm 5.29$
Sonication (present work)	$219^{b} \pm 4.93$
Commercial kit - Chemagen AG, Germany - Streptavidin PVA coated magnetic beads	30 – 50

Results are mean ± S.E.

Values with superscript a and b are significantly different ($P < 0.001$)

Table 23 provides the biotin-binding capacities of magnetic streptavidin particles immobilized with two different methods. The biotin-binding capacity of streptavidin particles with shaking method was significantly lower than compared to sonication method. As mentioned earlier, this possibly indicates

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

The biotin binding activity obtained with the present magnetic streptavidin nanoparticles is at least 4 - 5 times higher than compared to streptavidin PVA (polyvinyl alcohol) coated magnetic beads available commercially from chemagen AG, Baesweiler, Germany. The biotin binding capacity reported by chemagen streptavidin particles is using biotinylated protein. Since in the present study also biotinylated enzyme has been used to determine biotin-binding capacity, chemagen streptavidin particles are better comparison candidate. There also exists possibility that this large biotinylated probe (ALP – molecular weight 138000 daltons) may give lower biotin-binding values due to steric hindrance. Such low values have been reported in case of magnetic streptavidin particles 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 (Technote, Sera-Mag Magnetic Streptavidin microparticles from Seradyne Inc. Indianapolis, USA). Thus, it could be speculated that the biotin-binding capacity reported in the present study may also be under-estimated, since it was determined using large probe i.e. biotin-ALP. The confirmation on these lines could be done with the use of small probes such as biotin-fluorescein.

From the literature it appears that, when the binding capacity is determined using free biotin or biotinylated-oligonucleotide then it is expected to be at

least 5 fold higher than reported with biotinylated-protein. Therefore, according to this statement the free biotin binding capacity of magnetic streptavidin nanoparticles of the present study should be at least 1000 pmoles. This value is higher than reported by commercially available particles from different companies, which includes Spherotech, Dynal Biotec, Scipac and Cortex Biochem. The higher binding capacity obtained for streptavidin immobilized to magnetic nanoparticles is possibly attributed to the nano-size of the particles. Furthermore, from the above results it can be summarized that sonication method is better for streptavidin immobilization than shaking method.

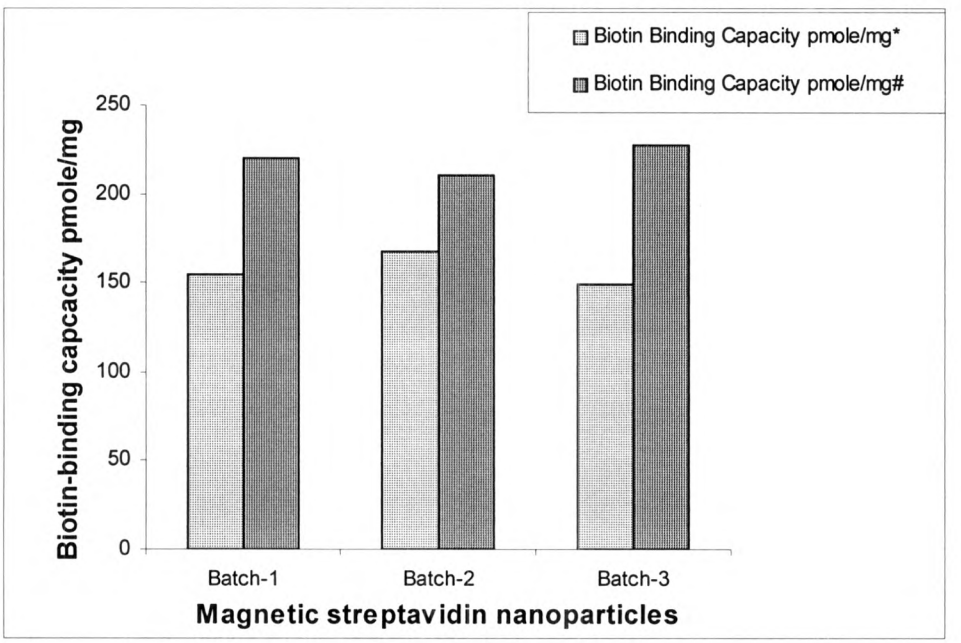

Figure 42: Comparison of biotin-binding capacity of magnetic streptavidin nanoparticles immobilized with shaking and sonication method, respectively. *shaking method, #sonication method.

The reproducibility of the BALP assay can be seen in Figure 42. Manufactured batches of streptavidin magnetic nanoparticles were examined for biotin-binding capacity. Thus, it can be concluded that if performed correctly, the precision and reproducibility of the BALP assay is satisfactory.

4.2.3 Confirmation of streptavidin binding with FTIR spectroscopy

As seen with ALP immobilization, the two characteristics bands were seen at 1540 and 1648 cm^{-1} in case of streptavidin alone & streptavidin magnetic nanoparticles confirming the presence of protein (streptavidin) onto magnetic particles (Fig. 43).

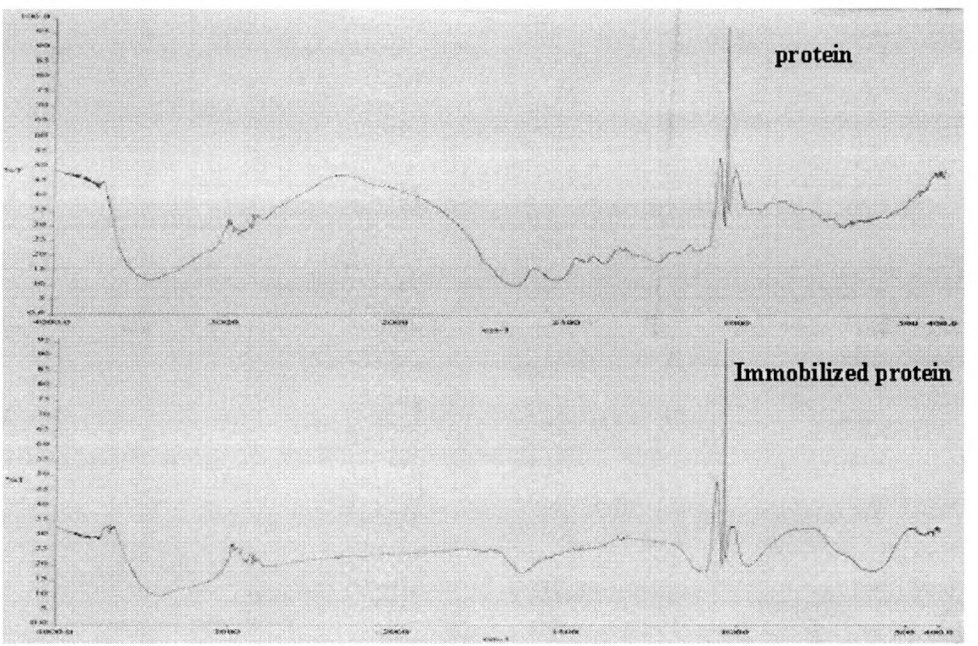

Figure 43. FTIR spectra of pure streptavidin (top) and streptavidin bound to magnetic nanoparticles (Fe_3O_4) (bottom).

4.2.4 Stability of immobilized streptavidin

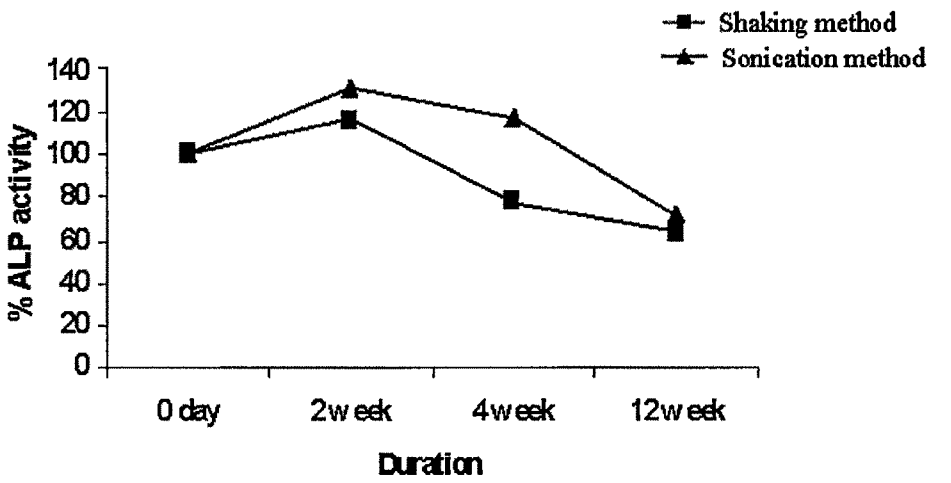

Figure 44: Storage stability of magnetic streptavidin nanoparticles using shaking and sonication method, respectively

In order to use the immobilized streptavidin for long-term purposes, the storage stability of immobilized preparation was evaluated. The streptavidin that was immobilized using both the methods (shaking and sonication) was found to be stable for at least 12 weeks at 4°C. Figure 44 shows the storage stabilities of immobilized streptavidin with shaking and sonication method, respectively. Each data point is the result of three independent observations done in duplicates. The trend of results was found to be similar with both the immobilization methods. There was a slight increase in the biotin binding ability although not significant (as determined from ALP activity), followed by a gradual decrease, finally by 12th week activity reducing to 70% to the basal level (zero day). This indicates a possible conformational change or

denaturation in the immobilized protein resulting in loss of the activity at 12th week.

4.3 SUMMARY

To conclude, ALP and streptavidin were successfully immobilized using both the shaking and sonication methods. Sonication method for immobilization appears to be quick and retains higher amount of functional activity compared to shaking method in both cases. However, sonication method is preferable if the immobilized ALP is to be used only for short-term purposes, while shaking method is the method of choice 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. The immobilized ALP was also used as a solid phase reactant for application in molecular cloning experiment; successful plasmid dephosphorylation has been shown in the present study. The method described for streptavidin immobilization is also efficient, as naked particles offer greater capacity to immobilize streptavidin and thereby provides higher biotin binding capacity; therefore lesser particle quantity is required per assay. Thus, the immobilization method described here can be used for preparation of streptavidin magnetic nanoparticles, which could be used for bioapplications such as nucleic acid purification, cell separation and isolation, and purification of biomolecule based on the use of avidin-biotin system.

The direct coupling method described here is simple, quick, and 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 procedure time and allow repeated use of enzymes and proteins.