CHAPTER 1

INTRODUCTION

1.1 Overview

Alkaline phosphatase (orthophosphoric monoester phosphohydrolase, alkaline optimum, EC 3.1.3.1) is a biomarker routinely used in the diagnosis and monitoring bone diseases[1]. Alkaline phosphatase is a glycoprotein and shows extensive intra- and inter-tissue charge heterogeneity and variations of the apparent molecules mass of the purified enzyme. Molecular mass values ranging between 130 and 220 kD have been reported after butanol extraction, 128 kD after solubilization with the detergent Emulgophen B C 720[2]. The primary structures of human alkaline phosphatase contain a sequence of 36 amino acids conformational close to the active center.

Alkaline phosphatase is comprised a group of pure isoenzymes encoded by at least four different gene loci. The most common ALP isoenzyme forms are intestinal, placental, germ cell and tissue-nonspecific[3, 4].

An intestinal isoenzyme is localized in the brush border of the mucus membrane with a possible variant in the renal tubules. Placental isoenzyme is derived from placental syntrophoblasts then this discernible by electrophoresis in the serum of pregnant women[5]. The germ cell isoenzyme is shown in testis, cervix, thymus and lung[6]. ALP from the tissue-nonspecific locus is observed in many tissues, including bone, liver and kidney[3,6,7,8].

Normally, the multiple groups originating from single generate loci should be called “isoforms”. So, a single amino acid has been removed or a carbohydrate has been added to or detected from the parent isoenzyme[5].

Although, measurement of alkaline phosphatase is widely used for monitoring bone diseases. Several ALP isoforms lead to lack specificity. The two major and clinically relevant isoforms in human serum are bone and liver ALP. Bone ALP is a glycoprotein found on the cell surface of osteoblasts. Bone ALP measurements provide a sensitive and accurate assessment of bone turnover. Numerous studies support the clinical utility of this marker in various disorders in which bone metabolism is either directly or indirectly affected. These disorders include bone metastases and osteoporosis. In osteoporosis, a chronic disorder where bone metabolic changers are sometimes subtle, bone ALP has proven to be an effective tool in long-term patient management.

A variety of methods have been developed that specifically isolate and measure the circulating bone alkaline phosphatase from the liver and kidney alkaline phosphatase isoforms. Several of these methods rely on the physicochemical properties of the enzyme and on the instability of the enzyme in the elevated temperatures such as heat-inactivation[1] and electrophoresis on agarose gels followed by precipitation with neuraminidase[6,9]. High-performance liquid chromatography[6,10] has also been used to separate the liver and bone isoforms. However, these methods are tedious, time –consuming and not practical in the setting of routine clinical laboratory testing. Immunoassays using monoclonal antibodies are highly specific for bone ALP, but it still involves long time-consumption and is very expensive. For examples, the commercial immunoassay; a two sites immunoradiometric assay (Tandem-R Ostase, Tandam-MP Ostase)[6,11,12] requires an overnight incubation; Metra Biosystems, (Alkphase-B[1]) which employs a single monoclonal antibody to capture bone ALP followed by reactivity with the substrate p-nitrophenyl phosphate to assess

enzyme activity requires a 3-h incubation. Although, other techniques such as the precipitation[13,14], affinity electrophoresis[13,15] and affinity chromatography[16] methods with wheat germ lectin are very reproducible with excellent resolution of the bone and liver isoenzymes (approximately 90% of the bone ALP is precipitated)[17], readily permit their quantification and can be carried out in any diagnostic biochemistry laboratory, but they have the same limitation of long time consuming.

Wheat germ (Figure 1.1) agglutinin is a lectin, a highly specific carbohydrate-binding proteins of plant origin, that interacts preferentially with bone ALP. Approximately 90 %[17] of the bone ALP can be selectively precipitated with wheat germ agglutinin.

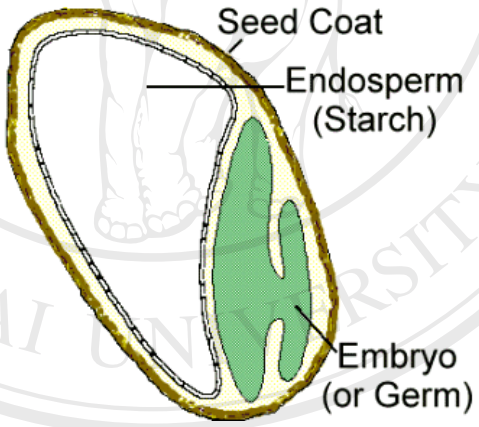

Figure 1.1 Composition of wheat seed

The bead-injection (BI) technique is the combination of the use of beads with a flowing stream of solution in a flow injection (FI) system[18]. Beads act as movable surfaces for immobilization of wheat germ lectin. The flowing stream of solution is used to carry beads through the system. The BI-FI system can be constructed using common materials that can be found in the laboratory. A simple, cost-effective

configuration of the flow cell and simple, unidirectional pumps can be used. Wheat germ lectin coated beads were flowed and retained in the flow cell. Specific lectin on the bead surfaces interacted with bone ALP in solution that was passed through the flow cell. Bone ALP was detected by p-nitrophenol phosphate. In this research, flow injection–bead injection system will be applied for determination of bone ALP in human serum. The proposed on-line bone ALP assay with a simple automatic BI-FI system is expected to be developed further for routine analysis of bone ALP in human serum which will be useful for screening and diagnosis of bone diseases.

1.2 Bone alkaline phosphatase

Many biochemical markers are available for studies of bone turnover, bone diseases and for monitoring response of treatment regimes in osteoporosis. Examples of these markers are alkaline phosphatase, procollagen (PICP/PINP) and osteocalcin (OC). Bone alkaline phosphatase is one of the most used biomarker of bone diseases.

1.2.1 Bone Alkaline phosphatase

Bone Alkaline phosphatase is a glycoprotein and function as an enzyme linked to the osteoblast membrane and released in the circulation during the bone forming phase of the remodeling process[19, 20].

The biochemical function of bone ALP is unknown and the mechanism of releasing is poorly understood. However, the precise function of bone ALP has still to be elucidated.

Both bone ALP and liver ALP are existed in the tissue-nonspecific ALP gene locus. So, approximately 95% of the total ALP activity in human serum is obtained

from bone and liver which occur in a ratio of 1:1 in healthy adults. Although, liver and bone ALP are derived from a common gene. However, the differences between the two isoenzymes are apparently ascribable to post-translational modifications of the carbohydrate side chain. O-glycosylation is one of the differences that bone ALP bears some O-linked carbohydrates whereas liver ALP does not. In addition, bone ALP has more fucose and sialic acid (N-acetylneuraminic acid) residues compared with liver ALP[20].

Bone ALP catalyses the hydrolysis of phosphate esters at the osteoblast cell surface to provide a high phosphate concentration of the bone mineralization process as part of the osteoblast cell role in bone remodeling. This results led to bone ALP levels raised in the circulation during period of active bone formation and bone growth. There are two age-dependent physiological peaks of high bone ALP activity as follows; infancy and at the time of puberty when bone growth is accelerated by the effects of sex steroid [6, 21]. The activities in various groups of subjects are shown in Table 1.1.

Table 1.1 Total ALP and bone ALP activities in various groups of subjects[22]

	Healthy blood donor			Patients	
	Male	Female	Children	Male	Female
No.	40	40	20	117	205
Age, yr	20-60	20-60	6 d-14 y	18-81	24-88
Mean ± SD	37±12	37±14	not reported	53±16	59±13
Total ALP, U/L	50-137	53-155	189-622	62-467	40-694
Mean ± SD	102±17	96±29	338±131	142±62	127±71
Bone ALP, U/L	27-96	32-102	120-544	19-117	22-109
Mean ± SD	61±14	56±18	288±136	62±21	61±20

1.2.2 Methodology for determination of bone alkaline phosphatase

Measurement of bone ALP activity in serum can provide an index of the rate of osteoblastic bone formation. Various methods have been proposed for specifically isolating and measuring bone ALP isoform of ALP and distinct it from the liver and kidney isoforms.

1.2.2.1 Heat inactivation [3, 6, 9]

This method for ALP measurement relies on the physicochemical properties of the enzyme. Bone ALP activity is identified and quantified by differential ALP isoforms sensitivity to heat inactivation. Placental ALP is stable to heat at 65 ^{o}C whereas other ALP isoenzymes can also be differentiated on the basis of their stabilities at temperatures lower than 65 ^{o}C. At 52-56^{o}C, liver ALP is more stable

than the bone ALP[23]. Incubation time of heat inactivation depends on temperature, see Table 1.2.

Table 1.2 Relationship of incubation time and temperature was used in heat inactivation

Temperature (^{o}C)	Incubation time (min)	Ref.
53-54	30	9
56	10-20	6
57	15-25	13

Inactivation rate is modified by slightly changing temperature. Heat-resistant ALP activity is determined by using the enzyme-substrate reaction, p-nitrophenyl phosphate as substrate. Heat resistant ALP activity is also calculated as the percentage of total ALP activity as follows;

Total ALP activity (%) = Bone ALP activity (%) + Liver ALP activity (%) (1.1)

1.2.2.2 Chemical inhibition[2,23]

Specific chemical inhibitors have been used to characterize ALP isoform in serum such as L-phenylalamine and levamisole. L-Phenylalamine inhibits intestinal and placental isoforms when present at a concentration of 5 mM but has less effect on the isoenzymes of bone or liver. Levamisole preferentially inhibits bone and liver ALP and is effective at much lower concentration.

1.2.2.3 Electrophoresis [4, 23]

Electrophoresis is a common technique used for analysis of ALP isoforms in serum. A variety of supporting media include agarose, cellulose acetate membrane and polyacrylamide gel. Densitometric scaning technique is employed for estimating ALP isoforms separated by electrophoresis method. However, liver and bone zones usually overlap to some extent which leads to difficulty in distinguish between these two isoforms, see Figure 1.2. Due to this problem, wheat germ agglutinin has been incorporated into gel or buffer before electrophoresis to ensure reliable discrimination of liver and bone isoenzyme[24].

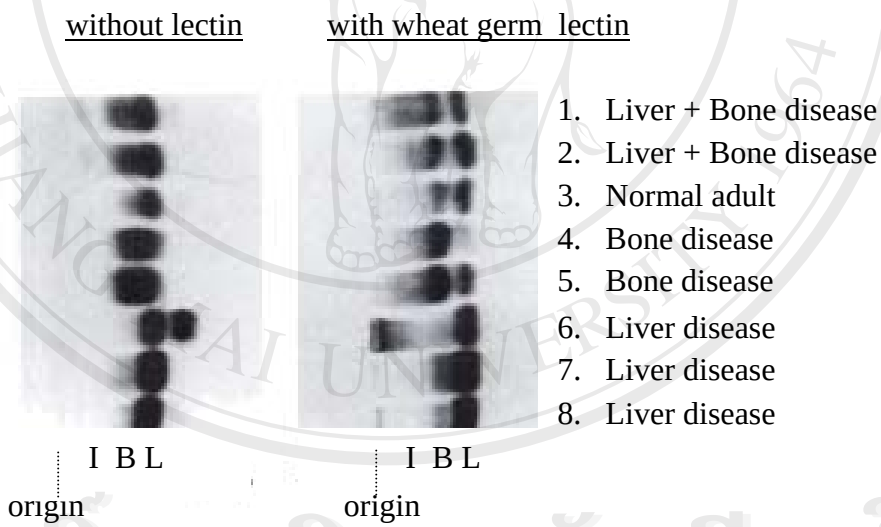

Figure 1.2 Separation of serum samples containing intestinal (I), bone (B) and liver (L) ALP isoforms by affinity electrophoresis with wheat-germ lectin and by conventional electrophoresis[13]

1.2.2.4 Chromatographic technique[10,23]

High performance liquid chromatography (HPLC) has been used for separating ALP. At least six different ALP isoforms in serum from healthy adults: three bone ALP (B/I, B1 and B2) and three liver ALP (L1, L2 and L3) isoforms (Figure 1.3)[25].

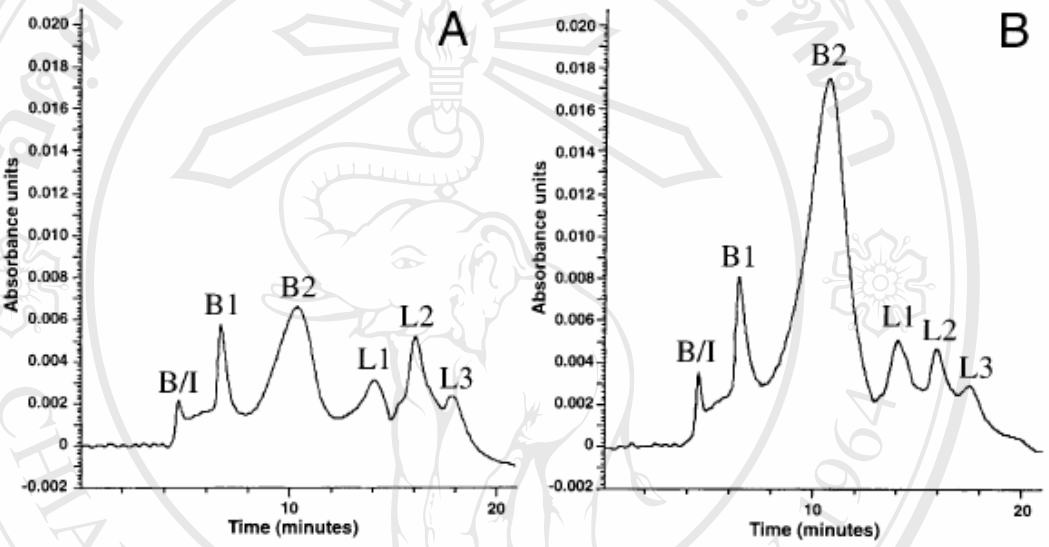

Figure 1.3 Serum ALP isoform profiles from a healthy male (A) and a prostate cancer patient with bone metastases (B)

Magnusson et. al.,[10] developed HPLC with post-column reaction detection for increase separation and performances of ALP iosoforms measurement. The isoenzyme-substrate complex was detected on-line into a packed-bed post column reactor.

1.2.2.5 Immunoassay

Immunoassays have been developed as means of achieving a higher level of specificity and sensitivity by using monoclonal antibodies to react with bone ALP. Measurement of bone ALP is based on mass and activity assays. Some

characteristics of the commercial immunoassay methods of bone ALP are presented in Table 1.3.

Table 1.3 Immunoassay method for bone alkaline phosphatase [6, 12, 14]

Commercial Immunoassays	Characteristics
Tandem-R Ostase	Two-site immunoradiometric assay (IRMA) Standard : dimeric bone ALP from SAOS-2 cell Over night incubation Reporting unit: μg/L
Tendem-MP Ostase	Enzyme immunoassay (EIA) Standard : dimeric bone ALP from SAOS-2 cell 60 min incubation Reporting unit: μg/L
Alkphase-B	Enzyme immunoassay (EIA) Standard : dimeric bone ALP from SAOS-2 cell 90 min incubation Reporting unit: U/L

A Two-site immunoradiometric has two monoclonal antibodies directed against the human bone ALP. This assay requires an overnight incubation and appears to provide reasonable sensitivity and specificity for the determination of bone ALP. The cross reactivity with liver ALP was reported to be 6%. Sensitivity of this assay was found at 2 μg/L. Within- and between-run CVs were reported at 4.0% and 8.5%, respectively[6,11].

In enzyme immunoassay (Tendem-MP Ostase), a single biotinylated monoclonal antibodies coated microwell is applied to bind bone ALP. Bone ALP is detected by the addition of p-nitrophenyl phosphatase. This activity assay uses a 60 min incubation time at room temperature. Precision was found to be 2.3-6.1% with a detection limit of 0.6 μg/L. Cross-reactivity with liver was reported at 16.8%.

Another enzyme immunoassay system is Alkphase-B. A monoclonal antibody coated on the strip is employed to capture bone ALP from sample followed by adding the substrate p-nitrophenyl phosphate to assess enzyme activity. This assay requires a 3 h incubation. Within- and between-run CVs were found at 3.6-4.2% and 3.6-7.7%, respectively. Cross reactivity with liver ALP appeared to be 3-8% and sensitivity was 0.7 U/L.

1.2.2.6 Lectin precipitation

Wheat germ lectin is known to bind specifically to N-acetylglucosamine and N-acetylneuraminic acid (sialic acid) residues of glycoproteins[13, 21] such as ALP. This characteristic of wheat germ lectin on being preferentially bound to bone ALP isoenzyme provides the isolation and quantitative measurement of ALP isoenzymes originating in liver and bone. Bone ALP activity was calculated by measuring ALP activity in the precipitate. Activity of liver ALP can be estimated by subtracting the activity of the bone ALP isoenzyme from total ALP activity.

1.3 Wheat germ lectin

Lectins are structurally diver carbohydrate-binding proteins of non-immune origin. Many lectins contain two or more sugar binding sites and can agglutinate cells

and/ or precipitate complex carbohydrate conjugates[26]. The lectin interaction is demonstrated in Figure 1.4

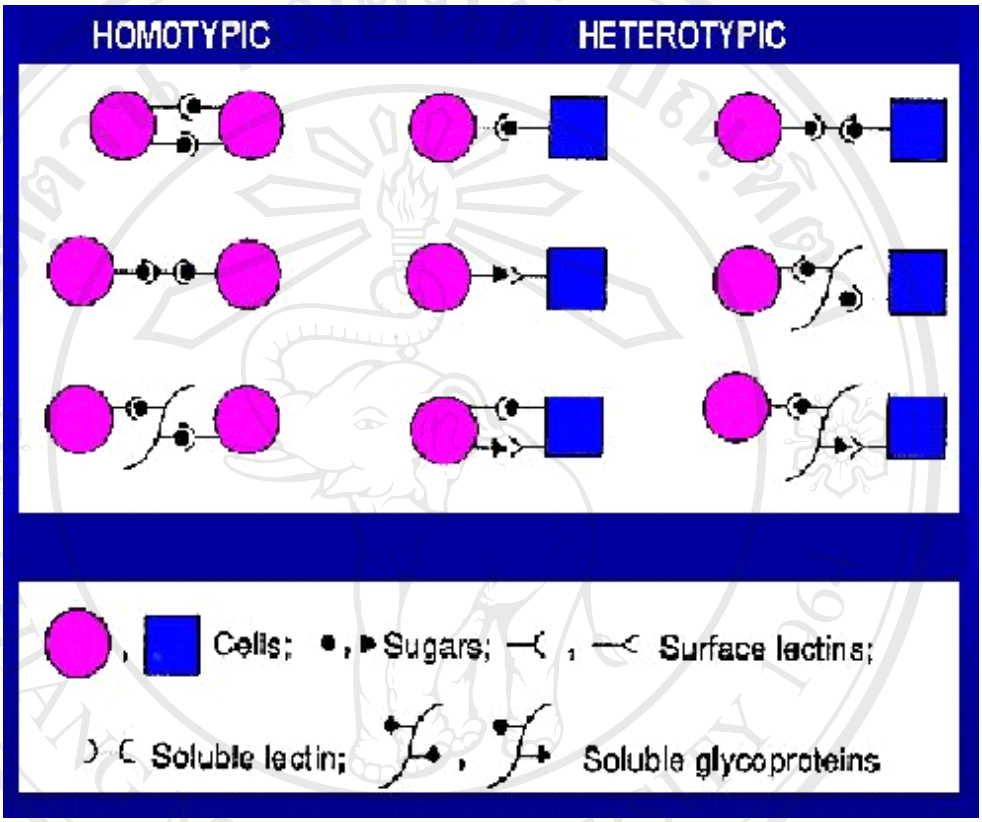

Figure 1.4 Lectin mediated interactions (Homotypic refer of the same type or form; corresponding to the other one of two paired organs or parts, Heterotypic refer of a different or unusual type or form)

Wheat germ lectin is lectin isolated from *Triticum vulgaris* (wheat germ). The structure model of wheat germ lectin is shown in Figure 1.5.

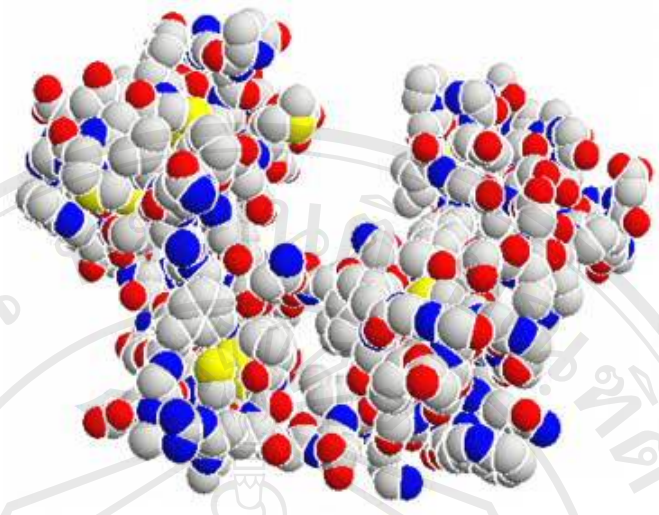

Figure 1.5 3 D structure model of wheat germ agglutinin[27]

Wheat germ lectin can bind to N-acetylglucosamine and N-acetyl neuraminic acid residues of bone ALP leading to isolation of bone ALP from other isoforms. Wheat germ lectin has been applied by mixing with gel as media in electrophoresis[13], and using as affinity column (wheat germ lectin affinity chromatography)[16] (Figure 1.2) for improving the performance isolation.

1.4 Flow injection-bead injection for determination of bone ALP

Flow injection analysis[28] is based on the injection of a solution sample into a carrier flowing stream of a suitable liquid. The analytical zone is transported to a detector where the signal is recorded simultaneously. Several detection techniques that have been used include spectrophotometry techniques such as fluorescence, atomic absorption, inductively couples plasma-atomic absorption spectrometry and electrochemistry. A simple configuration of the flow injection system is presented in Figure 1.6, which consists of a pump to propel the carrier stream through a thin tubing, an injection port for injection a volume of sample solution to a carrier stream,

and a mixing coil where the sample zone disperses and may react with the component of carrier stream, yielding a species which is monitored by a flow through detector. A typical signal is in a form of a peak whose height and area relate to the concentration of analyte.

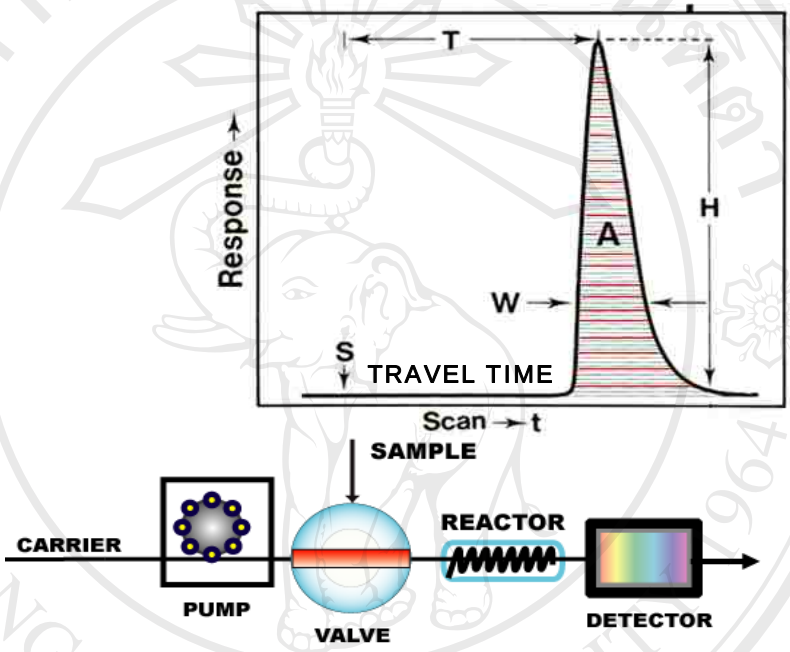

Figure 1.6 A simple FIA manifold with a typical recorded output as obtained from a flow-through detector. H, peak height: S, sample: and T, residence time[28]

Flow injection analysis is a combination of a sample injection, controlled dispersion and a reproducible timing of sample zone movement. This permits a highly reproducible readout even when the mixing is incomplete, the chemistry may not reach equilibrium and the signal is transient.

Bead injection technique (BI) technique[18,28] is the combination of the use of beads with a flowing stream of solution in a FI system. Beads are utilized as solid surfaces to preconcentrate or extract the analyte or to accommodate a chemical reaction. The flowing stream of solution is used to carry beads trough the system. There is no need to regenerate the bead surfaces because they are discarded after each use and are replaced by fresh ones. This helps to reduce the risk of contamination, denaturation and system fouling and also makes it possible to operate BI in the continuous flow system even if no suitable eluent for bead cleaning is found possible. The example of beads is shown in Figure 1.7.

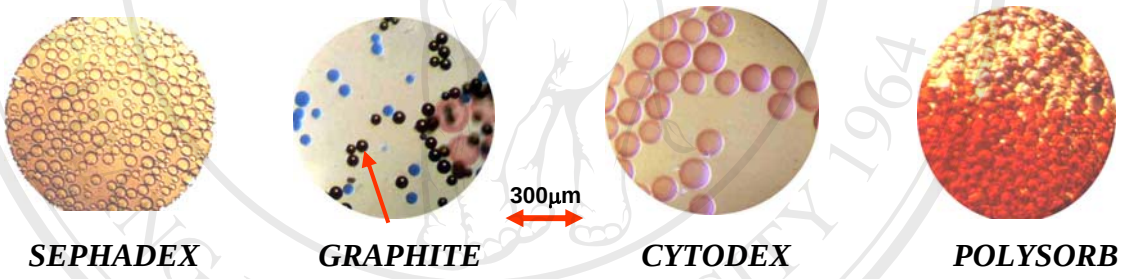

Figure 1.7 Beads material for supporting media[28]

Flow injection methods offer several advantages for automatic immunoassays. First, the many washing steps required are inherent in a flowing system. Second, the flow channel is enclosed and thus less prone to contamination. Finally, the flow based assays can be carried out rapidly. However, common drawback of many flow based methods is the necessity of using columns packed with antibody-coated beads, which serve as the retention surfaces. This retention surfaces cannot be discarded after each use, but must be regenerated prior to the next assay. Regeneration requires breaking

the antibody bound to the analyte without altering in any way the immobilized antibody, since changes in the surface reactivity will result in different binding characteristics and ultimately erroneous results[29].

Most methods used to determine bone ALP in human serum is manual operated. They are difficult, long time and sample consumption per analysis run. HPLC as online technique is not practical in the setting of routine clinical laboratory testing due to its expensive instrumentation.

We attempt to develop a simple FI-BI system for a lower cost and faster system to perform bone ALP assay.

1.5 Research objectives

a) To develop the FI-BI system for on-line assay of bone alkaline phosphatase

b) To apply the developed system for determination of bone alkaline phosphatase in human serum samples