



Doctor of Philosophy in
Chemistry

SAMPLE SPINE

POLYAMIDOAMINE (PAMAM) FUNCTIONALIZED MAGNETIC NANOPARTICLES FOR PROTEIN SEPARATION

by

Mine DEMİR

June 2015



POLYAMIDOAMINE (PAMAM) FUNCTIONALIZED MAGNETIC
NANOPARTICLES FOR PROTEIN SEPARATION

by

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Ph.D. Thesis – Chemistry
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Thesis Supervisor: Prof. Abdülhadi BAYKAL

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ABSTRACT

This dissertation deals with functionalized nanoparticles in combination with protein and their usage for protein separation. In order to synthesise superparamagnetic nanoparticles (SPIONs) amine and carboxylic acid functional PAMAM dendrimers, co-precipitation route was used for synthesizing superparamagnetic nanoparticles. The surface of SPIONs was modified with amine and carboxylic acid functional poly (amidoamine) (PAMAM) dendrimers with different generations from G1 to G5 was obtained. X-Ray Diffractometry (XRD) and Fourier Transform Infrared Spectroscopy (FT-IR) was used for approving the the formation of SPIONs, PAMAM modified SPIONs and carboxylic acid modified PAMAM-SPIONs. In addition, TEM and Thermal Analysis were studied for morphology and particle size and to investigate the stability of functionalized SPIONs respectively. Magnetic separation technique was chosen because of the ease and cheapness. Therefore, BSA solutions were mixed with functionalized SPIONs and supernatants were removed by using a suitable magnetic separator. Herein, Cu^{2+} ions were used for chelation of the surface of the modified SPIONs. For the evaluation of protein adsorption and desorption behavior of SPIONs, Bovine Serum Albumine (BSA) which is used commonly as model protein was chosen among the other proteins. The effect of various parameters such as concentration of BSA, ionic strength, pH, temperature, different metals and reusability of functionalized SPIONs were investigated. The highest adsorption behaviour was observed on the G5 of amine functional and carboxylic acid functional nanoparticles (55.31 mg/protein) and (55.00 mg/protein), respectively.

Keywords: Immobilization; Magnetic Nanoparticles; Magnetic Separation; Metal Chelation; Protein Assay; Protein Separation.

POLİ (AMİDOAMİN) (PAMAM) İLE FONKSİYONALİZE EDİLMİŞ MANYETİK NANOPARÇACIKLARIN PROTEİN AYRIŞTIRMASI İÇİN KULLANIMI

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ÖZ

Bu tez fonksiyonelize edilmiş süperparamanyetik parçacıklara protein immobilizasyonu ve proteinin manyetik ayrıştırılması ile ilgilidir. Amin ve karboksilik asit PAMAM dendrimer ile fonksiyonelize edilmiş Süperparamanyetik nanoparçacıkları sentezlemek için, birlikte çökeltme yöntemi kullanıldı. Süperparamanyetik nanoparçacıkların yüzeyi, amin ve karboksilik asit fonksiyonel PAMAM dendrimer ile modifiye edilerek, G1-G5 arası tüm jenerasyonlar elde edildi. X-Işını Difraktometresi (XRD) ve Fourier Transform Infrared Spektroskopisi, SPION, SPION-PAMAM, SPION-PAMAM-COOH oluşumlarının kanıtlanması için kullanıldı. Buna ek olarak fonksiyonelize edilmiş SPION'un morfolojisi ve parçacık büyüklüğü için sırasıyla TEM analizi, stabilitesinin incelenmesi için de Termal Analiz çalışıldı. Basit ve ucuz olmasından dolayı, manyetik ayırma tekniği seçildi. Bu sebeple, BSA çözeltileri fonksiyonelize edilmiş süperparamanyetik nanoparçacıklar ile karıştırıldı ve sıvı kısımlar manyetik ayırıcı kullanılarak ayrıldı. Burada, modifiye edilmiş süperparamanyetik nanoparçacıkların yüzeyinin şelasyonu için Cu^{2+} iyonu kullanıldı. Modifiye süperparamanyetik nanoparçacıkların, protein adsorpsiyon ve desorpsiyonu üzerindeki davranışlarının değerlendirilmesi için, Sığır Serum Albumini (BSA) model protein olarak kullanıldı. pH değeri, BSA konsantrasyonu, sıcaklık, farklı metaller, iyonik direnç ve tekrar kullanılabilirlik gibi çeşitli parametrelerin etkileri incelendi. En yüksek BSA adsorpsiyonu sırasıyla, G5 amin fonksiyonel ve karboksilik asit fonksiyonel (55.31 mg/protein ve 55.00 mg/protein) nanoparçacıklarda gözlenmiştir.

Anahtar Kelimeler: Immobilizasyon; Manyetik Nanoparçacıklar; Manyetik Ayırma; Metal Şelasyonu; Protein Analizi; Protein Ayrıştırılması

To my parents and my spouse

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LIST OF SYMBOLS AND ABBREVIATIONS

SYMBOL/ABBREVIATION

ACAC	Acetyl acetate
APTMS	Aminopropyl trimethoxysilane
BCA	Bicinchoninic acid
$C_6H_5N(NO)O^-$	N-nitrosophenylhydroxylamine
ELISA	Enzyme-Linked Immunosorbent Assay
ESA	Equine serum albumin
FC	Field cooling
kDa	Kilo Dalton
MCA	Monochloroacetic acid
M_xCup_x	Metalcupferronates
MNPs	Magnetic nanoparticles
MPTMS	Mercaptopropyl trimethoxysilane
MRI	Magnetic resonance imaging
OD-VSM	Quantum design vibrating sample magnetometer
Oe	Oersted
PAA	Polyacrylic acid
PAMAM	Poly (amidoamine)
PBA	Poly butyl (acrylate)
PLA	Poly lactic acid
RSA	Rat serum albumin
SPION	Superparamagnetic iron oxide nanoparticle
TEOS	Tetraethoxysilane
TMAOH	Tetramethylammonium hydroxide
W/O	Water-oil
ZFC	Zero field cooling

CHAPTER 1

INTRODUCTION

1.1 OVERVIEW

Due to the multitude of applications of magnetic nanoparticles are great interests by researchers. Smaller nanoparticles which are between 10-20 nm are more suitable and easy available.

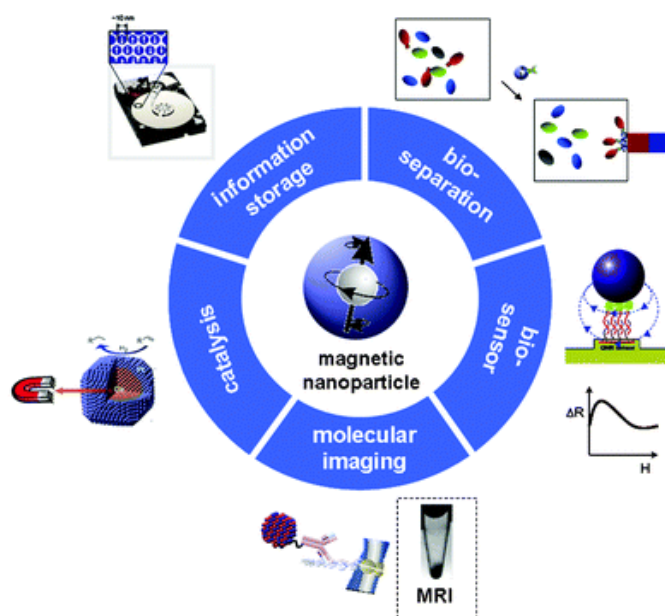


Figure 1.1 Applications of Magnetic Nanoparticles (Reproduced from [Jun et al., 2007] with permission of The Royal Society of Chemistry.

Above the blocking (T_c) temperature, they turn into super paramagnetic state and show superparamagnetic properties so every particle becomes single domain.

There are many applications of nanoparticles such as biotechnology (bio-separation, bio-sensor etc.), magnetic recording media (MRI), catalyst etc [An-Hui et al., 2007; Andra et al., 2007; Pankhurst et al., 2009; Jordan et al., 1999].

Nanoparticles are used for magnetic separation which is a quick and simple method that is used to capture proteins or other biomolecules in Biotechnology. Thus, superparamagnetic nanoparticles which are magnetized rapidly at the presence of external magnetic field and demagnetized at the absence of magnetic field are used to separate proteins or enzymes.

Metallic nanoparticles which are uncoated are easily oxidized and chemically active, in air and to reduce their energies then they tend to come together. Due that reason, nanoparticles are coated with silica, carbon, surfactant or polymer [Wu et al., 2008].

Nanoparticles can be synthesized by various methods. These are co-precipitation, micro-emulsion, hydrothermal synthesis, thermal decomposition etc. Fe^{2+} and Fe^{3+} ions are mixed in an appropriate ratio generally 2:1 and they are precipitated in a basic medium such as NaOH, TMAOH etc. under vigorous mixing in co-precipitation method. The sizes of the particles obtained by this method is affected by Fe^{2+}/Fe^{3+} ratio, pH, stirring speed, temperature, base type, ionic strength and the quantity of media. Precipitation process can be realized at room temperature or elevated temperature [Wu et al., 2007]. Micro-emulsion is the other technique for synthesizing nanoparticles. The stability of two liquid phases is provided by forming micelles. Droplets which are in oil in water micro-emulsion are wrapped by surfactant and disperse into the liquid. The particles are grown in the micelles and precipitated by adding solvent such as acetone, ethyl alcohol. Sizes of micelles change according to the molar ratio of water and surfactant [Solans et al., 2005]. $FeCl_3$, ethylene glycol which is high boiling solvent, sodium acetate and polyethylene glycol which is surfactant are mixed vigorously to form clear solution then put in a reactor or autoclave in hydro-thermal synthesis that known solvothermal method. Then reaction carried out at 200 °C for 8-72 hours [Liu et

al., 2007]. Mono dispersed nanoparticles which are between 200-800 nm are obtained with this method. Thermal decomposition is organic based method [Sun and Zeng, 2002]. Shortly, in the presence of oleic acid, complexes of iron-oleate reflux in high boiling point. Complex, decomposes thermally and meanwhile, the released iron turns to magnetite in the presence of oxygen and it grows not too much due to the surfactant in medium [Ozel, 2009].

The thesis presents the uses of functionalized magnetic nanoparticles for protein separation especially magnetic separation and application of Bio-Rad Assay for the Bovine Serum Albumin (BSA) determination.

1.2 MAGNETIC NANOPARTICLES (MNP's)

Magnetic nanoparticles are manifested in the presence of a magnetic field. They include generally Fe, Ni, Co type magnetic elements and their compounds. Their size is between 1-100 nm. Size of magnetic nanoparticles which are between 10-20 nm is suitable for many applications such as biologic, biochemical etc [Gubin et al., 2000].

The most common application area of MNP's is biotechnology, medicine and magnetic recording media [Niemeyer et al., 2001]. In Biotechnology, MNP's are used for magnetic separation. Magnetic separation is simple and quick method for catching proteins or other biomolecules [Saiyed et al., 2003]. Superparamagnetic particles were used for this process because the particles are magnetized immediately when applied external magnetic field and the magnetic field can be dispersed again immediately removed. Another application area is hyperthermia therapy which is considered as an additional treatment against chemotherapy and radiotherapy [Ramchand et al., 2001]. Magnetic nanoparticles are heated by magnetic hysteresis losses when exposed to changing magnetic fields. Tumor cells are very sensitive to heat and are also broken at 41 °C. Another use of the magnetic nanoparticles in the pharmaceutical industry is as drug carriers. Drug that attached to nanoparticles is injected into the tissue and is moved to area required under the guidance of these particles by applying an external magnetic field. The particles are retained therein until therapy is completed and removed when therapy is completed [Safarikova et al., 2001].

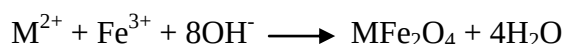
Magnetic nanoparticles are classified to three groups. These are oxides, metallic and metallic with shell. The most common one is ferrite nanoparticles which are oxides and they are generally used for biomedical applications. Gold nanoparticles which are type of metallic are used for technical applications. Cobalt magnetic nanoparticles are example for metallic with shell. They have higher magnetization and stability. Magnetic nanoparticles which are naked are chemically active and they are unstable in water (at neutral pH) or in physiological fluids, so oxidize easily by air then agglomerated and precipitated quickly [Lu et al., 2007; Kango et al., 2013]. Due to that reasons, to stabilize MNP's surface in solution they can be coated with organic or inorganic substance such as surfactants, polymers, silicones, carbons etc.

Co-precipitation [Ünal et al., 2010], micro-emulsion [Baykal et al., 2009], thermal decomposition [Baykal et al., 2015] and hydrothermal [Baykal et al., 2014] are methods for synthesizing nanoparticles.

1.3 SYNTHETIC METHODS

1.3.1 Co-precipitation Route

One of the facile and convenient ways to synthesize metal oxides and ferrites which are MNP's from aqueous salt conditions is co-precipitation route. By addition of NaOH which is a base at room temperature or at elevated temperature under inert atmosphere this method is achieved.



Where M can be Fe^{2+} , Mn^{2+} , Co^{2+} , Cu^{2+} , Mg^{2+} , Zn^{2+} and Ni^{2+} .

Co-precipitation process should be completed at a pH levels between 8 to 14, with a stoichiometric ratio of 2:1 (Fe^{3+}/M^{2+}) in a non-oxidizing oxygen environment. There are some parameters effects of size, shape and composition of magnetic nanoparticles.

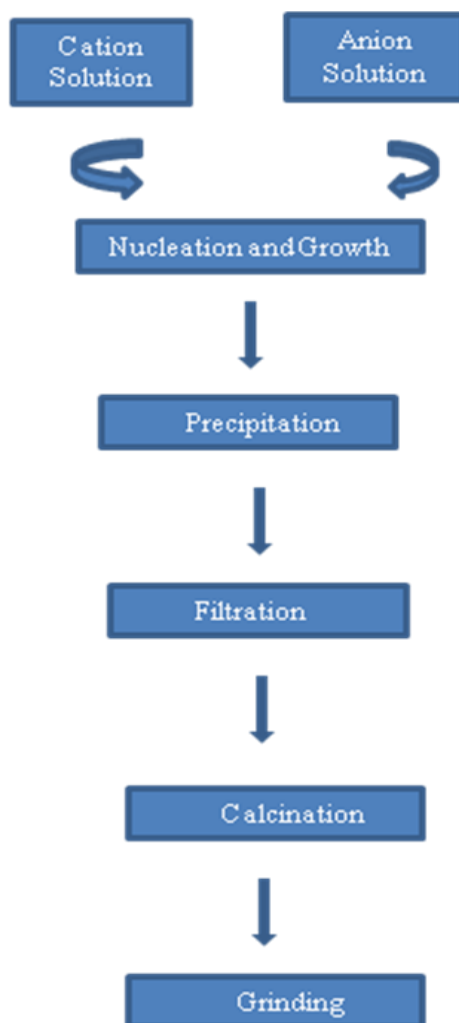


Figure 1.2 Flow diagrams for co-precipitation method.

These are salt type (e.g. chlorides, sulfates and nitrates), temperature, pH, ratio of metals, base type, bubbling of nitrogen gas, ionic strength of media, [Schüth et al., 2007; Toprak et al., 2010; Baykal et al., 2011].

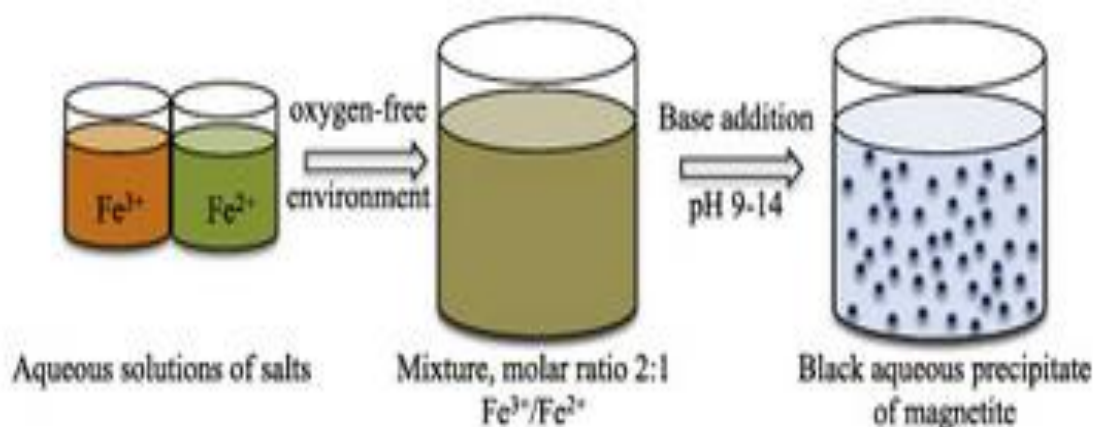


Figure 1.3 Schematic representation of co-precipitation method [Gautier et al., 2013].

1.3.2 Microemulsion Route

The microemulsions in the formation of nanoparticles are used as nanoreactors. The most widely used emulsions are water-oil (W/O) emulsions. They are isotropic, thermodynamically stable. The stability of the two liquid phases is achieved by forming micelles. Water, oil and surfactant are three components of micro-emulsions [Langevin, 1992]. Droplets which are in water-oil micro-emulsion disperse by wrapping surfactant. Particles are enlarged in micelles and precipitated by adding solvent such as acetone, ethyl alcohol. The size of micelles varies according to the molar ratio of water and surfactant. By mixing two identical microemulsions containing desired reactants the microdroplets will continuously collide, coalesce and break again and finally a precipitate forms in the micelles [Gupta and Gupta, 2005].

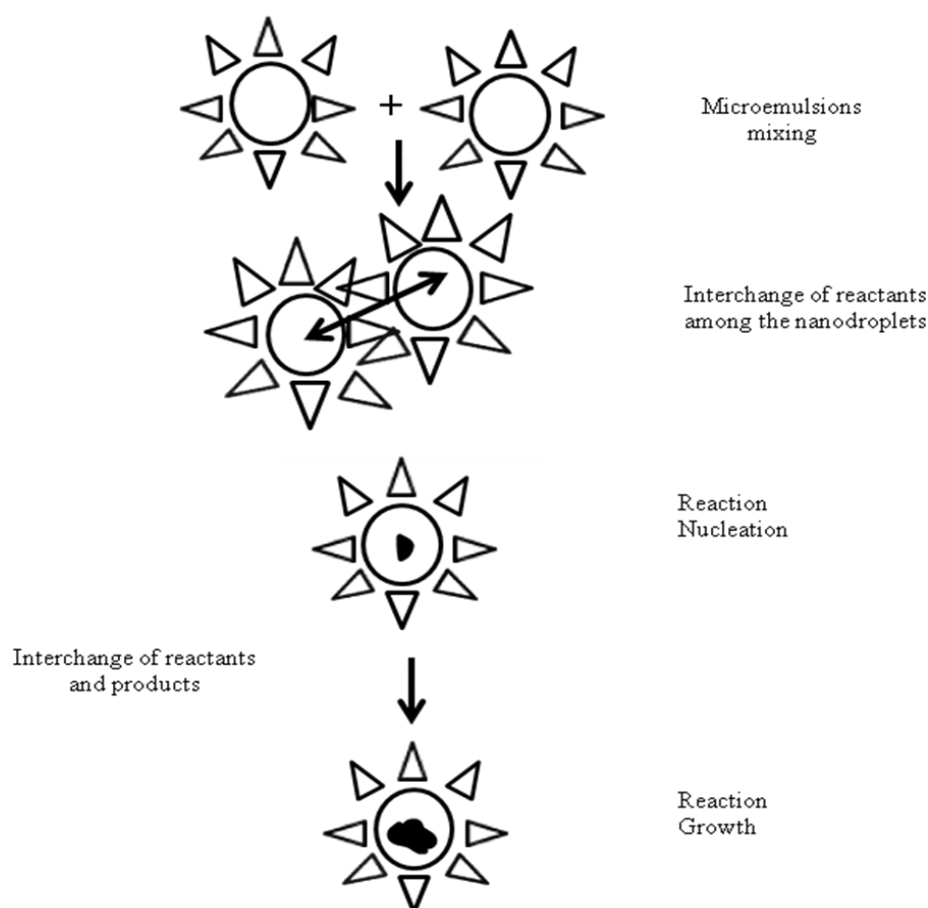


Figure 1.4 Mechanism of formation of nanoparticles in microemulsion [Lopez-Quintela, 2003].

1.3.3 Thermal Decomposition Route

This route is based on organic. Briefly, iron oleate complexes such as acetylacetonates $[M(acac)_n]$, ($M=Fe, Mn, Co, Ni, Cr$; $n=2$ or 3 , $acac=acetylacetonate$), metalcupferronates $[M^xCup_x]$, ($M=metal\ ion$; $Cup=N$ -nitrosophenylhydroxylamine, $C_6H_5N(NO)O^-$) were refluxed with stirring in the high boiling organic solvent in the presence of oleic acid [Rockenberger et al., 1999; Baykal et al., 2008]. Also by using carbonyls, which are organo-metallic precursors MNP's can be prepared [Farrell et al., 2003]. In this method surfactants such as fatty acids [Jana et al., 2004], oleic acid, and organic solvents as hexadecylamine are often used. Complex thermally decomposes in the meantime iron which is exposed transforms to iron oxides in the presence of oxygen environment and remains not too much growth. Nanocrystals which are obtained with

this method are good monodispers and they are well in organic solvents such as chloroform, hexane. Particle size varies from 5 to 22 nm depending on the boiling time and temperature.

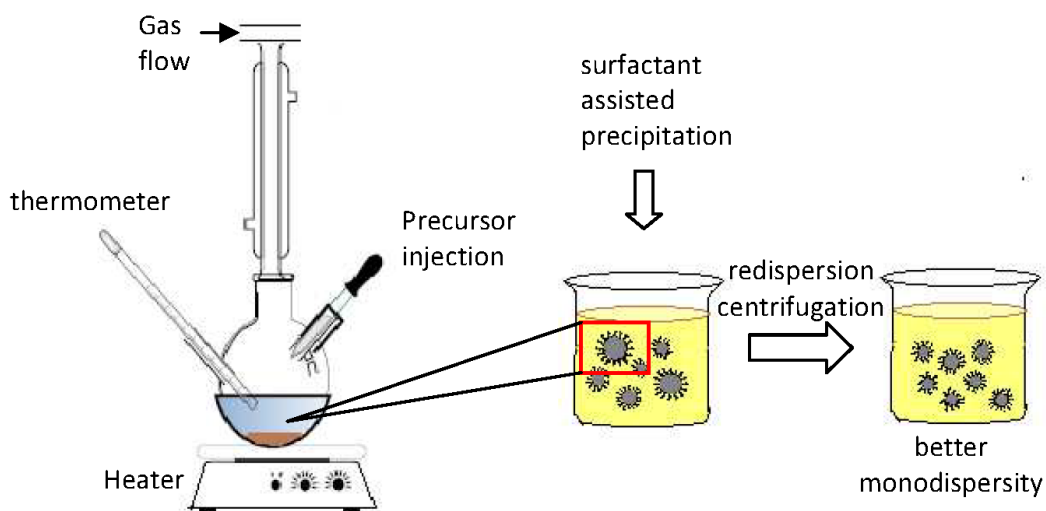


Figure 1.5 Synthesis of nanoparticles by thermal decomposition method [Umut, 2013].

1.3.4 Hydrothermal Route

Hydrothermal route is named as solvothermal method. MNPs and ultra fine powders can be prepared by using this method. [Baykal et al., 2013; Baykal et al., 2009; Baykal et al., 2008; Baykal et al., 2007].

Reactors or autoclaves are used to complete the reactions. Pressure and temperature in the reactors or autoclaves, can be higher. Nickel ferrite NPs by using ethylene glycol as solvent and sodium acetate as electrostatic stabilizer synthesized by Wang-et-al [Wang et al., 2009].

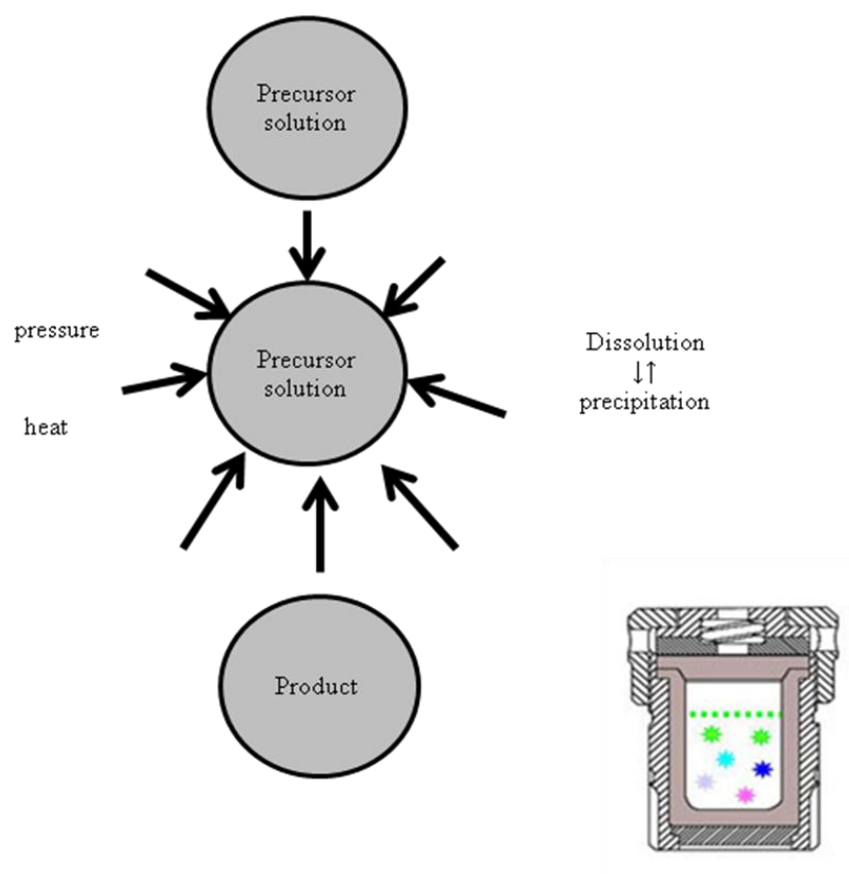


Figure 1.6 Synthesis of nanoparticles by hydrothermal route and high temperature and pressure vessel [UTPA, 2011].

Table 1.1 Summary comparisons of the synthetic methods [Lu et al., 2007].

Synthesis of Method	Co-precipitation	Thermal decomposition	Micro-emulsion	Hydrothermal synthesis
Synthesis	very simple, ambient conditions	complicated, inert atmosphere	complicated, ambient conditions	simple, high pressure
Temperature of Reaction (°C)	20-90	100-320	20-50	220
Period of Reaction	mins	hrs-days	hrs	hrs-days
Solvent	water	organic compound	organic compound	water-ethanol
Surface-capping agents	needed, added during or after reaction	needed, added during reaction	needed, added during reaction	needed, added during reaction
Distribution of Size	relatively narrow	very narrow	relatively narrow	very narrow
Shape Control	not good	very good	good	very good
Yield	high / scalable	high / scalable	low	Medium

1.4 SURFACE MODIFICATION OF MAGNETIC NANOPARTICLES

It is difficult to maintain stability of MNPs for a long time due to agglomeration and precipitation despite the important developments in the MNPs synthesis. The most important issue for application of magnetic nanoparticles is stability. Like Fe, Co, Ni metals which are pure metals and their metal alloys are sensitive, easily oxidize in air. Due to that reason, chemical stability of magnetic nanoparticles is necessary. It can be provided by protecting magnetic particles layer and in this condition oxygen does not affect the magnetic particles surface. To protect the magnetic nanoparticles oxidation by oxygen or erosion by acid or base are some strategies. On principle, the naked magnetic nanoparticle as a core is coated by a shell in all protection strategies [Baykal et al., 2012; Temizel et al., 2011; Yalçiner et al., 2011]. There are two ways to realize the coating of MNPs. Surfactants and polymers [Larsen et al., 2008; Liu et al., 2004; Hong et al., 2005; Sahoo et al., 2001; Kim et al., 2005] are organic coatings and silica [Kobayashi et al., 2003], carbon [Lu et al., 2005], precious metals (such as Ag [Sobal et al., 2002], Au [Liu et al., 1998; Lin et al., 2001]) or oxides are inorganic coatings.

1.4.1 Coating of Magnetic Nanoparticles

To prevent agglomeration during and after the synthesis, magnetic nanoparticles surface is passivated with surfactant or polymers. For this purpose, acids such as oleic acid, lauric acid, trioctyl phosphonic acid and bases as pyridine are used as surfactant and dextran, chitosan, starch, gelatin and gum arabic which are natural polymers and PEG, PBA, PLA, PMMA and PAA which are synthetic polymers are used as polymers. The organic based stabilizer with functional terminal groups can keep the particles dispersed in solution by the introduction of electrostatic or steric repulsions between these organic molecules as previously described by Papell in 1965 [Papell, 1965]. In fact, the net surface charge [Massart, 1981] and the nature of surfactant molecule [Raj and Moskowitz, 1990; Decuyper and Jonaiu, 1991; Wooding et al., 1992] adsorbed on the surface of ferromagnetic nanoparticles are the major factors affecting their colloidal stability in solutions. Due to co-precipitation of Fe^{2+} and Fe^{3+} in ammonia or NaOH solution MNPs are generally negatively charged in the synthesis of magnetic nanoparticles by co-precipitation method. As a result, highly unstable colloidal particles form and agglomeration occurs. By adding of surfactant with aqueous TMAOH or aqueous perchloric acid, peptization of magnetic nanoparticle precipitate is provided. At

this time, this undesirable condition can be prevented. Because precipitate disperses and forms a colloid. So, magnetic nanoparticles redisperses rapidly through surface adsorption [Massart, 1981]. Another way is to re-disperse the particles in nitric acid which oxidises them to maghemite ($\gamma\text{-Fe}_2\text{O}_3$) [Zins et al., 1999]. Magnetic nanoparticles can be chemically adsorbed or anchored by surfactants or polymers, creating repulsive single or double layers [Shen et al., 1999; Sousa et al., 2001]. Protective layers which provide stability to particles keep the particles colloidal form by balancing Van der Waals Forces and steric repulsion.

Cornell and Schertmann reported that functional groups such as phosphate, carboxylate and sulphate of polymer can bind to the magnetite surface [Cornell and Schertmann, 1996]. Coating of MNPs can be performed with the poly(aniline), poly(methylidene malonate), poly(alkylcyanoacrylates), poly(pyrrole) which are suitable polymers or poly (glycolic acid), poly(lactic acid), poly(ϵ -caprolactone which are polyesters [Wan and Li, 1998; Butterworth et al., 1996; Tartaj et al., 2005; Barrat, 2003]. For drug targeting and as contrast agents for magnetic resonance imaging (MRI), MNPs are coated with biocompatible polymers [Harris et al., 2003; Thunemann et al., 2006]. Inverse micro-emulsion for the polymer coated magnetic nanoparticles was studied by Chu et al [Chu et al., 1999]. Magnetite nanoparticle (Fe_3O_4) was first formed in the reversed aqueous micelle. Mixture of methacrylic acid and hydroxyethyl methacrylate monomers which are oil soluble was used polymer coating on the magnetic nanoparticles. Under a nitrogenic environment, (*N*, *N'*-methylenebis (acrylamide)) which is crosslinker molecule and (2,2-azobis(isobutyronitrile)) that is initiator were added. The polymerization reaction was conducted at 55 °C. Coated magnetite nanoparticles were precipitated in the mixture of acetone-methanol (9:1 ratio) and then redispersed in toluene. The coated magnetic nanoparticles showed a superparamagnetic property with an average size of 80 nm. Baykal et al. [Baykal et al., 2013] reported that one-step hydrothermal synthesis of Fe_3O_4 nanoparticles coated with Polyethyleneglycol (PEG). By using Polyethylene Glycol, Esir et al. [Esir et al., 2014] synthesized CoFe_2O_4 nanoparticles. TREG stabilized nanoparticle synthesized by Günay et al. [Günay et al., 2013], Erdemi et al. [Erdemi et al., 2013].

Polyaniline can also be used for coating of magnetic nanoparticles in the presence of oxidant ammonium peroxodisulphate [Deng et al., 2002]. Nanoparticles

which were obtained the end of the reaction showed polydispersity with an average diameter of 20-30 nm and core-shell structure. Asher et al. [Asher et al., 2002] reported that iron nanoparticles with an average size 10 nm can be embedded in polystyrene using emulsion polymerization [Xu et al., 2001]. Zhang et al. [Zhang et al., 2002] used similar method for coating of MnFe_2O_4 nanoparticles with polystyrene. Radical polymerization method was used and nanoparticles which have smaller than 15 nm and core-shell structure was obtained [Zhang et al., 2002]. MnFe_2O_4 NPs were in this procedure, mixed with 3-chloropropionic acid which is aqueous initiator at pH 4.0. Then, the excess amount of initiator was washed out and MnFe_2O_4 nanoparticles were dried in air. Following drying, they were added to styrene solution under nitrogen. Then xylene solution which contains CuCl and 4,4-dinonyl-2,2-dipyridyl was added. The solution was stirred and kept at 130 °C for 24 h to give the polystyrene coated MnFe_2O_4 nanoparticles. In this free radical polymerization, $\text{K}_2\text{S}_2\text{O}_8$ was used as catalyst. Polyaniline- MnFe_2O_4 -CTAB nanocomposite was successfully synthesized by Shafiu et al. [Shafiu et al., 2014]. Sözeri et al. [Sözeri et al., 2013] used oxidative chemical polymerization of aniline in the presence of ammonium peroxydisulfate (APS) to synthesize (PANI)/Cobalt-manganese ferrite. Kurtan et al. [Kurtan et al., 2013] used similar method to investigate the electrical properties of $\text{PANI-Co}_{0.5}\text{Mn}_{0.5}\text{Fe}_2\text{O}_4$ nanocomposite.

1.4.1.1 Dendrimer Coating

Dendrimers which are well-defined chemical structure are complex and large molecules. Also, they are monodisperse (consistent size and form) macromolecules which have regular and three-dimensional architecture.

The first dendrimers were made by divergent synthesis approaches by Fritz Vögtle in 1978 [Wogtle et al., 1978], R.G. Denkewalter at Allied Corporation in 1981 [Denkewalter et al., 1981], Donald Tomalia at Dow Chemical in 1983 and in 1985 [Tomalia et al., 1985] and by George Newkome in 1985 [Newkome et al., 1985].

Core that provides for growing of generations, branches which contain amine group and trap drugs or imaging agents and end (surface groups) are three parts of dendrimers [Fig. 1.7].

Functional groups on the molecular surface provide to the dendrimers some properties. However, there are examples of dendrimers with internal functionality [Anthoni et al., 2009; McElhanon et al., 2000; Liang et al., 2005]. Dendritic encapsulation provides the active site for the isolation to functional molecules [Hecht et al., 2001; Frechet et al., 2002; Fischer et al., 1999]. Also, it is possible to make dendrimers water soluble, unlike most polymers, by functionalizing their outer shell with charged species or other hydrophilic groups.

Toxicity, chirality, crystallinity and tecto-dendrimer formation are other controllable properties of dendrimers [Nanjwade et al., 2009].

Dendrimers are produced sequence of reaction steps which are iterative and each additional iteration creates a higher generation dendrimer.

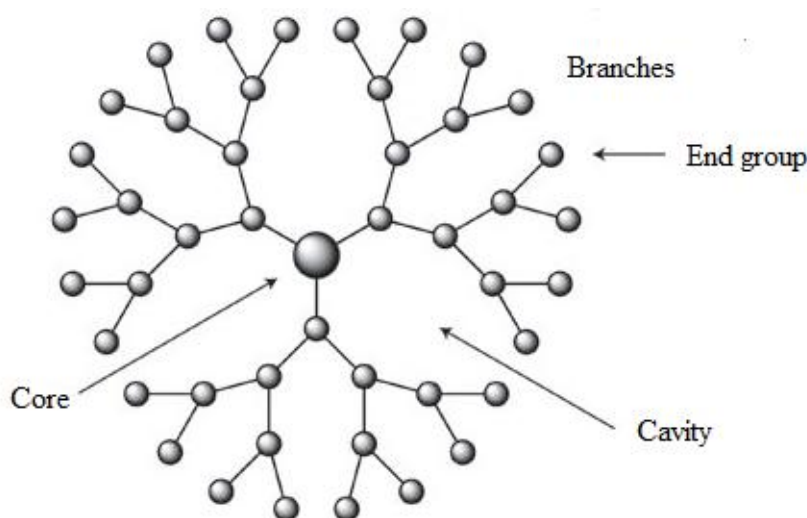


Figure 1.7 Dendrimer structure [Astruc, 2012].

Poly (amidoamine) (PAMAM) is a one of the member of the dendrimer family. This polymer has amide and amine functionality. Due to its functional group, it is biocompatible. Its trade name is 'Starburst' and first synthesized by Tomalia [Tomalia, 1985]. Synthesis of PAMAM dendrimers has some advantages. These are high level of

structural control, low polydispersity and greater biocompatibility which is due to interior amide bonds and the combination of surface amines. PAMAM dendrimers have wide range applications such as drug delivery, molecular encapsulation, gene therapy, building blocks for nanostructures, micelle mimics as decontaminating agents [Bosman et al., 1999]. For these materials, to be of maximum use for such diverse application, it is essential to obtain a comprehensive understanding of their structure and dynamics. There are some properties such as generation, surface area, distribution of monomer and terminal groups to affect the shape and size of the dendrimers [Maiti et al., 2004]. Kurtan et al. [Kurtan et al., 2014] synthesized amino-silane modified nanoparticles by coating with PAMAM dendrimer. The next study was sulfamic-acid functionalized magnetic Fe_3O_4 nanoparticles by coating with PAMAM [Kurtan et al., 2014].

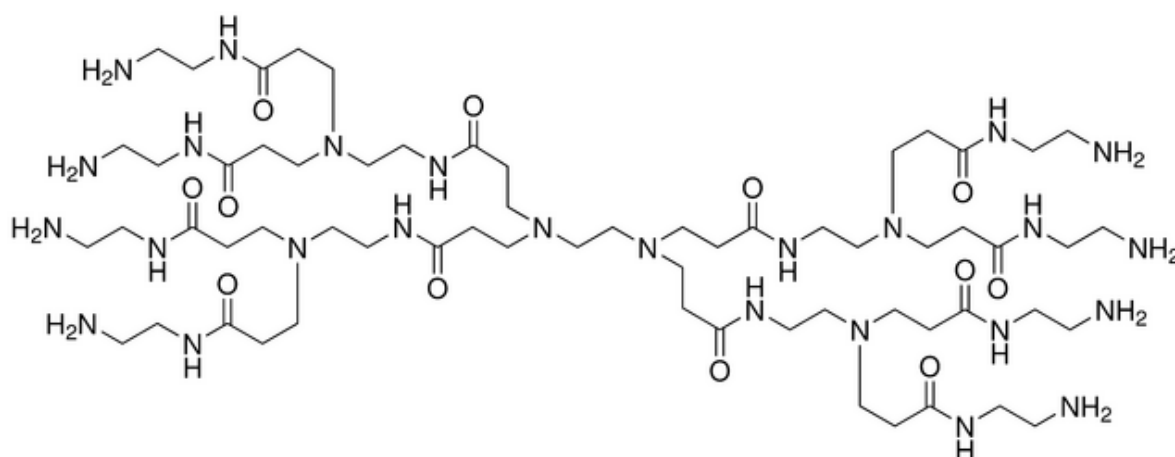


Figure 1.8 PAMAM dendrimer structure (Gen 1 PAMAM, Starburst Dendrimer) [Arthur, 2006].

1.4.1.2 Silica Coating

Coating of magnetic nanoparticles with silica protects magnetic core by preventing the direct contact of the magnetic core with additional chemical species [Ma et al., 2006].

Under a wide pH range, the silica shell shows great stability. In addition, functionalization of its surface allows for carrying chemical or biochemical species. For preparing silica-coated magnetic nanoparticles Stöber method can be used [Stober et al., 1968; Tago et al., 2002; Lu et al., 2002; Graf et al., 2003; Philipse et al., 1994]. Changing the ammonium hydroxide concentration and tetraethoxysilane (TEOS) to water ratio can be adjusted the thickness of silica shell. Also, organic additives which have various functional groups can easily be attached on the silica shell [Ulman, 1996]. This allows additional functionality such as bio-labelling, drug targeting, and drug delivery etc. Magnetic iron oxides which are hematite (Fe_2O_3) and magnetite (Fe_3O_4) can be coated with silica [Ohmori et al., 1992; Correa-Duarte et al., 1998; Ohmori et al., 1993].

Xia and co-workers have synthesized ferrofluids coated with silica shells by controlled hydrolysis of tetraethoxysilane TEOS [Lu et al., 2002]. TEOS was added to the ferrofluid with different amount. Silica coating was completed at room temperature within 3 hours. Kobayashi et al. [Kobayashi et al., 2003] reported that the synthesis of magnetic cobalt nanoparticle encapsulated in amorphous silica shell. In this method, TEOS and 3-aminopropyl trimethoxysilane (APTMS) were used which are as the silica precursors.

Stöber method is mainly used for nanoparticles surface modification. In this method, synthesis can take place in solvents which are hydrophobic or hydrophilic and silica shell that prevents the nanoparticles from coalescing but can also generate functional surfaces for further modification. Moreover, biocompatibility of nanoparticles increases due to the low toxicity of silica coating [Liz Marzan et al., 1996]. Liz Marzan reported the silica coating of gold nanoparticles using the Stöber method.

Stöber method cannot applied directly for the nanoparticle which synthesized in hydrophobic solvent and usually a primer which is silane-based agents such as APTMS or MPTMS that make surface of nanoparticle hydrophilic is required for the surface activation [Shen et al., 2008].

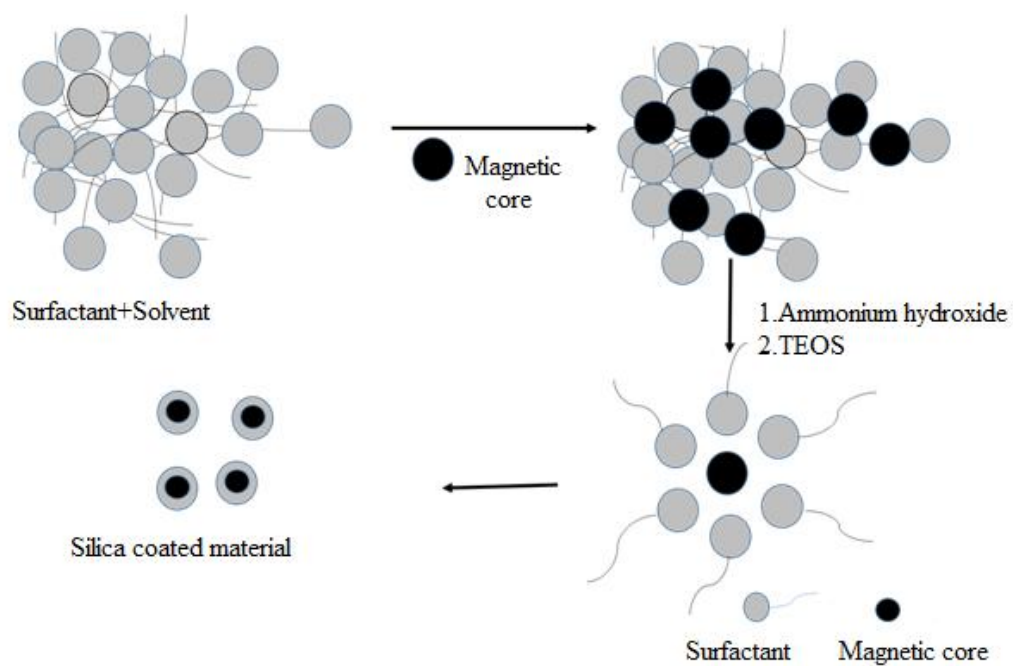


Figure 1.9 Schematic representation of preparation of silica-coated magnetite [Liane et al., 2012].

1.5 PROTEIN SEPARATION

1.5.1 Proteins

Proteins are polypeptides which are formed by covalent bonding of amino acids that are certain type, number and specific arrangement. Proteins are consisting of amino acids which are smaller units and they attaches to one another in long chains. Different types of amino acids combine and form protein. The sequence of amino acids determines each protein's unique 3-dimensional structure and its specific function.

In all cells and in all parts of the cells have proteins. In a bacteria cell, there are approximately 4,000 protein species. Each type of species is responsible for many functions. Proteins have many vital functions. Proteins which are large, complex molecules are important for body because of their functionalities. They are responsible the regulation of the body's tissues and organs and the work in cells [Altınışık, 2009].

Proteins are composed of long chains which contain amino acid groups that form peptide bond binding together with covalent bonds. In proteins, 20 kinds of α -amino acids are present and they show difference in the structure of R groups which may be hydrophilic or hydrophobic, acidic, basic or neutral.

There are four distinct levels of protein structures (Fig. 1.10). These are amino acid residues, primary, secondary, tertiary and quaternary structures. Each α -amino acid forms from side chain and back-bone part in all the amino acid types. Primary structure consists of the linear sequence of amino acids in the polypeptide chain. Primary structure can be kept by peptide bonds which are covalent bonds that are made during the protein biosynthesis process or translation. Regular polypeptide chains form secondary structure of protein wherein polypeptide chains are ordered sequentially, repetitively and one dimensionally. Linus Pauling and coworkers were suggested that the alpha helix and the beta strand or beta sheets in 1951 [Pauling et al., 1951]. They are two main types of secondary structures. There are hydrogen bonds between the main-chain peptide groups in these secondary structures. The folding of long polypeptide chains on each other creates three-dimensional tertiary structures. Quaternary structure is the three-dimensional structure of a multi-subunit protein and how the subunits fit together. Quaternary structure plays important role in enzymatic activity. In this

context, stabilization of the quaternary structure occurs with the same non-covalent interactions and disulfide bonds as the tertiary structure [Nelson, 2000].

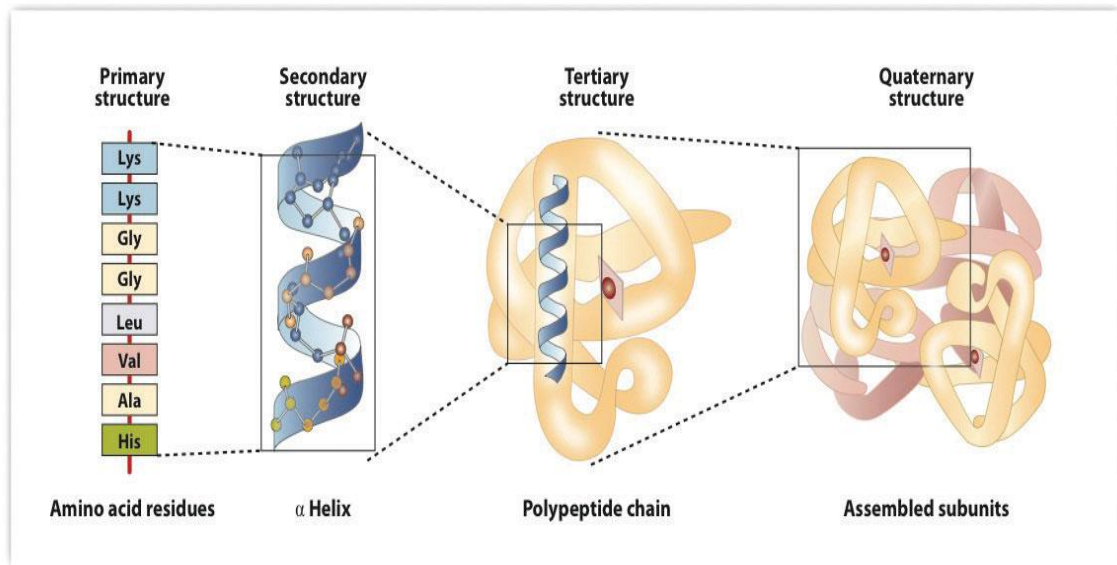


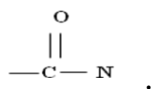
Figure 1.10 Levels of structure in proteins [Nelson and Cox, Lehninger Principles of Biochemistry (3rd edition)].

Figure 1.10 shows the protein structure levels. As seen from the figure, the specific amino acid sequence creates primary structure of proteins. Secondary structures are formed by twisting of peptide chain into a α -helix or beta sheet which are types of secondary structures. Incorporation of this helical segment forms the tertiary structure of protein which is the folded polypeptide chain. The single polypeptide chain is a subunit that constitutes the quaternary structure of a protein, such as hemoglobin that has four polypeptide chains.

There are covalent bonds and non-covalent bonds in the structure of proteins. Peptide and disulfide bonds are covalent bonds; hydrogen, ionic and hydrophobic bonds are non-covalent bonds of protein [Brandon et al., 1991].

Covalent Bonds

Bonds between α -carboxyl carbon of one amino acid and α -amino nitrogen of the other amino acid are peptide bonds which are symbolized as follows;



Disulphide bonds are S-S bonds which are formed resulting loss of sulfhydryl groups (Thiol, S-H) between two cystein residues. Disulphide bonds have a significant impact on the formation and protection of shape of protein molecule [He et al., 2006, Sevier et al., 2002].

Non-covalent Bonds

If the proton is shared between a proton acceptor (O, C and N) and proton donor (NH and OH), hydrogen bonds form [Hubbard et al, 2010]. Ionic bonds are formed by electrostatic attraction force ($\text{COO}^- \cdots \cdots \text{H}_3\text{N}^+$) which is developed between electronegative and electropositive groups. Hydrophobic bonds are formed with the weak attractive force which is known as Van der Waals-London [Karshikoff, 2006].

1.5.1.1 General Features of Proteins

Proteins have many features [NCS Pearson, 2014]. These are;

- a) Denaturation: Proteins become denaturated with various effects such as heat, X-ray or UV rays, effect of acid, organic solvent, etc. So, these parameters change the chemical and physical properties of proteins. For example, solubility of protein is drastically reduced and biological activity is lost.
- b) Amphoteric substance: Proteins are amphoteric agents, in other words amphoter electrolyte or ampholyte and they act both acid and base.
- c) Hydrolysis: Proteins become hydrolyzed the result of destruction of peptide bonds in polypeptide chains with water inlet.

1.5.1.2 Classification of Proteins

Proteins are divided into two groups according to their composition; simple and complex structure (conjugated) [Nelson, 2005].

Simple Proteins

They are only composed of amino acids that when they are hydrolyzed and the one who make chain of polypeptide.

Globular and fibrillar proteins are simple proteins. Globular proteins are divided into sub-groups; albumins, globulins, glutelins, prolamins, protamins, histones. Skleroproteins, fibrinogens and myosins are fibrillar proteins.

Conjugated Proteins

They are composed of amino acids but polypeptide chain binds to prosthetic group and when they hydrolyzed they give other chemical substances which have different character.

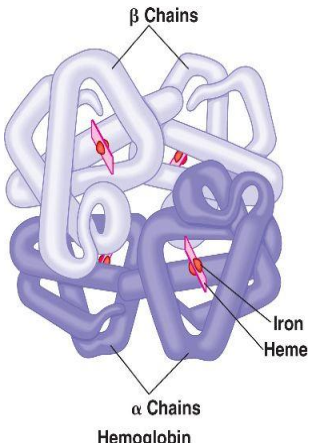
Type of protein	Simple		Conjugated
	Fibrous	Globular	
Shape			
Example of this protein	Collagen	Myoglobin	Hemoglobin

Figure 1.11 Classification of proteins.

Glycoproteins, proteoglycans, lipoproteins, phosphoproteins, nucleoproteins and chromoproteins are conjugated proteins [Nelson, 2005].

1.5.2 Bovine Serum Albumin (BSA)

Albumins are type of globular proteins which are soluble in water and the aqueous solution. In an aqueous solution which is generally saturated by ammonium sulfate, they precipitate and denature by heat. Their molecular weight is generally below 100,000.

Serum albumin which is in blood plasma with a concentration of 50 g/L has been the best model of protein for many years. It carries many endogenous and exogenous substances due to that reason it is named functional transport protein. Additionally, it regulates blood osmotic pressure and adjusts pH in blood so it has an important role in body [Carter et al., 1994].

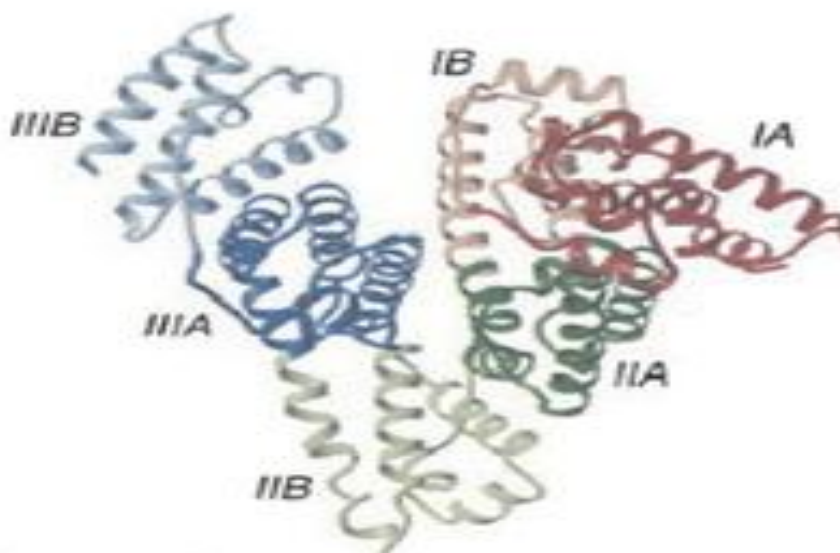


Figure 1.12 Domain structure of serum albumin [Huang et al., 2004].

Fig. 1.12 shows the heart-shaped serum albumin molecule. As seen from the figure, there are three homologous α -helical domains (I, II, III). Two sub-domains (A and B) which are in each domain share common structural motifs. BSA, ESA, RSA are type of albumin.

Bovine serum albumin which is a serum protein is same as blood serum albumin. BSA was the earliest primary sequence that was determined [Brown, 1975]. From the calculation of amino acid composition, its molecular weight is 66,411 g/mol and in a single peptide chain, it includes 583 types of amino acids [Peters, 1996]. Also, BSA has high content of residues of Asp, Glu, Ala, Leu and Lys. In the BSA structure, 17 disulfide bridges and one free SH group are found and this groups form dimmers which are linked covalently [Foster, 1977]. In biochemical applications such as immunohistochemistry, ELISAs and immunoblots, BSA has been widely used. Also, it has also been used as a nutrient in cell and microbial culture media.

1.5.3 Protein Properties for Separation Technics

Proteins are composed of different number and sequences of amino acids. These distinct characteristics provide many chemical and physical properties to protein. As a result, single protein can be purified from a mixture of thousands of proteins. Hence, properties of proteins determine the methods of separation technics. These properties are; such as metal and ligand binding, density, size, solubility, shape, hydrophobicity, charge distribution, isoelectric point and charge etc. [Burgess, 2006].

Table 1.2 Type of separation process according to the basis of separation [Burgess, 2006].

Separation Process	Basis of separation
Precipitation	
AMMONIUM SULFATE	Solubility
ACETONE	Solubility
POLYETHYLENEIMINE	Charge, size
ISOELECTRIC	Solubility, pI
Phase partitioning (e.g. with polyethylene glycol)	Solubility
Chromatography	
ION EXCHANGE (IEX)	Charge, charge distribution
HYDROPHOBIC INTERACTION (HIC)	Hydrophobicity
REVERSE-PHASE HPLC	Hydrophobicity, size
AFFINITY	Ligand-binding site
DNA AFFINITY	DNA-binding site
LECTIN AFFINITY	Carbohydrate content and type
IMMOBILIZED METAL AFFINITY (IMAC)	Metal binding
IMMUNOAFFINITY (IAC)	specific antigenic site
CHROMATOFOCUSING	pI
GEL FILTRATION/SIZE EXCLUSION (SEC)	Size, shape
Electrophoresis	
GEL ELECTROPHORESIS (PAGE)	Charge, size, shape
ISOELECTRIC FOCUSING (IEF)	pI
Centrifugation	Size, shape, density
Ultrafiltration	Size, shape

1.5.4 Type of Separation Methods

1.5.4.1 Ammonium Sulfate Precipitation (separation according to the solubility)

By resolution, the proteins can be separated or purified. The oldest method which is commonly known ammonium sulfate or sodium sulfate precipitation for separation or purification of protein. Globulin precipitates at 38 °C, 22.2% (saturated) and half saturated concentration of Na₂SO₄ (ammonium sulfate) solutions, albumin precipitates again in ammonium sulfate solution but which is only fully saturated concentration. During the separation process, for getting rid of the denaturation of proteins,

precipitation steps are realized in a cold environment. Another way to improve the method is to modify pH of the solution. At lowest pH, on solubility of the protein is equal to the isoelectric point of the protein solubility [Altınışik, 2009]. As seen from the Figure 1.13 all of the proteins are soluble at low ionic strength, the least soluble proteins and further proteins precipitate with the increasing of ionic strength.

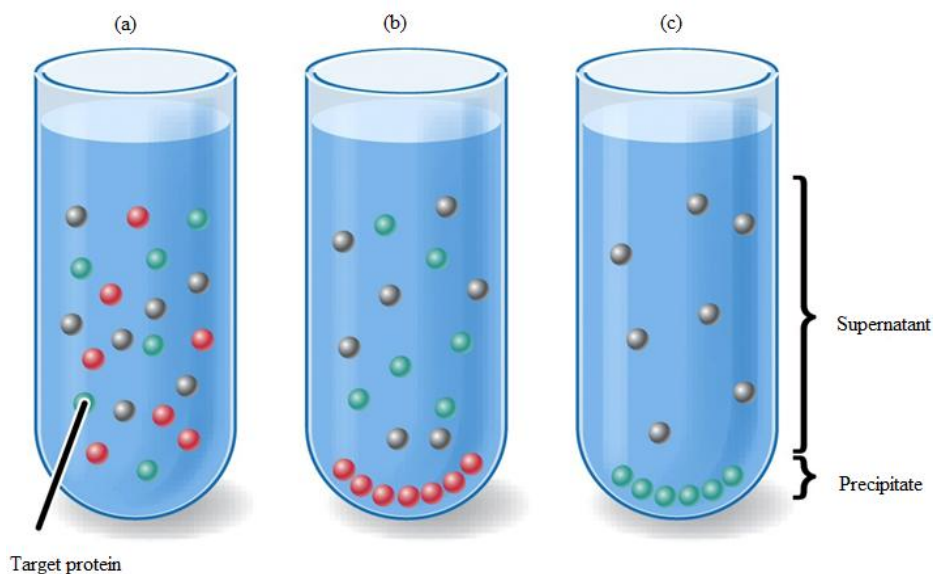


Figure 1.13 Ammonium sulfate precipitation (salting out), a) At low ionic strength, b) increasing of ionic strength, c) At higher ionic strengths [Nehete et al., 2013].

1.5.4.2 Separation on the Base of Electrical Charge

1.5.4.2.1 Electrophoresis

The method of electrophoresis depends on proteins different migration speeds. Because of this different migration speeds, proteins have different pH from the isoelectric point of the electrical field.

Electrophoresis process contains displacing of proteins toward the anode in buffer. Proteins which are separated according to the different migration speeds, on a carrier material are visualized by staining. The evaluation of the electropherogram is

obtained quantitatively. Cellulose acetate layer, agar gel, paper, starch gel, polyacrylamide gel are carrier materials used in electrophoresis. Depends on the carrier material type, there are some electrophoresis methods [Altınışık, 2009] which can be one dimensional (i.e. one plane of separation) or two dimensional. To separate the most routine protein and nucleic acid, electrophoresis is applied one dimensionally.

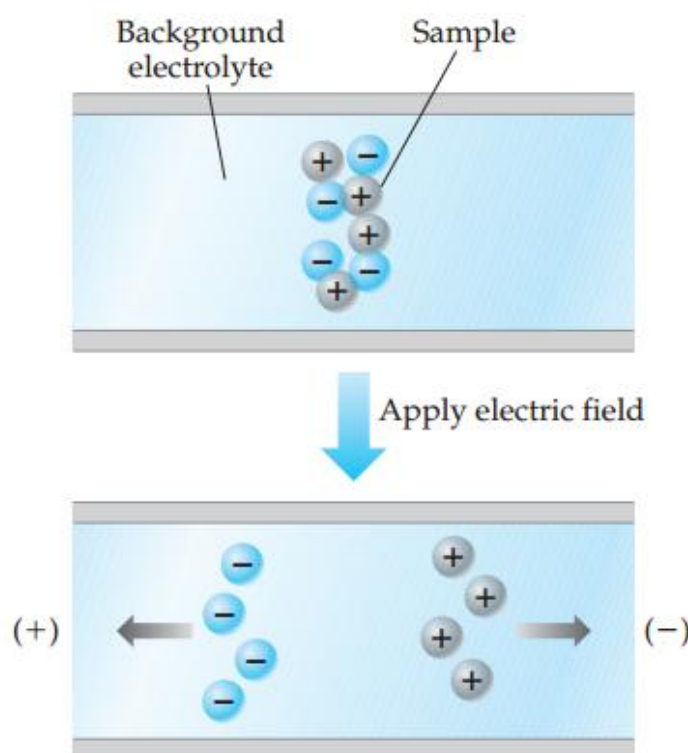


Figure 1.14 Separation of positively and negatively charged analytes in a sample by electrophoresis [Riekkola et al., 2004; Blanc et al., 1987; Inczedy et al., 1997; Karger et al., 1973].

For finger printing, proteins are separated by using two-dimensional electrophoresis. So, electrophoresis is classified two main categories as Gel and Capillary electrophoresis. Other gel electrophoresis are SDS-PAGE (Sodium dodecyl sulfate polyacrylamide gel electrophoresis), IEF (Isoelectric focusing), and 2-D (2-dimensional) [Righetti and Gelfi, 2000].

1.5.4.2.2 Isoelectric Focusing Method

Isoelectric focusing method is based on isoelectric point of protein. Separation of proteins depends on differences of the isoelectric point. By using artificial electrolyte in the electrical field, pH gradient is created. At pH gradient in the electrical field, every protein molecule is moved to suitable place depends on its isoelectric point and immobile where the forces are equal two sides and pH is equal to isoelectric point.

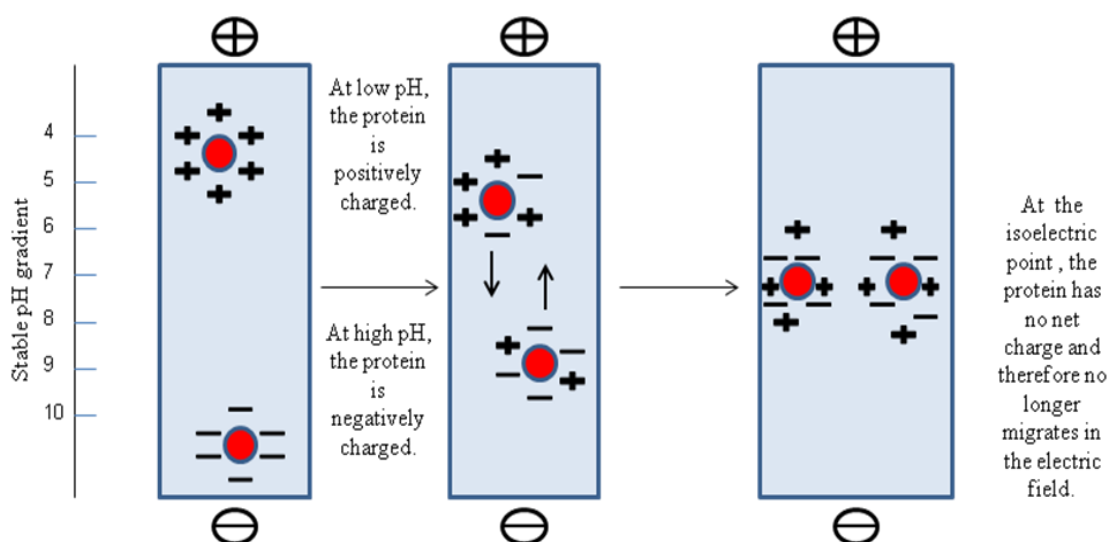


Figure 1.15 Separation of protein molecules by isoelectric focusing [Motifolio Inc., Molecular Cell Biology, 2015].

1.5.4.2.3 Ion Exchange Column Chromatography

Ion exchange chromatography contains two phases. In the stationary phase, there is cellulose in the column. Cellulose is equipped positively charged diethylaminoethyl and carboxymethyl groups. Mobile phase contains counterions. Addition of protein to mobile phase, displacing of proteins with the counterions is realized condition of charge and retained on the column by stationary phase. Separation process is achieved with changing of pH of mobile phase and setting to the isoelectric point of protein. Separation of protein is completed by passing through eluate with the opening of the

faucet. Determination of protein concentration of eluate is measured with by using UV instruments.

1.5.4.3 Separation on the Base of Molecular Size

1.5.4.3.1 Ultra-Centrifugation

In an ultra-centrifugation method, large molecules are sedimented against gravitational field.

Analytical ultra-centrifugation has important role in proteins and their complexes characterization [Schachman, 1992]. It is based on density difference between the particles/macromolecules. Molecules are dispersed in the medium where subjected to gravitational fields [Koolmann and Roehm, 2005]. Quantum-dot binding protein characterized by ultracentrifugation [Wervie et al., 2014].

1.5.4.3.2 Gel Filtration (Size-exclusion) Chromatography

In the size-exclusion chromatography that is abbreviated SEC which is a type of chromatographic method, molecules which in solution are separated basis of size and molecular weight. This chromatographic method is generally used for proteins and industrial polymers which are large molecules or macromolecular complexes.

Gel filtration medium is used in SEC. To form a packed bed, it is packed into a column. Due to chemical and physical stability and inertness, porous matrix is chosen as medium which is in the form of spherical particles. Stationary phase is the liquid which is inside the pores. Mobile phase is particles which are in equilibrium with liquid which is inside and outside [Dauphin et al., 2007].

Briefly, proteins which are small and medium-sized enter the hollow of gel particles which behaves molecular sieve. By using suitable solvent, the column is eluted and protein/s is/are obtained. For proteomics analysis, SEC which plays a practical role has been used [Lecchi et al., 2003].

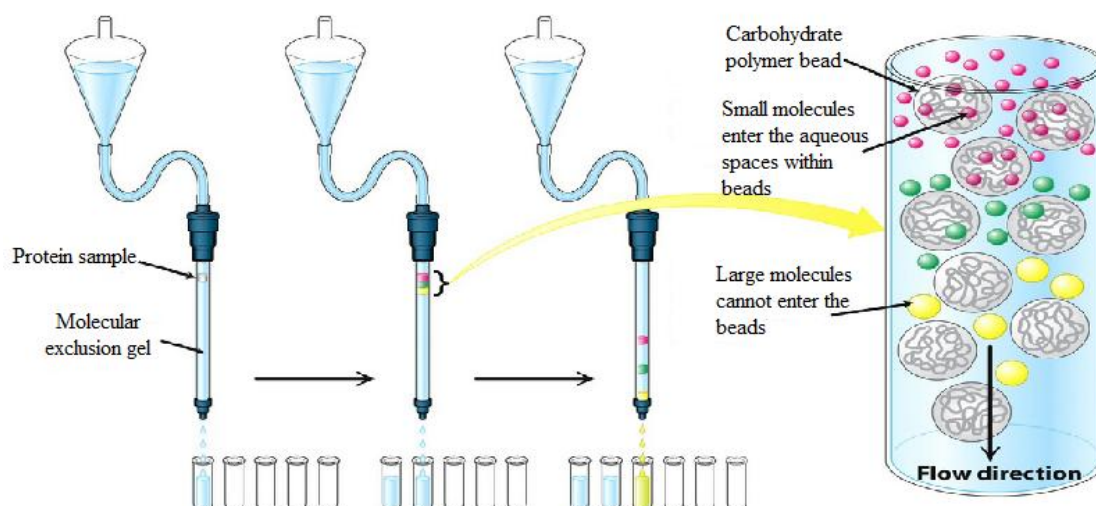


Figure 1.16 Gel filtration methods [Berg et al., 2012].

1.5.4.3.3 Affinity Chromatography

In the affinity chromatography, separation process realizes with the interaction between a protein or group of protein and a specific ligand in the chromatography matrix. High capacity, high selectivity and high resolution are some advantages of this technique. In the affinity chromatography, biomolecule is purified according to its chemical structure and biological function which are important properties make unique this chromatographic method among the other purification technology. Because of time-consuming, difficulty and impossibility of other techniques purification processes can often be easily achieved with affinity chromatography. Furthermore, by using affinity chromatography, isolation of pure substances at low concentration in large volumes of crude sample, separation of biomolecules from denaturated or functionally different forms, and removing of specific contaminants can be performed [Amersham Biosciences AB, 2002].

In the affinity process, proteins bind to small molecules specifically. At that time, substrate molecule attaches polysaccharide such as agarose which is carrier material by a chemical reaction and then is placed in a column. While other proteins are passing through the column, substrate catches its specific proteins in the mixture when a protein

mixture is passed through the column. Then, protein is removed from the column by passing substrate and then is divided in eluate.

Porath [Porath, 1992] performed immobilized metal ion affinity chromatography under very mild and nondenaturing conditions. Applications of affinity chromatography in proteomix were studied by Lee and Lee [Lee and Lee, 2004].

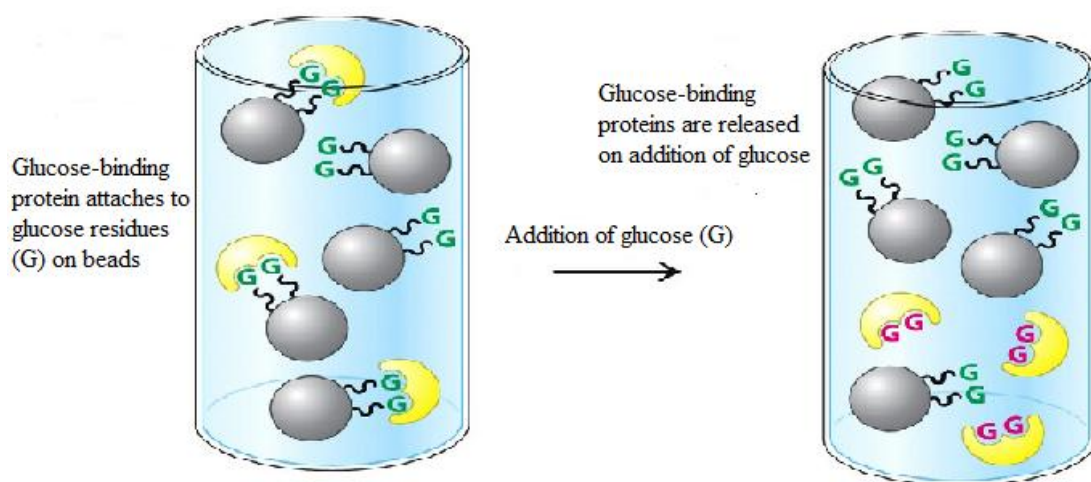


Figure 1.17 Affinity Chromatography [Berg et al., 2012].

1.5.4.4 Magnetic Techniques for Protein Separation/Purification

In the magnetic techniques, magnetic carriers which bear magnetic biopolymer, hydrophobic or immobilized affinity ligand or ion-exchange groups, with target compound(s) are mixed. Waste from food and fermentation industry, crude cell lysates, cultivation media, whole blood, urine, plasma, milk, ascites fluid are examples of target compound. After the magnetic particles binding to the target molecule, using a suitable magnetic separator, magnetic complex is easily and rapidly removed from the sample [Safarik and Safarikova, 2004]. After washing out the contaminants, the purified target compound(s) can be eluted. Then, it can be used for further work (Fig. 1.18 and 1.19).

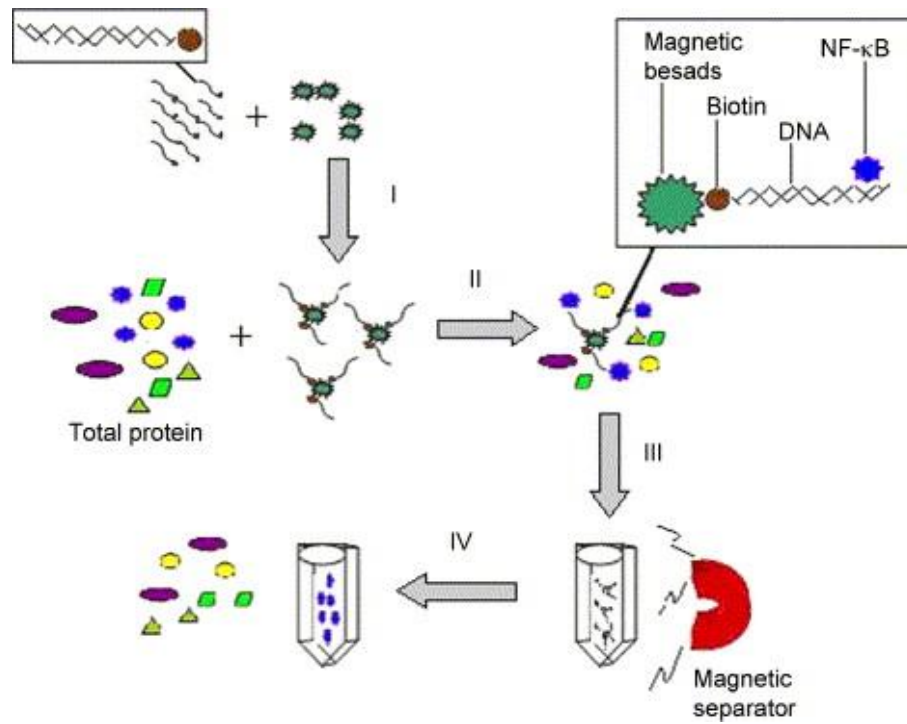


Figure 1.18 Protein separations by magnetic techniques [Gubin, 2009].

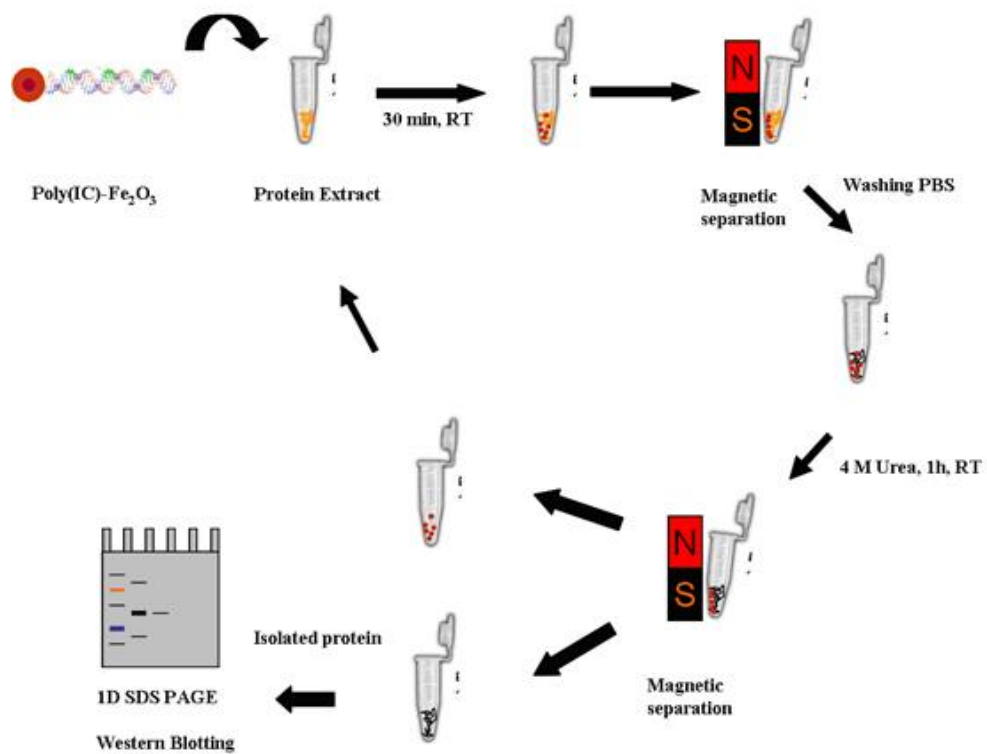


Figure 1.19 Protein separations by magnetic techniques [Shukoor et al., 2011].

There are some advantages of magnetic separation techniques when compared with other separation methods. Due to a few handling steps magnetic separation process is simple. One single test tube or another vessel is enough the purification procedure for all steps. This technique eliminates the pretreatment such as filtration, centrifugation etc. So, no need to use costly systems which are filters, centrifuges, liquid chromatography or other instruments. Direct separation can be performed in crude samples which contain suspended solid material. The total separation time is shortened by entegrating all the steps which contain the disintegration and separation for isolating intracellular proteins [Schuster et al., 2000]. Magnetic adsorbents which have magnetic properties can be easily removed from the sample. Magnetic separation is suitable method for recovering of small magnetic particles which have 0.1-1.0 μm in diameter in the presence of biological debris and other fouling material of similar size. In addition, at large scale operation, magnetic separation is useful, efficient and powerful procedure. The magnetic separation is fundamental technique for many automated procedures, especially magnetic-particle based immunoassay systems for the determination of a variety of analytes, among them proteins and peptides. Recently, there are several automated systems to separate nucleic acids and proteins. Magnetic separation is usually very gentle for separating target proteins or peptides. In traditional column chromatography, protein complexes which are large tend to be broken up. In contrast, when gentle magnetic separation procedure is used, large protein complexes may remain intact [Hoffmann et al., 2002]. Separation process is affected positively by the higher protein concentration and the reduced shearing forces throughout the isolation process.

While target proteins are separated in the standard chromatography techniques formation of diluted protein solution which have large volume is observed. Instead of pretreatments such as precipitation, ultrafiltration, etc. suitable magnetic particles can be used for decreasing its concentration [Alche and Dickinson, 1998].

In the isolation of protein and peptides, protein G, streptavidin, protein A, antibodies which are available immobilized affinity ligands with particles for magnetic affinity adsorption are often used. Immobilized ligands that contain magnetic particles which are mentioned above can also serve as generic solid phases to which native or modified affinity ligands such as biotinylated molecules in the case of immobilized

streptavidin, antibodies in the case of immobilized protein A, protein G or secondary antibodies can be immobilized.

Gelatin, nitriloacetic acid, trypsin inhibitor, glutathione and trypsin which are affinity ligands are already immobilized to commercially available carriers. Standard procedures which are applied in affinity chromatography can be used to immobilize the affinity ligands with magnetic particles which are commercial and laboratory made. There are some available functional groups which are generally -NH_2 -OH or COOH , on the surface of magnetic particles for immobilizing and in some cases; it is possible to use epoxyactivated, tosylactivated magnetic particles which are in the activated form [Safarik and Safarikova, 2004].

Surface of magnetite, maghemite or ferrites which are magnetic particles can be silanized for modification in the laboratory. In this step, surface of inorganic particles is modified and this enables easy immobilization of affinity ligands because of the available of the suitable [Weetall and Lee, 1989]. Exposition of iron oxides with the usage of magnetic nanoparticles can decrease enzymatic activity in exceptional cases. At this condition, having an outer layer of pure polymer which is encapsulated microspheres will be safer.

1.6 PROTEIN ASSAY METHODS

According to the type of chemistry, protein assay methods which are generally colorimetric can be classified based on copper and dye. Copper based protein assay involves the chelation of protein-copper and dye based protein assay contains color change of the bound dye.

1.6.1 Copper-Based Protein Assay Methods

1.6.1.1 *Peptides and the Biuret Reaction*

Among the other spectrophotometric (colorimetric) methods, the least sensitive and oldest assay for total protein analysis is Biuret assays. The biuret assay has some advantages. These are easy preparation of reagent, having less sensitive chemical interference and easy operation which make the Biuret assay more applicable. This assay contains chelation process of cupric ion (Cu^{2+}) with polypeptide in strong basic condition [Gornall et al., 1949; Itzhaki and Gill, 1964].

For analyzing protein both quantitatively and qualitatively, the biuret reaction can be used. The presence of peptide bonds in the protein provides the availability of this method. As a result of interaction of Cu^{2+} with the peptide complex in a basic condition, the color of solution changes to purple. Quantitative measurement can be performed spectrophotometrically in the visible region. Alkaline copper sulfate solution is generally used as biuret reagent. The color intensity depends on the number of peptide bonds which reacts with biuret reagent. Biuret assay can not be applied to amino acids and also di-peptides which have only one peptide bond but do with tri-, oligo-, and poly-peptides. As understood clearly that if at least two peptide bonds such as HN-CO , $-\text{HN-CH}_2-$, $-\text{HN-CS-}$ in the molecule, biuret reaction can be done.

The Biuret reaction takes its name from biuret which is obtained by heating urea then reaction of cupric ions give a similar colored complex [Hortin and Meilinger, 2005].

1.6.1.2 Bicinchoninic Acid (BCA) Protein Assays

In the bicinchoninic assay, cupric ion (Cu^{2+}) is reduced to the cuprous ion (Cu^+) by protein in the basic condition. During the process, chelation occurs and color of the solution changes. Bicinchoninic acid (BCA) reagent is responsible of the part of process [Smith et al., 1985].

There is similarity between the bicinchoninic acid (BCA) and Lowry assay due to depends on the conversion of Cu^{2+} to Cu^+ under alkaline conditions. These assays have same sensitivity but due to the stability of BCA under alkaline conditions, BCA carries out one-step process while Lowry assay is two. The reaction results in the development of an intense purple color with an absorbance maximum at 562 nm. The production of cuprous ion (Cu^+) depends on concentration of protein and time of incubation. Protein content of unknown samples can be measured to compare with known protein standards by using spectrophotometer. In Lowry assay, some compounds can interfere the reaction but BCA assay is more tolerant to the presence of such compounds then this property is another advantage of the BCA. Furthermore, BCA is not affected urea and guanidinium chloride which are detergents and denaturing agents although it is more sensitive to the presence of reducing sugars [Walker, 2002].

1.6.1.3 Lowry Protein Assays

To determine the total protein which in solution, Lowry assay which is biochemical assay can be used. In this assay, the ratio of protein concentration plays important role on the sample by changing the color. Using colorimetric techniques, the total concentration of protein can be measured [Lowry et al., 1951]. This assay took the name by Oliver H. Lowry who is biochemist and developed Folin-Ciocalteu reagent in the 1940s.

1.6.2 Dye-Based Protein Assay Methods

1.6.2.1 Coomassie Dye (Bradford) Protein Assay

Dr. Marion Bradford is a scientist who described using of Coomassie G-250 dye which is colorimetric reagent to detect the total protein in 1976. Brilliant blue G-250 is the other name of the Coomassie dye which binds to protein molecules in acidic pH. There are two groups in the structure of Coomassie dye. While triphenylmethane group provides the binding of nonpolar structures of protein, the anion sulfonate groups are responsible with the interacting of arginine and lysine side chains which are cationic side chains of protein in acidic pH [Lovrien et al., 1995].

In the mechanism, dye binds to proteins and solution of color changes that provides total protein measurement [Bradford, 1976; Sedmak and Grossberg, 1977]. Coomassie dye is sensitive to the detection of albumin and globular protein. Bradford assay is the most popular and used method due to its sensitivity and simplicity.

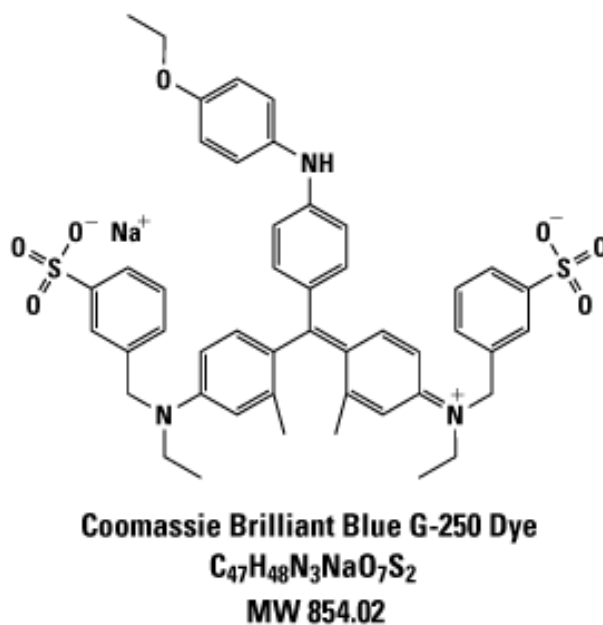


Figure 1.20 Chemical structure of coomassie dye [Weiler, 1917].

1.6.2.2 Pierce 660nm Protein Assay

Pierce 660nm protein assay is similar to Coomassie-based assay. It was introduced by the Thermo Scientific in 2008. For most detergents, Pierce 660nm protein assay is suitable and more linear response curve can be obtained. In Pierce 660nm assay, the dye-metal complex which is found binds to protein in an acidic buffer. The dye-metal complex is shifted at 660nm which is maximum absorption. The color of reagent before the binding is reddish-brown then turns to green when protein is bound.

Due to amino acid groups which are positively charged and dye-metal complex which is deprotonated negatively at low pH, the color of the solution changes [Antharavally et al., 2009]. In the assay, stability of color increases with increasing protein concentrations.

1.6.2.3 Bio-Rad Protein Assay

Bio-Rad Protein assay is similar with Bradford assay and total protein concentration is measured spectrophotometrically. Acidic dye is added to protein solution and measurements are performed at 595 nm by using microplate reader or spectrophotometer in this assay.

Color changes with the different protein concentrations in the Bio-Rad Protein assay which is a dye-binding assay [Bradford, 1976]. Coomassie Brilliant Blue G-250 dye which is reagent that used in this assay shifts from 465 nm to 595 nm. At 595 nm which is maximum absorbance dye binds to protein [Reisner et al., 1975; Fazekas de St. Groth et al., 1963; Sedmak et al., 1977]. The binding of Coomassie blue dye to arginine which is primarily basic and aromatic amino acid residue was reported by Compton [Compton et al., 1985].

CHAPTER 2

EXPERIMENTAL

2.1 MATERIALS

Bovine Serum Albumin (BSA) was obtained from Sigma Aldrich. Ferrous chloride tetrahydrate ($\text{FeCl}_2 \cdot 4\text{H}_2\text{O}$), ferric chloride hexahydrate ($\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$), 3-amine-propyl trimethoxysilane (APTMS, $\text{NH}_2(\text{CH}_2)_3\text{-Si}(\text{OCH}_3)_3$), ethylenediamine ($\text{C}_2\text{H}_8\text{N}_2$), hydrochloric acid (HCl), sodium chloride (NaCl), sodium hydroxide (NaOH), methylacrylate ($\text{C}_4\text{H}_8\text{O}_2$), ethanol ($\text{C}_2\text{H}_5\text{OH}$), methanol (CH_3OH), toluene (C_7H_8), tetramethyl ammonium hydroxide ($\text{N}(\text{CH}_3)_4^+\text{OH}^-$), and mono chloro acetic acid ($\text{C}_2\text{H}_3\text{ClO}_2$) were purchased from Merck. All other chemicals used were of the highest purity grade.

2.2 INSTRUMENTATION

Huber JSO-Debyeflex 1001 Diffractometer which is operated at 40 kV and 35 mA using Cu K_α radiation ($\lambda = 1.54178 \text{ \AA}$) was used for X-Ray powder diffraction (XRD) analysis.

Fourier Transform analysis was performed by using FT-IR Spectrometer (Bruker ALPHA) which is scanned the range of $4000\text{--}400 \text{ cm}^{-1}$ to investigate the modification of superparamagnetic iron oxide nanoparticles (SPIONs) with Poly (amidoamine) dendrimers (PAMAM).

Transmission electron microscopy (TEM) (FEI Tecnai G2 Sphera microscope) was used to confirm the particle size and for characterizing of morphology of the

prepared materials. First sample was placed into column of TEM instruments then a drop of sample which in alcohol was dripped to TEM grid. The size distribution was determined by measuring at least 200 particles from five micrographs.

TGA measurement was performed by Perkin Elmer Instruments model, STA 6000 under N_2 in the temperature range of 30–800 °C with an increasing heating rate of 108 °C/min to determine the thermal stability. For thermo gravimetric analysis, 5 mg of powder sample was placed to TGA instrument.

Quantum Design Vibrating sample magnetometer (QD-VSM) was used for VSM analysis. The magnetization of sample measurements was performed up to 10 kOe at room temperature. Measurements of ZFC (zero field cooling) and FC (field cooling) were carried out at 100 Oe and the blocking temperature was determined from the measurements.

Biotek Microplate Reader (Biotek Instruments, Power Wave XS) was used for the determination of immobilized BSA proteins from the supernatant. Gen5 Data Analysis Software programme integrated with biotek instruments was used. Experimental procedure for protein adsorption measurements was selected from Gen5 (Temperature: 37 °C, Incubation time: 4 min. and Absorbance: 595 nm).

2.3 SYNTHESIS

2.3.1 Polyamidoamine (PAMAM) Functionalized SPION Synthesis

2.3.1.1 *Synthesis of Fe_3O_4 Nanoparticle (SPION)*

Preparation of an aqueous suspension of SPION which has an average particle size between 8-10 nm were realized by using controlled co-precipitation method as reported earlier [Mikhaylova et al., 2004]. Shortly, under nitrogen gas which prevents oxidation during the reaction, a solution mixture 25 ml of 1 M $FeCl_3 \cdot 6H_2O$, 0.5 M $FeCl_2 \cdot 4H_2O$ and 0.4 M HCl was prepared. The co-precipitation of SPION was carried out in a reactor with high-speed mechanical stirring (2000 rpm). First, iron solution which was preheated to 80 °C before the co-precipitation reaction was added to NaOH

that was 250 ml of 0.5 M. To adjust to pH to 11, TMAOH solution (5 wt. %) was added drop by drop into the solution with mixing. By using external magnetic field, black powder was collected (Fig. 2.1) and washed several times with water and ethanol. In Fig. 2.1, SPION synthesis is shown.

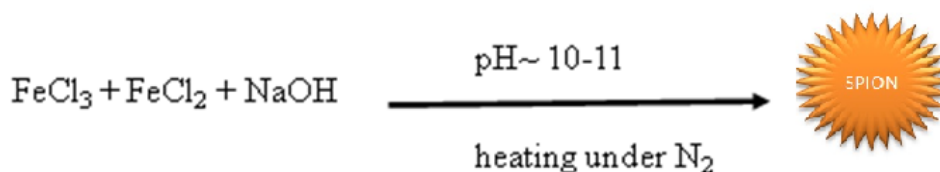


Figure 2.1 Schematic representation of synthesis of SPION.

2.3.1.2 Surface Modification of SPION with APTMS

APTMS-coated SPION was dispersed in toluene/methanol with a concentration of 1:1 v/v (Fig. 2.2, step 1). For the modification of SPION, the following procedure was performed. Methanol was used for magnetic separation of SPION. The 50 ml SPION ethanol solution was prepared and was diluted to 150 ml by ethanol. A 10 ml amount of 3-aminopropyltrimethoxysilane ($\text{NH}_2(\text{CH}_2)_3\text{-Si}(\text{OCH}_3)_3$, APTMS) was added into the SPION-ethanol solution which was placed in two-necked flask with water-cooled condenser and temperature controller. The silanization was performed at 60 °C for 7h with rapid stirring. The product was washed with methanol for several times (Fig. 2.2, step 1] and dried into powder at room temperature under vacuum. G0 represents the SPION modified only with APTMS. Silanization process proceeds through particle surfaces which bears the hydroxyl groups in the organic solvent at that time APTMS acts as a coupling agent.

2.3.1.3 Surface Modification of SPION with PAMAM Dendrimer

Generation of dendrimer was initiated with dispersing G0-SPION in 50 ml methanol solution. The addition of 20 ml methylacrylate to the solution, the suspension

was immersed in an ultrasonicated water bath at room temperature and stirred for 7h. The particles were collected magnetically and washed with methanol 5 times by magnetic separation. Then 4 ml ethylenediamine was added and the suspension stirred at room temperature for 3h in an ultrasonicated water bath. The particles were again rinsed with methanol five times by magnetic separation.

The addition of methylacrylate and ethylenediamine was repeated until the desired number of generations (G1-G5) was achieved on the SPION surface. The PAMAM-SPION was then washed 3 times with 25 ml methanol and five times with 25 ml water by magnetic separation.

Surface modification of SPION with PAMAM dendrimer is shown in Fig. 2.2 step 2, step 3, step 4, and step 5.

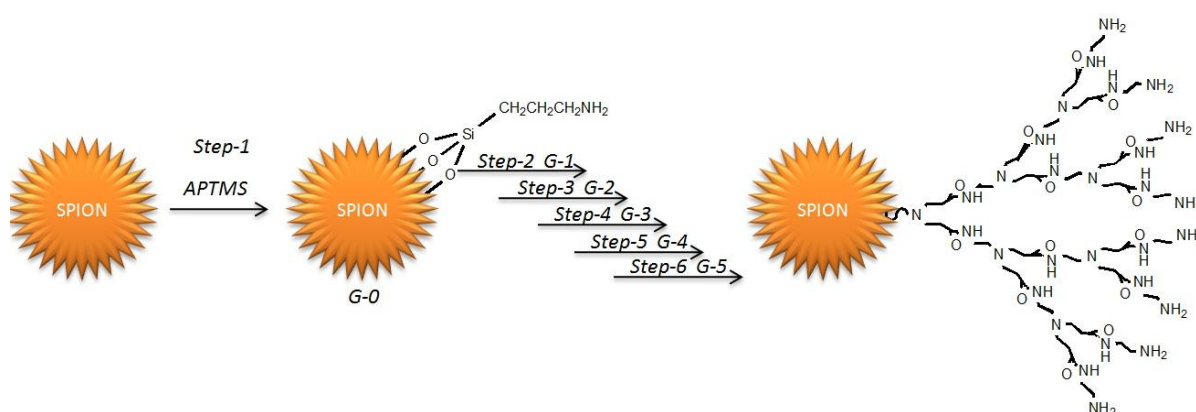


Figure 2.2 Synthesis of PAMAM modified SPION.

2.3.1.4 Modification of PAMAM-SPION with Chloro Acetic Acid

To modificate PAMAM-SPION with chloro acetic acid ($\text{C}_2\text{H}_3\text{ClO}_2$), 0.5 M chloro acetic acid solution (9.45 gr / 200 ml) was prepared. The mixture of G5 dendrimer (0.5 gr) and chloro acetic acid solution (100 ml) was put in a 500 ml one necked round-bottom flask and placed in a condenser at 30 °C stirred for 1 week. Then, the result solution was washed three times with water and methanol once by magnetic separation.

The product was dried into vacuum at 70 °C for 1.5 h to obtain chloro acetic acid modified G5. After the drying, modified product was obtained. Modification of PAMAM-SPION with chloro acetic acid is presented in Fig. 2.3.

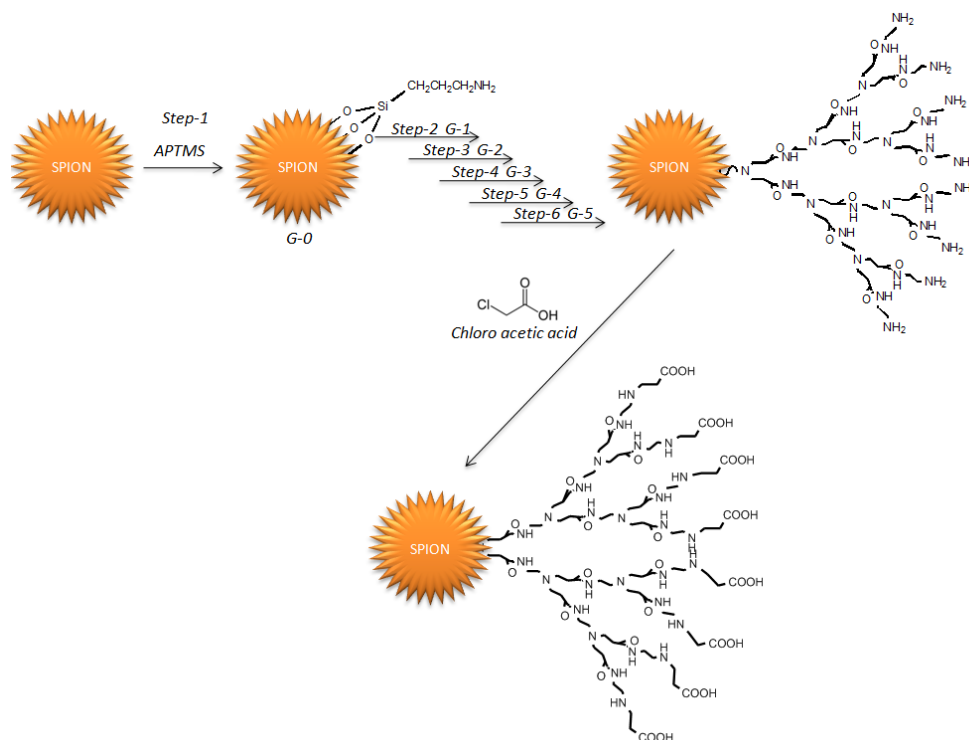


Figure 2.3 Modification of G5 with MCA [Baykal et al., 2014].

2.4. BSA ADSORPTION STUDIES

2.4.1 Metal Chelation

Metal ions (Cu^{2+} , Zn^{2+} , Co^{2+} , Ni^{2+}) which contain 50 ppm metal ions in 2 ml solutions and modified SPION was mixed for chelation.

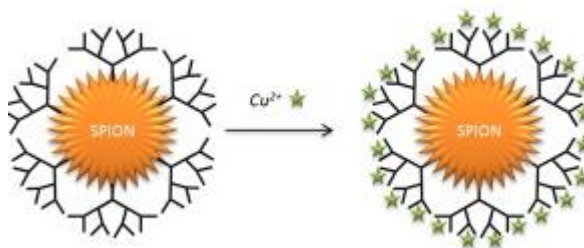


Figure 2.4 Chelation of SPION-PAMAM [Baykal et al., 2014].

This step was performed by using HCl and NaOH to adjust pH 5.0. The solution was shaken at 25 °C for 2h by using orbital shaker. Chelation of SPION-PAMAM dendrimer is shown in Fig. 2.4.

2.4.2 BSA Adsorption (immobilization)

Immobilization of BSA on PAMAM modified SPION is shown in Fig. 2.5. All adsorption of BSA studies was performed in the range of pH 3.0 and 8.0. Acetate and phosphate buffer solutions which are 0.05M were used for this reason. Initial enzyme concentration was taken 1.5 mg/mL. All BSA adsorption studies were performed at 25 °C for 3 h while shaking continuously in an orbital shaker. Removing of protein adsorption beads and washing steps were realized by using same buffer solution.

Separation of mixtures was made by using suitable magnet. Protein detection with SPION-PAMAM was achieved by Biotek Microplate Reader at 595 nm from the supernatant solution. BSA adsorption amount was calculated by the following formula below.

$$Q = (C_0 - C_e) V / m$$

In this equation Q is the adsorbed BSA (mg/g), C_0 refers the concentration of BSA in the initial solution and C_e is the equilibrium concentrations in the solution after the measurement., V is the solution volume (mL) and m is the mass of PAMAM modified SPION (g).

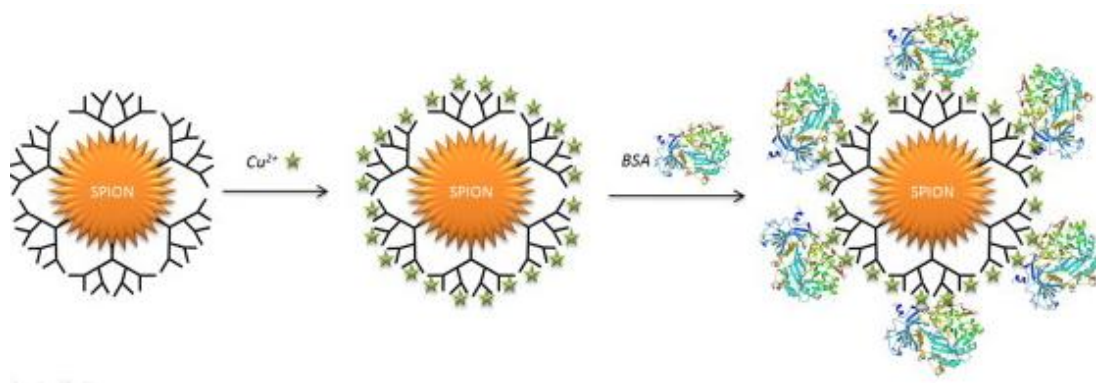


Figure 2.5 Schematic representation of BSA immobilization on PAMAM modified SPION BSA immobilization on PAMAM modified SPION [Baykal et al., 2014].

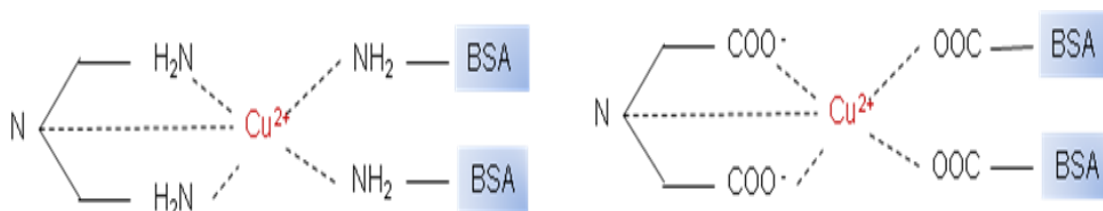


Figure 2.6 Binding mechanisms of Cu^{2+} ions and BSA molecules.

2.4.3 Properties of Immobilized BSA Protein

2.4.3.1 Effect of Generation

To find the more effective generation, immobilization of BSA was realized on to the G1-G5 generations. Measurements of BSA adsorptions were done at 595 nm.

2.4.3.2 Effect of pH

pH of the medium has a significant effect on protein adsorption because proteins are neutral at their isoelectric points (pI), below and above at their isoelectric points they are positively and negatively charged. In addition, pH also plays a big role in conformational changes of soft protein. Soft proteins such as BSA adapt with the structural changes when they contact with the surface [Nakanishi et al., 2001].

Acetate and phosphate buffer which are 0.05 M and in the pH range of 3.0-5.0 and 6.0-8.0 respectively were used for determining different pH effect on adsorption capacity of BSA.

2.4.3.3 Effect of BSA Concentration

Concentrations which are taken between 0.2–2.0 mg/mL to investigate the BSA concentration effect on BSA adsorption.

2.4.3.4 Effect of Temperature

Another important factor affects the protein adsorption is the temperature. Due to the temperature difference, proteins are exposed to a conformational change between primary and quaternary structure. Even proteins denature with the temperature [Vidal et al., 2010]. Mishina et al. stated that BSA is approximately denatured the temperature between 50-55 °C [Mishina et al., 2008]. The effect of temperature on BSA adsorption was studied in the range of 25-75 °C at pH 5.0.

2.4.3.5 Effect of Salt Concentration

NaCl was chosen as salt source among the others. Different concentrations of NaCl which were taken between 0.05-0.25 M was used to consider the effect of ionic strength on adsorption amount of BSA.

2.4.3.6 Effect of Different Metals

Metals which are used as chelating agents have a functional role for adsorbing of enzyme. Due to the usage of important feature, they act as linker in BSA enzyme adsorption.

In the batch experiment that deals with adsorption of enzyme, pH and metals which are chosen should be suitable. In this adsorption process, acetate and phosphate solutions which are 0.05 M were used as buffer in the pH range of 3.0–8.0. The chelation effect of Cu, Zn, Cr and Ni metals on BSA adsorption capacity was considered with the use of Biotek instrument.

2.4.3.7 Effect of Reusability

Reusability is the most important property and advantage for support material in the magnetic separation processes. The effect of reusability on BSA adsorption amount was considered after the repeating of 5 cycle of adsorption and desorption. Desorption of BSA immobilized SPION-PAMAM was realized by using 1.0 M NaCl solution.

2.5 PROTEIN ASSAY

For the determination of adsorption capacity of BSA, Bio-Rad Protein Assay which is similar to Bradford method was used. In this assay, acidic dye is added to protein solution and measurement is done at 595 nm by using microplate reader. For all BSA determinations, Standard Procedure of Microtiter Plate Protocol was applied. A typical procedure is as followed; first, dye reagent is prepared by diluting 1 part Dye Reagent Concentrate which is Coomassie Blue Reagent G-250 with 4 parts distilled water. This diluted reagent is stored in the refrigerator at 4 °C during the analysis. Five Standard protein dilutions which is representative of the protein solution to be tested are prepared. The linear range of this microtiter plate assay is 0.05 mg/mL to approximately 0.5 mg/mL. Our standard protein solutions are prepared between 0.1-0.5 mg/mL and assayed in triplicate. Sample solution and 10 µL of each standard protein solution are pipetted into the microtiter plate wells then 200 µL of dye solution is added to each well. Then this plate is placed into instrument. Gen5 programme integrated with Biotek Instrument is opened and procedure is created for the experimental condition which is temperature, incubation time and wavelength of absorbance. All measurements were at 37 °C and 595 nm.

CHAPTER 3

RESULTS AND DISCUSSIONS

3.1 CHARACTERIZATION OF PAMAM MODIFIED SPION

3.1.1 Characterization

3.1.1.1 FT-IR Analysis

Fourier Transform Infrared Spectroscopy was used to characterize SPION-APTMS and SPION-PAMAM. Figure 3.1 represents respectively FT-IR results of SPION and SPION-PAMAM (G1 to G5). The bands which are at 590 and 445 cm^{-1} show the metal-oxygen stretching vibrations of SPION at the tetrahedral and octahedral site [Wei et al., 2007; Nyquist et al, 1971; Bruce et al, 2004] clearly in Fig. 3.1. Also, there were organic vibration peaks which are observed for all PAMAM coated SPION (SPION-PAMAM G1 to G5) [Uzun et al., 2010; Wei et al., 2007, Bruce et al., 2004]. N-H stretching vibration peak which is broad band that seen from the Fig. 3.1 was observed at 3440 cm^{-1} . Covalent binding of APTMS on the surface of SPION was also confirmed by FT-IR. The band that can be assigned to vibration of Si-O group was observed at 995 cm^{-1} by the introduction of APTMS to the surface of SPION.

The band at 995 cm^{-1} which is Si-O stretching vibration at the surface of SPION-NH₂ was shifted to 1025 cm^{-1} . This shift can be explained by the presence of highly electronegative -CO-NH₂ groups in SPION-PAMAM [Mikhaylova et al., 2004; Toprak et al., 2007].

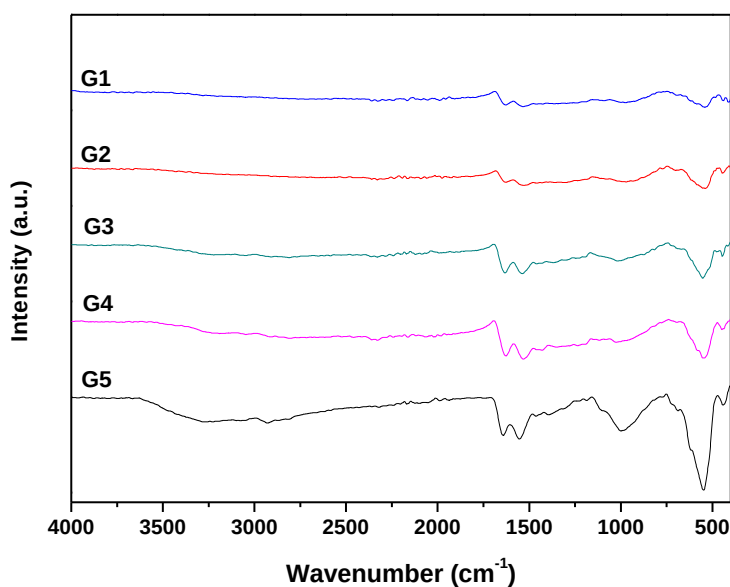


Figure 3.1 FT - IR spectrum of PAMAM dendrimer modified SPIONs from G1 to G5.

3.1.1.2 XRD Analysis

The XRD patterns of SPION-PAMAM were obtained using a Huber JSO-Debye flex 1001 diffractometer and presented in Figure 3.2. The data was collected in Debye-Scherrer geometry using a monochromated X-ray beam of Cu K α radiation ($\lambda=1.54178$). XRD pattern indicates that the product consists of magnetite, Fe $_3$ O $_4$, and diffraction peaks are broadened owing to very small crystallite size. All of the observed diffraction peaks are indexed by the cubic structure of Fe $_3$ O $_4$ (JCPDS no 19-629) revealing a high phase purity of magnetite.

By using line profile fitting method, the size of the crystallites was estimated from the diffraction pattern [Wejrzanowski et al., 2006; Pielaszek et al., 2003]. The line profile, shown in Figure 3.2, was fitted for observed 7 peaks with the following miller indices: (111), (220), (311), (400), (422), (511) and (440) [Qie et al., 2012].

The average crystallite size, $D \pm \sigma$, was obtained as 9 ± 1 nm as a result of this line profile fitting. Line Profile fitting is performed using a Profile Fitting Programme which uses JCPDS cards. Fingerprint of each crystalline is defined in this cards.

Diffraction patterns of each crystal depending on the atomic sequences in the substance are determined by using this programme.

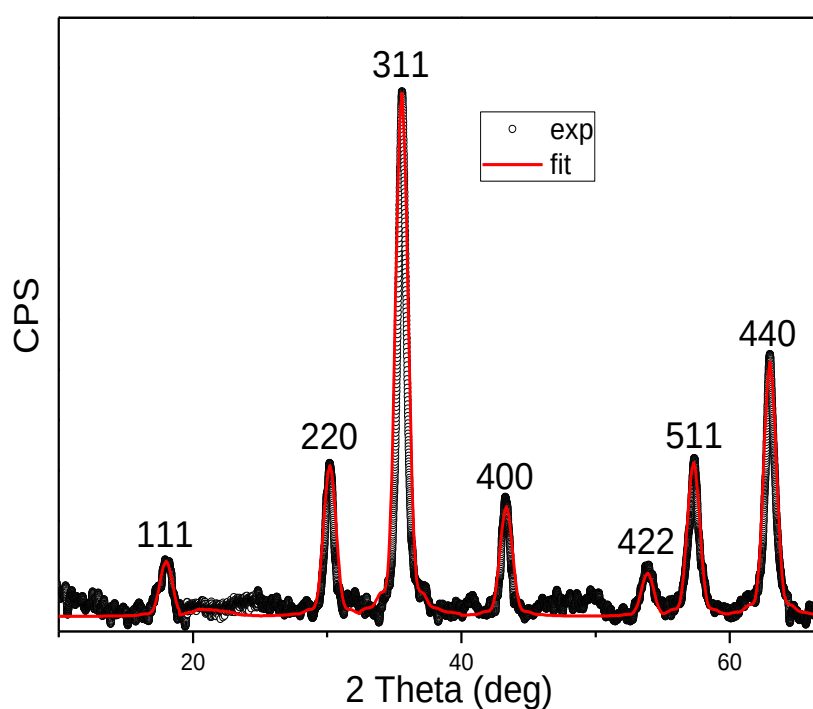


Figure 3.2 XRD powder diffraction pattern and line profile fitting of PAMAM dendrimer modified SPION-.

3.1.1.3 TEM Analysis

Fig. 3.3 a, b, c, d, e shows a typical transmission electron microscopic image of the SPION-PAMAM (G1 to G5). Morphology and particle size of SPION-PAMAM (G1 to G5) was investigated by using FEI Technai G2 Sphera microscope. As seen from the histograms and Table 3.1 particle size increases with increasing generation (from G1 to G5). Figure 3.3 shows that the Fe_3O_4 particles which are similar to spherical and polygonic and square morphologies are observed [Mikhaylova et al., 2004; Uzun et al., 2010].

Products which are interacting magnetically and have high surface area were agglomerated slightly [Mikhaylova et al., 2004; Tomalia et al., 1990]. Using log-normal fitting, the distribution of particle size was calculated by following equation for quantitative analysis of size distribution [Kim et al., 2007].

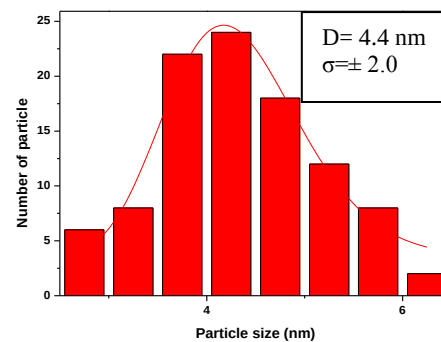
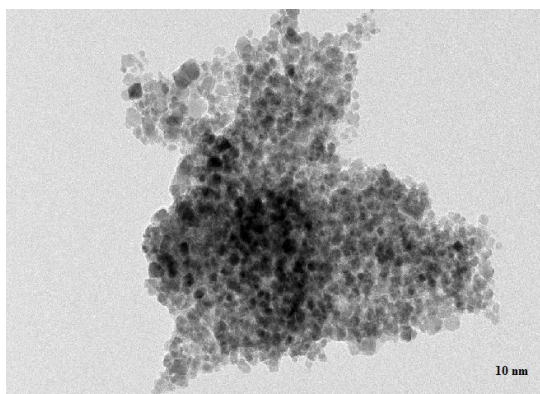
$$P(D) = \frac{A}{D\sigma_D\sqrt{2\pi}} \exp\left(-\frac{1}{2\sigma_D^2} \ln^2\left(\frac{D}{D_0}\right)\right)$$

Where P (D) is particle size distribution, σ_D is the standard deviation of the diameter and D_0 is the mean diameter.

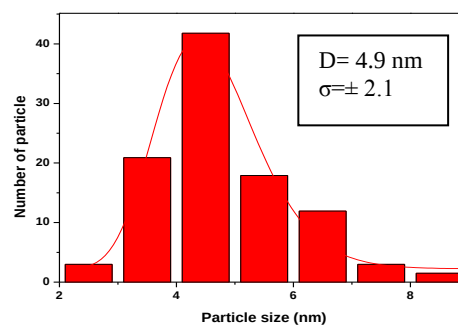
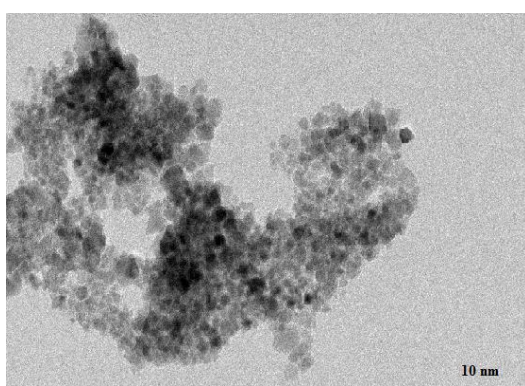
Spherical particles are visible when look at the TEM images of the figures and size distribution histograms of followings (Fig. 3.3 a, b, c, d, e). Crystallite size that obtained from XRD analysis as compared with the particle size which is calculated from as a result of the TEM analysis that is seen modified SPIONs which have single and also polycrystalline character.

Table 3.1 Particle size of dendrimers modified SPION–PAMAM G1 to G5.

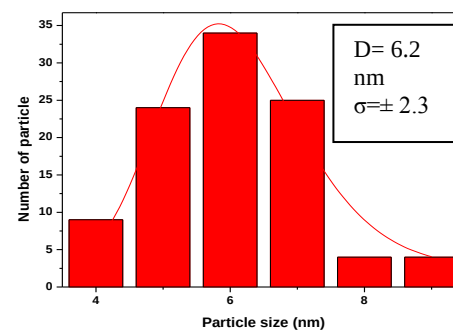
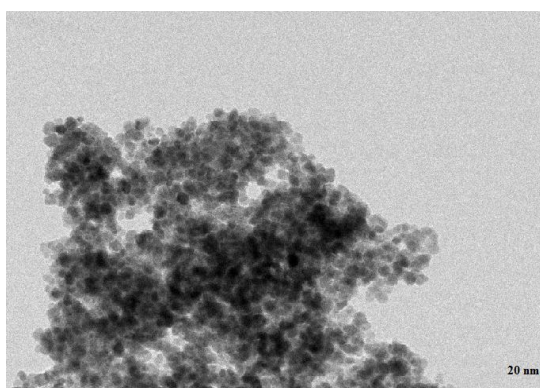
Product	Particle Size (nm)
SPION–PAMAM G1	4.4±2.0
SPION–PAMAM G2	4.9±2.1
SPION–PAMAM G3	6.2±2.3
SPION–PAMAM G4	8.5±2.1
SPION–PAMAM G5	9.1±2.0



(a)



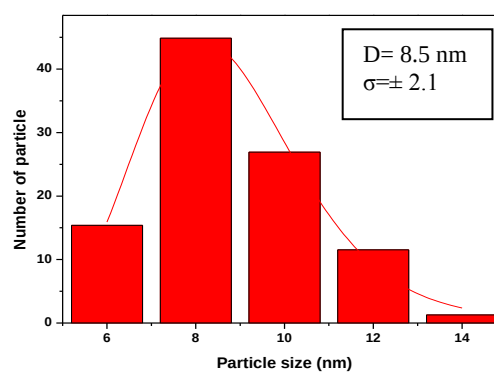
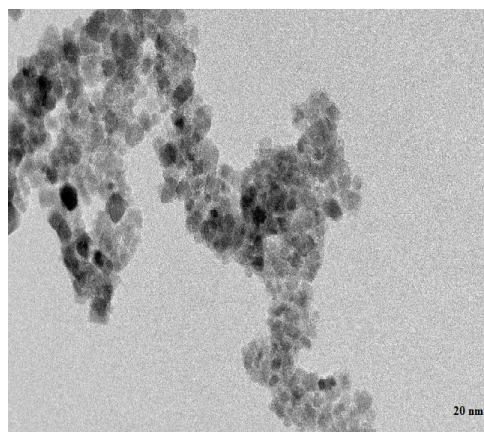
(b)



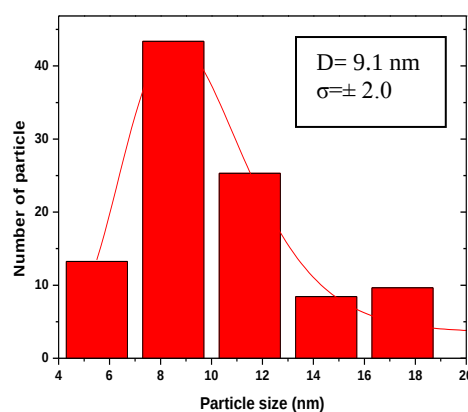
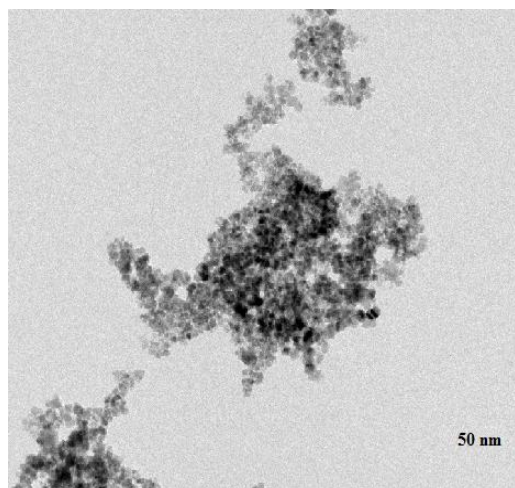
(c)

Figure 3.3 TEM images of PAMAM dendrimer modified SPIONs and their particle size histograms obtained from TEM data a) SPIONs-G1, b) SPIONs-G2, c) SPIONs-G3, d) SPIONs-G4 and e) SPIONs-G5.

Continued



(d)



(e)

Figure 3.3 TEM images of PAMAM dendrimer modified SPIONs and their particle size histograms obtained from TEM data a) SPIONs-G1, b) SPIONs-G2, c) SPIONs-G3, d) SPIONs-G4 and e) SPIONs-G5.

3.1.1.4 Thermal Analysis

The stability and inorganic loading of the modified SPIONs (from G1 to G5) was investigated by using TGA instrument. Figure 3.4 shows the thermogram of modified SPION (from G1 to G5). Previous studies which are related to synthesis of PAMAM

dendrimer show that similarity of TGA results [Tajabadia et al., 2013; Kavas et al., 2011; Shi et al., 2009; Baykal et al., 2012; Qie et al., 2012; Pan et al., 2005].

As seen from the Figure 3.4, weight loss increases between 50 and 800 °C with increasing dendrimer generation. The reason behind it, increasing of molecular weight of dendrimers and chain length of C-backbone.

There are three regions in the thermogram. These are between 200–400 °C and 410–750 °C. The weight loss in the first region depends on adsorbed water which is evaporated physically on the surface and other regions are based on decomposition of dendrimer layer.

The weight loss of each product (modified SPION from G1 to G5) was given in Table 3.2.

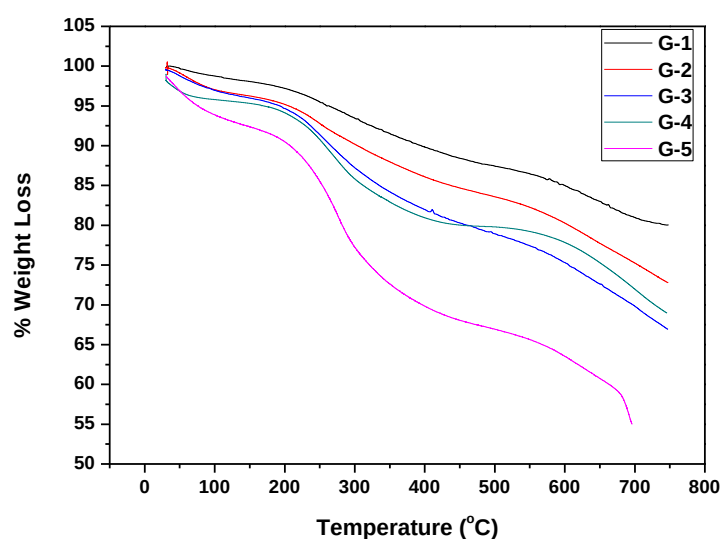


Figure 3.4 TGA curves of PAMAM dendrimer modified SPIONs from G1 to G5.

Table 3.2 Weight loss of dendrimers modified SPION–PAMAM G1 to G5.

Product	Weight Loss (%)
SPION–PAMAM G1	20
SPION–PAMAM G2	27
SPION–PAMAM G3	31
SPION–PAMAM G4	34
SPION–PAMAM G5	41

3.1.1.5 VSM Analysis

Figure 3.5 represents the magnetic property of modified SPION from G1 to G5. As seen clearly from the figure, magnetization of the modified SPIONs decrease while generation number increases as a result of spin canting and the existence of non-magnetic layer at the particle surface [Tajabadia et al., 2013; Faiyas et al., 2010]. The saturation magnetization value for modified SPION (G1) start from 54.1 emu/g. Increasing of each generation decreases the M_s value. As seen from the Table 3.3, all M_s values are smaller as compared with the theoretical value of bulk magnetite (i.e., 92 emu/g) [Cullity, 1972; Minjuan et al., 1994]. Superparamagnetic NPs have some properties. One of them reduced magnetization due to the surface spin disorder and spin canting effects (which spins are tilted by a small angle about their axis rather than being exactly co-parallel) [Kodama et al., 1996; Xavier et al., 2002], which happen while the particle size decreases, when the surface to volume ratio of particles increases. There are no coercivity and no remanence in all M-H hysteresis curves. These features show the modified SPIONs to be superparamagnetic. Also, Langevin theory describes well this magnetic behavior. According to the theory, magnetization is effected by external field as follows;

$$M = M_s \left\{ \text{Coth} \left(\frac{\mu H}{k_B T} \right) - \frac{k_B T}{\mu H} \right\}$$

Where M and M_s refer magnetization and saturation magnetization, μH represents the energy due to the external field and $k_B T$ (k_B is the Boltzmann constant and T is

temperature measured in Kelvin) stands for the thermal energy. Magnetic moment which is abbreviated μ depends on average size of particle and calculation can be performed to fit equation which is in the above according to the measurements in the M-H hysteresis curves. Thus, average sizes of the magnetic cores of SPIONs coated with PAMAM dendrimer (for G5) are around 9 nm.

Materials which their size is below 20 nm are called superparamagnetic. As seen from the results, all sizes that found in the studies (XRD, TEM etc) are below 20 nm which is superparamagnetic limit. Size which is calculated from TEM observations is slightly different from result obtained from XRD but this difference small and negligible. [Kodama, 1996; Battle et al., 2002; Murbe et al., 2008].

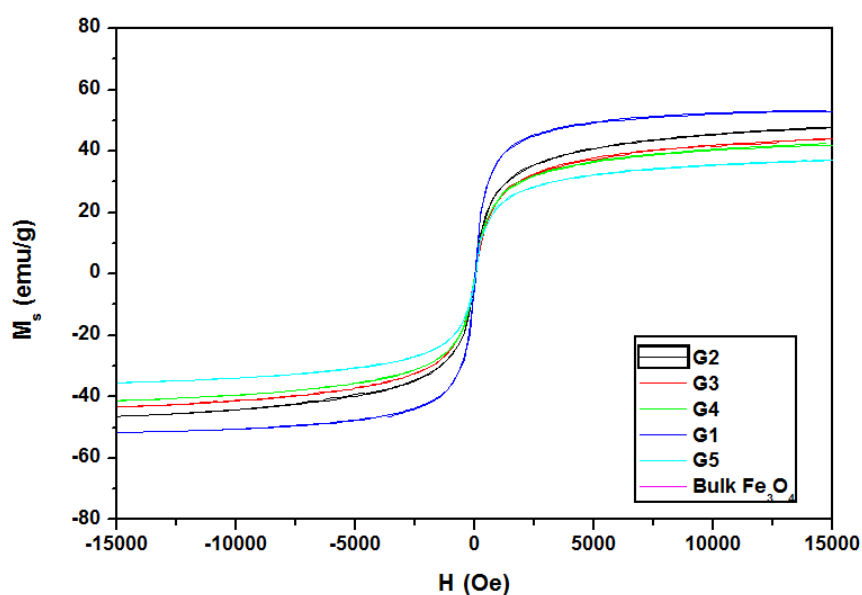


Figure 3.5 Room temperature M-H curve of bulk Fe_3O_4 NPs, and PAMAM dendrimer modified SPIONs (G1, G2, G3, G4 and G5).

Table 3.3 Saturation magnetization values of dendrimers modified SPION–PAMAM G1 to G5.

Product	Particle Size (nm)	Ms (emu/g)
SPION–PAMAM G1	4.4±2.0	54.1
SPION–PAMAM G2	4.9±2.1	48.8
SPION–PAMAM G3	6.2±2.3	45,1
SPION–PAMAM G4	8.5±2.1	43.3
SPION–PAMAM G5	9.1±2.0	37.6

3.1.2 BSA Adsorption Studies

3.1.2.1 Effect of Generation on BSA Adsorption

BSA was immobilized onto PAMAM modified SPIONs from G1 to G5 to investigate the effect of generation. Figure 3.6 shows the generation effect on the adsorbed BSA. According to Table 3.4, adsorbed amount of BSA on PAMAM modified SPION (G5) is 2.10 times great than that of on G1. Pan and co workers were studied about dendrimer modified MNPs for protein immobilization. Pan et al., reported that the BSA binding capacities of G1-G5 are 3.9-7.7 times great than G0 [Pan et al, 2005]. The reason for this increment is the increase in the available surface area of modified SPIONs (from G1 to G5) which are dispersed depending on the positive repulsive charges. While the generation number increases, the amount and concentration of surface amine also increase as a result increasing of dendritic structure which is conjugated to BSA.

As a result, increased adsorbed amount of BSA from G1 to G5 in our study confirms the effect of generation.

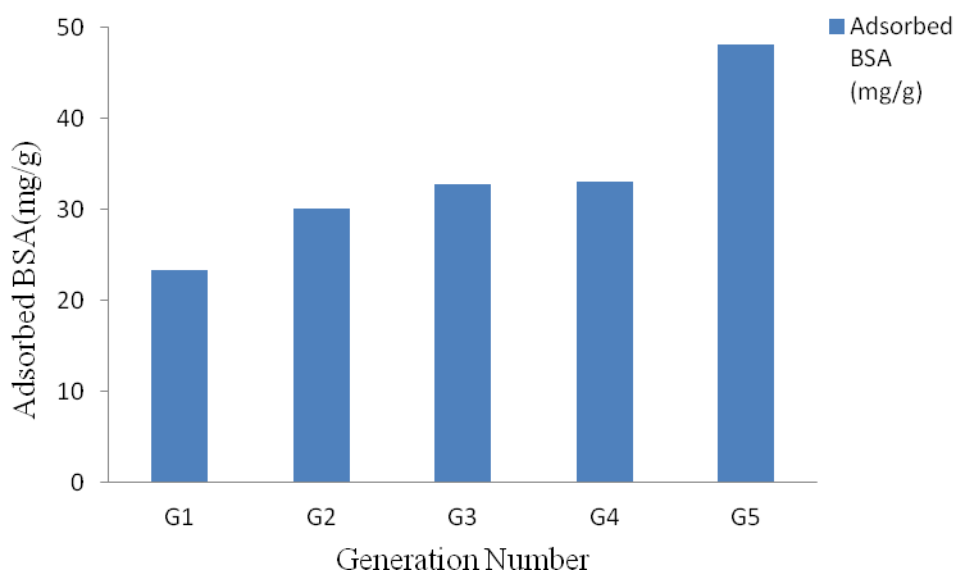


Figure 3.6 Generation effect on BSA immobilizing capacity of PAMAM modified SPIONs.

Table 3.4 Generation effect on adsorbed amount of BSA.

Generation Number	Adsorbed BSA (mg/g)
G1	23.37
G2	30.04
G3	32.77
G4	33.08
G5	48.08

3.1.2.2 Effect of pH on BSA Adsorption

pH is one of the most important parameters controlling the adsorption process. Acidic or basic medium affects the activities of the regions on the adsorbent surface that allow binding and thus, the adsorption capacity changes. To immobilize the BSA onto the modified SPIONs weighing 5 mg, pH was varied between 3.0 and 8.0. Figure 3.7 shows the adsorbed amount of BSA on the modified SPIONs. As it can be seen from Figure 3.7, the maximum adsorption was observed at pH 7.0. The amount of adsorbed BSA was 52.84 mg/g at this pH due to the some interactions between BSA and chelated Cu^{2+} . These are electrostatic interactions and coordination which result in a change in the conformational state of BSA and ionization states of several groups in the BSA structure on the amino acid chain. As seen in Figure 3.7, the adsorbed amount of BSA decreased at pH values below and above pH 7.0. The immobilization yield was high between pH 3.0 and 7.0. This was attributed to the repulsion between identical charged groups and some conformational changes depending on pH on the surface of the protein.

BSA has an isoelectric point of 5.2 where amino groups are protonated [Pan et al., 2005]. Pan et al. investigated interaction of PAMAM dendrimer-coated MNPs with BSA and observed the conformational change in BSA structure at pH 9.0. The maximum load of support was achieved at pH 7.0 where the net charge of BSA protein becomes negative in our work. The value of pH shifted two units towards acidic pH.

This situation may be due to the ionic interactions which occur between BSA structure (of negative groups) and Cu^{2+} (positively charged) presence on the surface.

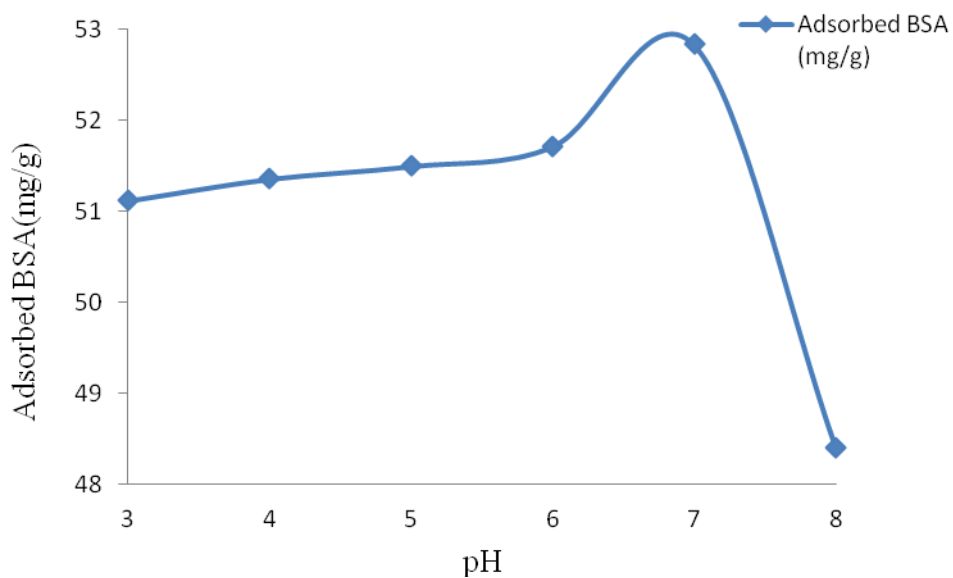


Figure 3.7 BSA adsorption capacity of PAMAM modified SPION in different pH; BSA concentration: 1.6 mg/mL, Incubation time: 3 h.

Table 3.5 pH effect in the range of 3.0-8.0 in the adsorbed amount of BSA.

pH	Adsorbed BSA (mg/g)
3.0	51.12
4.0	51.36
5.0	51.50
6.0	51.72
7.0	52.84
8.0	48.40

3.1.2.3 Effect of BSA Concentration on BSA Adsorption

Fig. 3.8 shows the effect of BSA concentration on the adsorption capacity of BSA. As seen from the figure, the adsorption capacity initially increases with increasing protein concentration and reaches saturation when the concentration rises to 1.6 mg/mL. This shows that the adsorption capacity of BSA on modified SPIONs was dependent on the equilibrium concentration of BSA. The figure shows two behaviors in the adsorption capacity: in the initial part of figure, there is concentration-dependent increase because of the enhanced interaction between Cu^{2+} chelated groups and BSA protein, but when the concentration of BSA is greater than 1.6 mg/mL, the capacity becomes constant. Our results reveal that Cu^{2+} chelation significantly increased the BSA adsorption capacity of the SPIONs up to 41.40 mg/g at pH 7.0 in phosphate buffer.

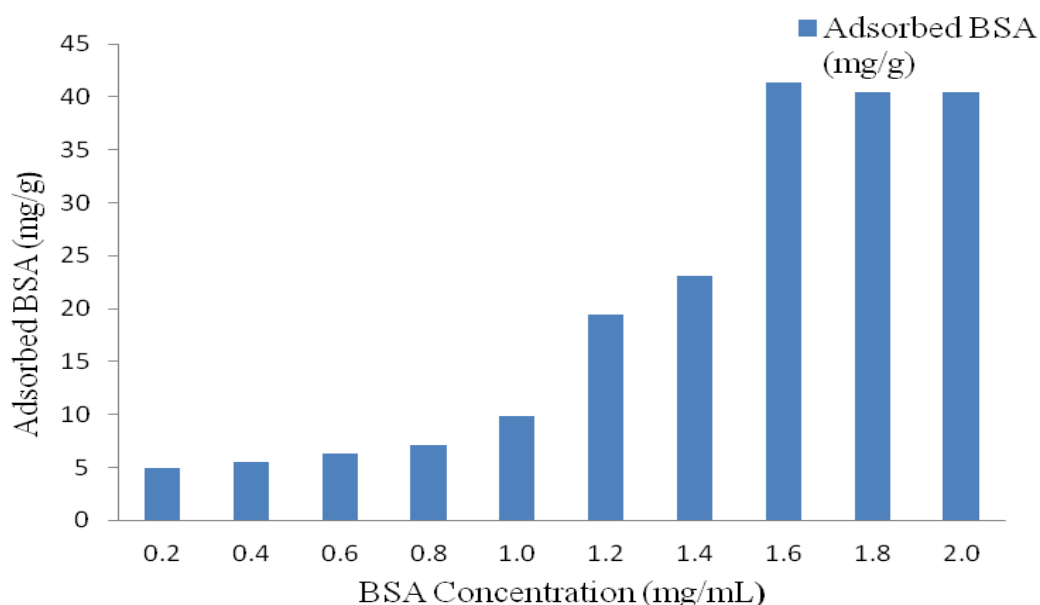


Figure 3.8 BSA adsorption capacity of PAMAM modified SPION in different BSA concentrations; Temperature: 25 °C, Incubation time: 3 h.

Denizli et al., synthesized copper (II) chelated PHEMA (poly (hydroxyethyl methacrylate) NPs for protein separation. They used human serum albumin (HSA) which is a plasma protein for separation and found that adsorption capacity of HSA on PHEMA NPs was dependent on HSA concentration [Denizli et al., 2009].

Table 3.6 BSA concentration effect in the range of 0.2-2.0 mg/mL in the adsorbed amount of BSA.

BSA concentration (mg/mL)	Adsorbed BSA (mg/g)
0.2	4.960
0.4	5.560
0.6	6.920
0.8	7.120
1.0	9.840
1.2	19.44
1.4	23.04
1.6	41.40
1.8	40.44
2.0	40.40

3.1.2.4 Effect of Temperature on BSA Adsorption

The effect of temperature on the adsorption of BSA is given in Figure 3.9. The temperature was varied between 25 °C and 75 °C. As seen from the figure, the adsorption of BSA on modified SPIONs decreased with increasing temperature. The maximum amount of adsorbed BSA on modified SPIONs was 35.86 mg/g at 25 °C. When the temperature was increased from 25 to 75 °C, the adsorption capacity reduced about 30%. This decrease may be due to physical changes which may occur on the surface of protein or adsorbent at elevated temperatures or the exothermic nature of adsorption. Invertase adsorption behavior at different temperatures on PAMAM modified SPIONs was investigated by Uzun et al., 2010. They reported that the invertase adsorption capacity was strongly dependent on temperature.

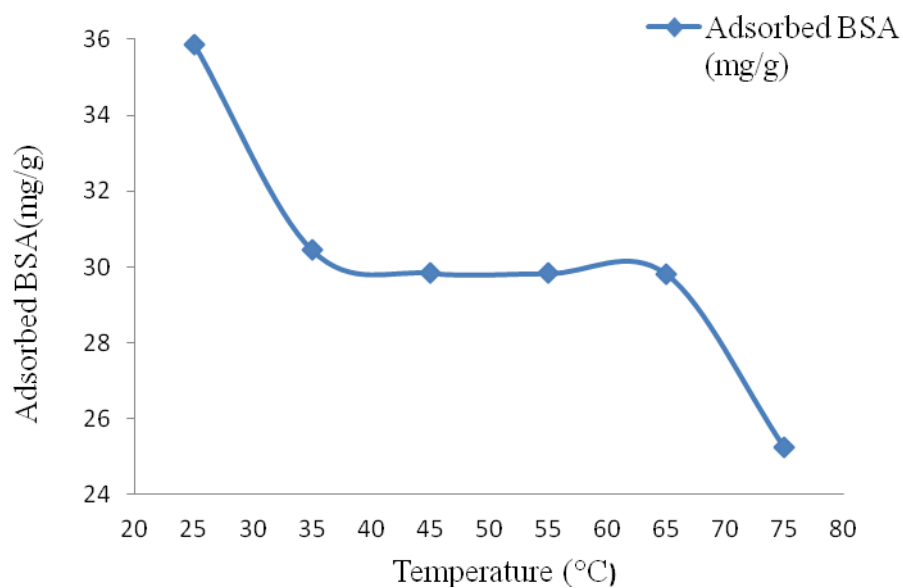


Figure 3.9 BSA adsorption capacity of PAMAM modified SPION in different temperature; BSA concentration: 1.6 mg/mL, Incubation time: 3 h.

Table 3.7 Temperature effect in the range of 25-75 °C in the adsorbed amount of BSA.

Temperature (°C)	Adsorbed BSA (mg/g)
25	35.86
35	30.44
45	29.84
55	29.83
65	29.82
75	25.26

3.1.2.5 Effect of salt concentration on BSA Adsorption

Salt concentration, also known as ionic strength is another very important parameter that affects the adsorption capacity of BSA. The effect of NaCl concentration on the BSA adsorption is given in Figure 3.10. Sodium chloride concentration was

varied between 0.05–0.25 mM. As can be seen from the figure, adsorption capacity of BSA increased with increasing salt concentration. The amount of adsorbed BSA increases with increasing concentration of NaCl from 0.05 to 0.25 mM. As stated before, at pH 7.0, since protein molecules are in basic condition while the BSA becomes negatively charged, the solubility of protein is elevated by each species repelling each other. High ionic strength cause the shielding effect on charged protein molecules. So the electrostatic repulsion is eliminated by molecules [Shmanai et al., 2000]. Hence, the solubility of protein decreases and the binding of protein molecules to magnetic supports increases. This is the reason why the BSA immobilization capacity increases with the increase of NaCl concentration at pH 7.0.

BSA protein adsorption was investigated on the amino silane-modified super paramagnetic silica support in work carried out by Liu et al in 2004 [Liu et al. 2004]. They reported that the adsorption capacity of BSA increases with the increasing concentration of NaCl from 0 to 0.6 M at pH 9.0.

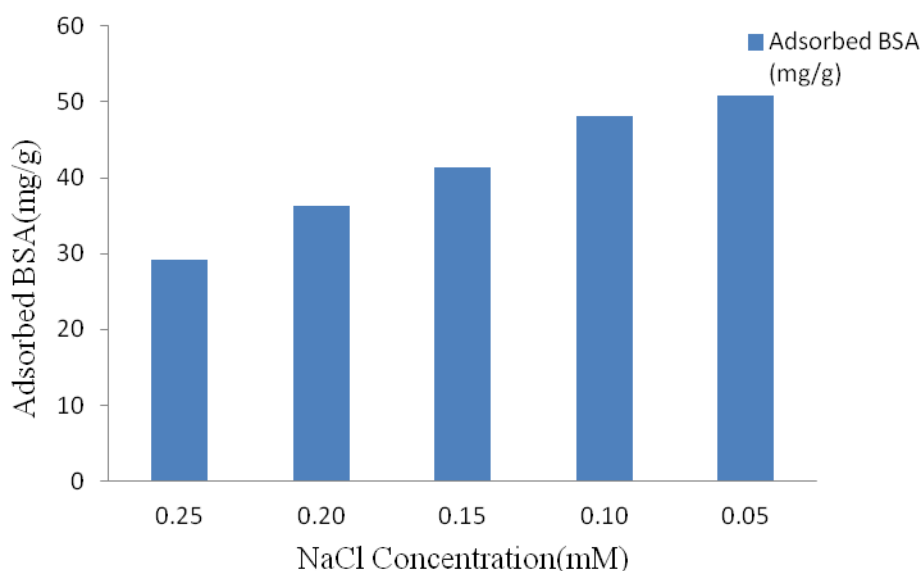


Figure 3.10 BSA adsorption capacity of PAMAM modified SPION in different NaCl concentrations; BSA concentration: 1.6 mg/mL, Incubation time: 3 h.

Table 3.8 NaCl concentration effect in the range of 0.05-0.25 mM in the adsorbed amount of BSA.

NaCl concentration (mM)	Adsorbed BSA (mg/g)
0.05	50.90
0.10	48.15
0.15	41.44
0.20	36.36
0.25	29.16

3.1.2.6 Effect of Different Metals on BSA Adsorption

The effect of different metals on adsorption capacity of BSA is shown in Figure 3.11. According to the Figure 3.11 and Table 3.9, the maximum and minimum adsorption capacities were observed for copper and nickel chelation, respectively. The amount of adsorbed BSA was 55.31 mg/g for copper chelation and 22.95 mg/g for nickel chelation. As seen from the figure, adsorption capacity decreased with the chelation of different metals ranging from copper to nickel. Metal ions which have higher chemical and physical stability and relatively low specificity show different affinity to proteins owing to the different number of sites of interaction. For example, copper metal requires one interaction while other metals require multi-point interaction sites.

Uzun et al. reported the effect of different metals on invertase adsorption capacity where magnetic support was polyvinylimidazole-grafted MNPs [Uzun et al., 2013]. Their findings where the copper metal had the highest adsorption while nickel had the lowest adsorption capacity support our results.

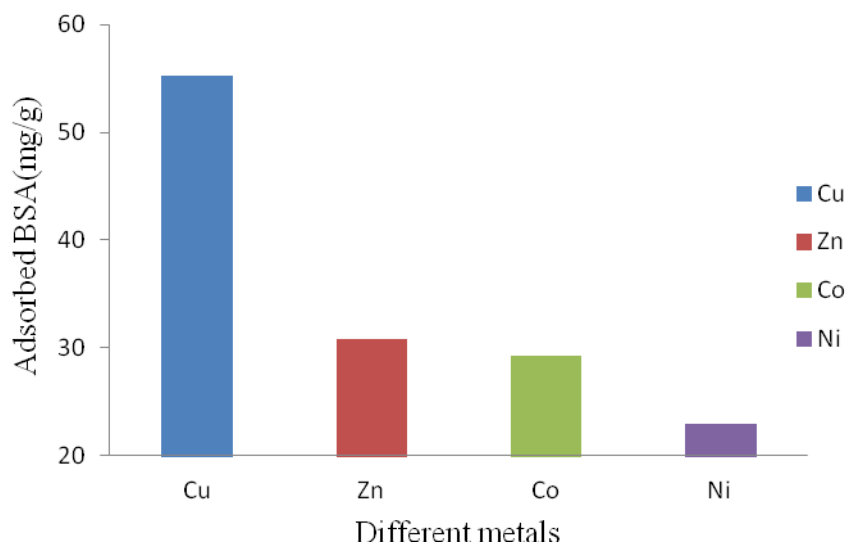


Figure 3.11 Effect of different metals on BSA adsorption capacity.

Table 3.9 Different metals effect on adsorbed amount of BSA.

Different Metals	Adsorbed BSA (mg/g)
Cu	55.31
Zn	30.77
Co	29.30
Ni	22.95

3.1.2.7 Effect of reusability on BSA adsorption

In the magnetic separation processes, reusability of the support material is an advantage. To investigate the effect of reusability, desorption studies were accomplished by incubation of BSA adsorbed modified SPIONs in 1.0 M NaCl solution for 1 hour. To determine the reusability of modified SPIONs on BSA adsorption, the same SPIONs were re-used for 5 times. As seen from Figure 3.12, there was no significant decrease in the adsorption capacity. These results demonstrate that modified SPIONs can be re-used in the adsorption of BSA without a significant change in the

adsorption capacity. The maximum adsorption of BSA was determined as 29.59 mg/g at pH 7.0.

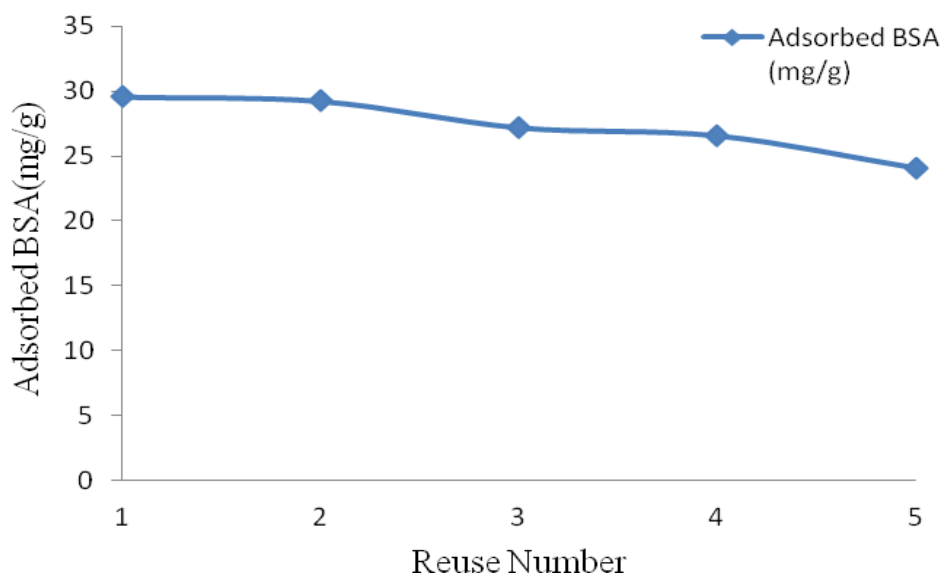


Figure 3.12 Effect of reusability of PAMAM modified SPION on BSA adsorption; BSA concentration: 1.6 mg/mL, Temperature: 25°C.

The slight decrease in the adsorbed amount of BSA may be due to the denaturation or the leakage of BSA from the prepared magnetic supports. Liang et al. found that the regenerated functionalized silica-coated magnetic MnFe_2O_4 nanoparticles obtained after the five repeated cycle still showed same performance without lowering the adsorption capacity [Liang et al., 2010].

Table 3.10 Effect of reusability on adsorbed amount of BSA.

Reuse Number	Adsorbed BSA (mg/g)
1	29.59
2	29.25
3	27.50
4	27.19
5	24.03

3.2 CHARACTERIZATION OF MONOCHLOROACETIC ACID (MCA) FUNCTIONALIZED PAMAM MODIFIED SPION

3.2.1 Characterization

3.2.1.1 FT-IR Analysis

SPION, APTMS modified SPION, PAMAM modified SPION and carboxylic acid modified PAMAM-SPION was characterized by using Fourier Transform Infrared Spectroscopy. FT-IR results of SPION, SPION-NH₂ (APTMS modified SPION-NH₂, G0-SPION), PAMAM coated SPION (dendrimers modified SPION-PAMAM) and carboxylic acid modified PAMAM coated SPION (SPION-PAMAM-COOH) were presented in Figure 3.13. Characteristic peaks which are same as the commercial one are seen from spectra. Metal-oxygen bands are characteristic peaks. Two peaks which are stretching vibrations of the metal at tetrahedral and octahedral sites at 590 cm⁻¹ and 445 cm⁻¹ in are observed in Figure 3.13 [Wei et al., 2007; Nyquist et al., 1971; Bruce et al., 2004]. New bands can be observed in spectrum of aminosilane-coated SPION. Due to the strong bonding of Si, their frequencies shifted to lower values. The band at 3440 cm⁻¹ shows the vibration of -NH₂ group in Figure 3.13. Si-O stretching vibration which is at the surface of aminosilane-Fe₃O₄ was shifted from 995 cm⁻¹ to 1025 cm⁻¹ due to the presence of -CO-NH₂ groups which are highly electronegative in SPION PAMAM G5. In addition, the presence of chloro acetic acid in SPION-PAMAM-COOH was confirmed by bands at 1716 cm⁻¹, 2845 cm⁻¹ and 2915 cm⁻¹ which are correspond to vibration of C-H stretching.

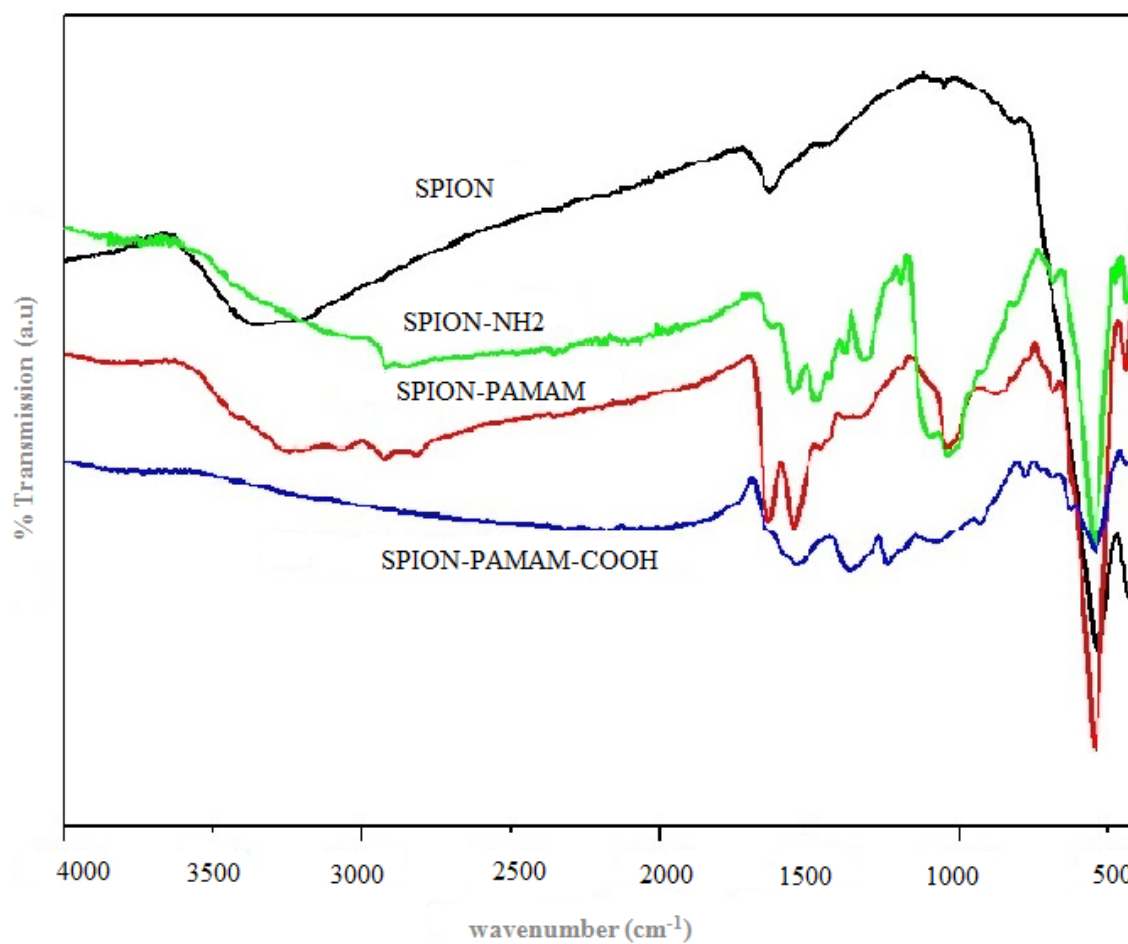


Figure 3.13 FT-IR spectrum of uncoated SPION, APTMS modified SPION, PAMAM modified SPION and carboxylic acid functional PAMAM-SPION.

3.2.1.2 XRD Analysis

XRD powder pattern of SPION-PAMAM was shown in Figure 3.14. XRD analysis showed that all XRD peaks (with hkl values of (111), (220), (311), (400), (422), (511) and (440)) found in XRD pattern match to Fe_3O_4 with cubic structure (JCPDS no 19-629). Estimation of crystallite size of SPION was done by using the line profile fitting method [Wejrzanowski et al., 2006] and the average crystallite size ($D \pm \sigma$) was calculated as 9 ± 1 nm.

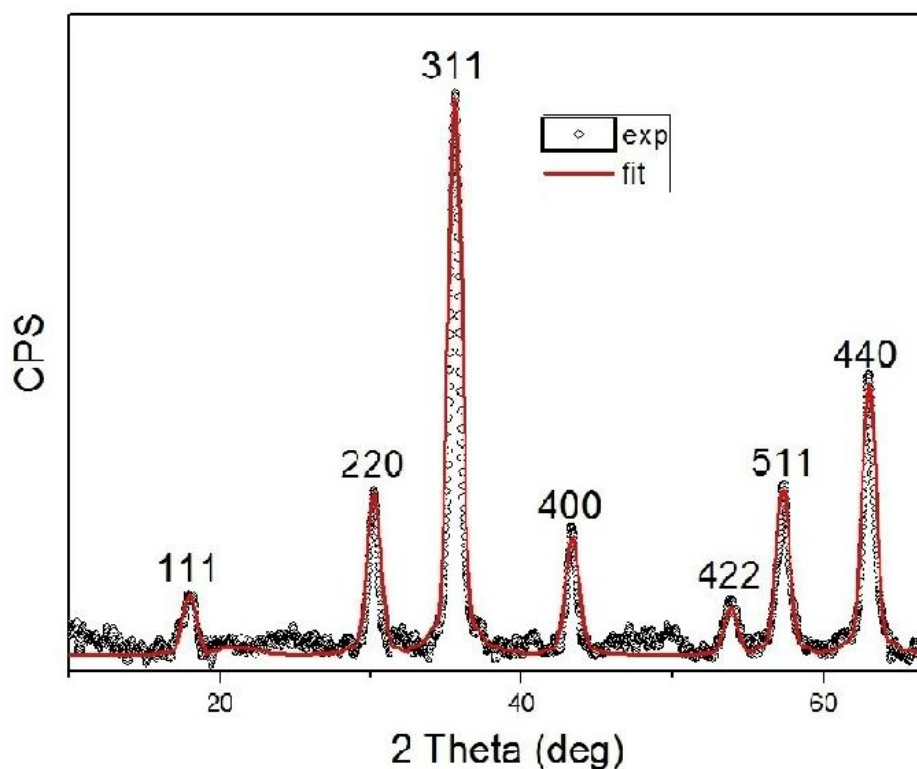
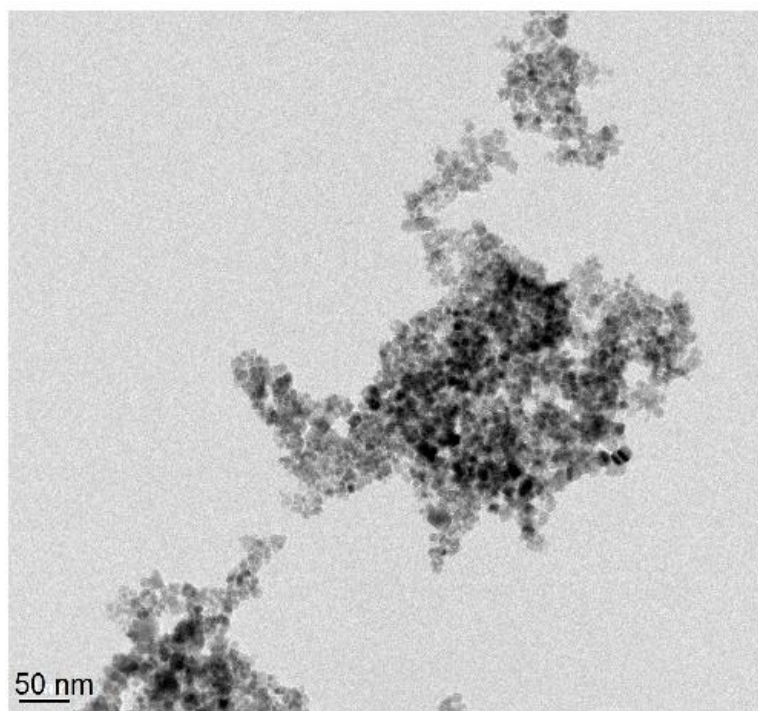


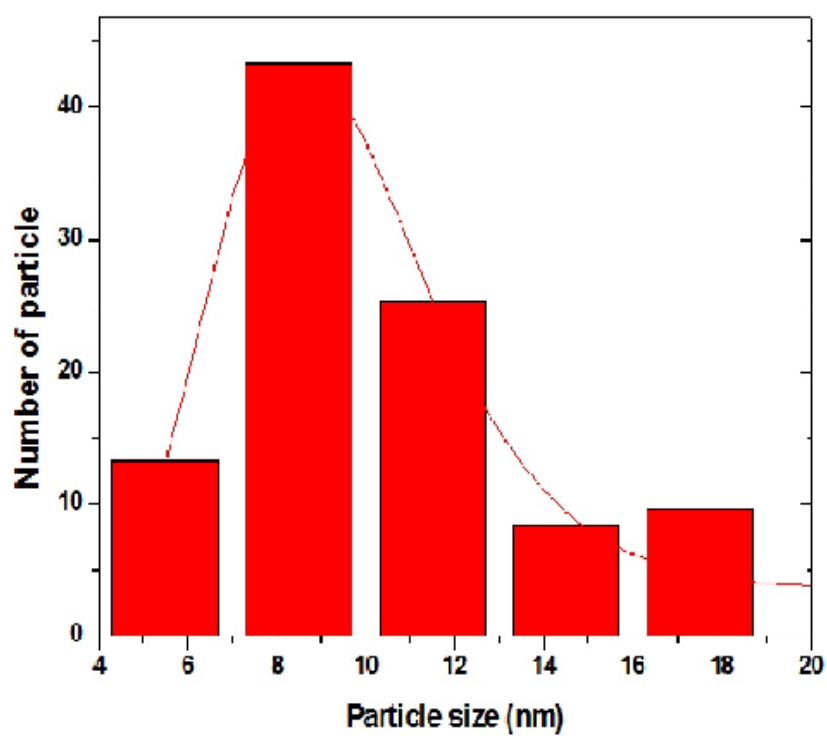
Figure 3.14 XRD powder pattern and line profile fitting of PAMAM dendrimer modified SPION-

3.2.1.3 TEM Analysis

TEM was used to characterize the morphology and size distribution of modified SPION. Fig. 3.15 shows the calculated histogram and micrographs at different magnifications. While Fe_3O_4 particles show dominantly near spherical morphology due to the agglomeration before the modification of dendrimer, some square and polygonic morphology were observed. Average particle size for the Fe_3O_4 nanoparticles was calculated as 9 ± 0.1 nm by using log-normal fitting to the size distribution histogram. While Fe_3O_4 particles show single crystalline structure in X-ray line profile fitting, under the TEM polycrystalline particles are observed.



a)



b)

Figure 3.15 a) TEM images of PAMAM dendrimer modified SPION (G5) and b) its particle size histogram obtained from TEM data.

3.2.1.4 Thermal Analysis

Thermal behavior of dendrimers modified with SPION-PAMAM was presented in Figure 3.16. Thermograph consists of three decomposition points. These are; at 200 °C, 200-400 °C and 410-750 °C, respectively. The reason of first decomposition was evaporation of water; the others were due to the decomposition of dendrimer layer. The weight percentage of organic and inorganic (dendrimer) for SPION-PAMAM G5 were calculated from the thermograph as 35% and 65%, respectively.

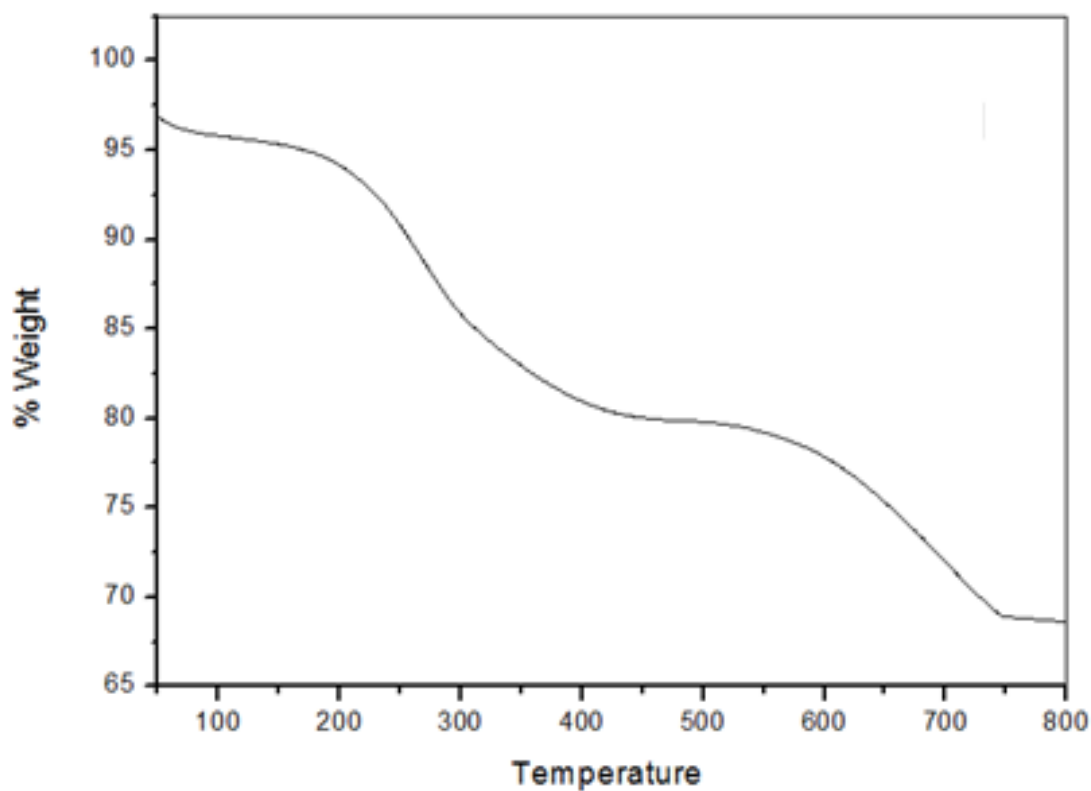


Figure 3.16 TGA curve of PAMAM modified SPION.

3.2.2 BSA Adsorption Studies

3.2.2.1 Effect of Generation on BSA Adsorption

The absorbance measurements in Biotek Microplate Reader of all supernatant samples at 595 nm were recorded as seen in Figure 3.17 to investigate the effect of generation on the immobilization of BSA on the SPION-PAMAM dendrimers. The increase of BSA immobilization is attributed to the contribution of dendritic structure. As a result, the increase of BSA binding to modified SPION-PAMAM dendrimers from G1 to G5 is partly due to increased available surface area since modified SPION-PAMAM dendrimers disperse very well by the repulsion of positive charges. The maximum adsorption was obtained for G5 (48.08 mg/g).

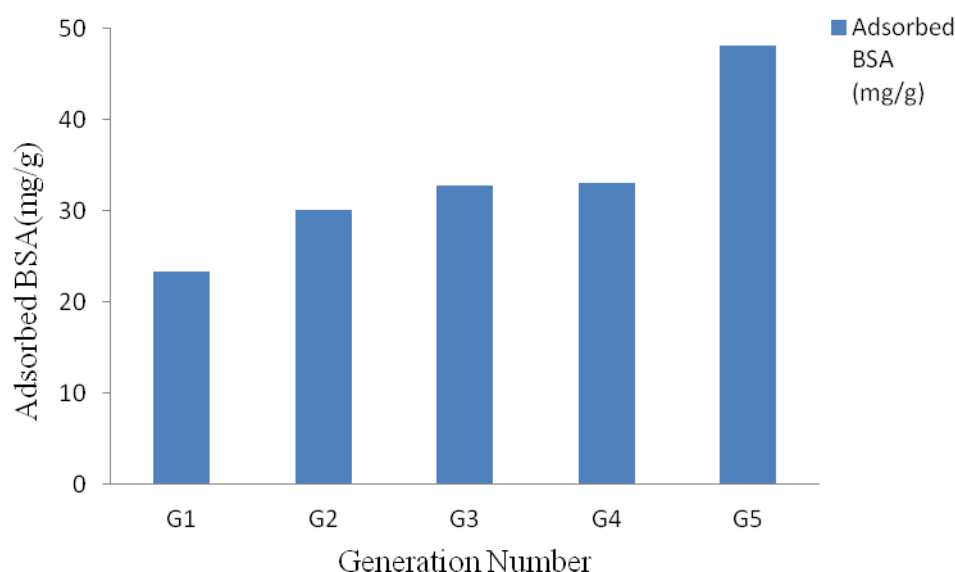


Figure 3.17 Generation effect on BSA immobilizing capacity of PAMAM modified SPIONs.

Table 3.11 Generation effect on adsorbed amount of BSA.

Generation Number	Adsorbed BSA (mg/g)
G1	23.37
G2	30.04
G3	32.77
G4	33.08
G5	48.08

A previous study by Pan et al. about protein separation by dendrimer-modified MNPs showed that G5 dendrimer was 7.7 times as much as that of MNPs modified only with aminosilane [Pan et al., 2005].

3.2.2.2 Effect of pH on BSA Adsorption

The amount of BSA adsorbed on to SPION-PAMAM-MCA as a function of pH was shown Fig. 3.18. To investigate the effect of pH on the immobilization of BSA on to modified (with chloroacetic acid) PAMAM-SPIONs (G5) was studied in the pH range 3.0-8.0 (modified nanoparticles were weighted as 5 mg). In this study, the maximum adsorption of BSA was observed at pH 3.0 and below pH 6.0, the adsorbed amount of BSA decreased. The maximum adsorption was 57.46 mg/g at pH 3.0. Pan et al. reported that the isoelectric point (pI) of BSA is 5.2 [Pan et al., 2005] and if the pH and pI of proteins are close to each other, the net charge of protein is zero and the protein will have maximum hydrophobic properties [Lienqueo et al., 2007]. At pH 3.0, the maximum BSA adsorption capacity was observed and this shows that there are maximum hydrophobic interactions when compared to other interactions between BSA and SPION-PAMAM-MCA. Significantly, a lower enzyme adsorption was obtained for modified G5 in the pH 6.0 as seen in Figure 3.18.

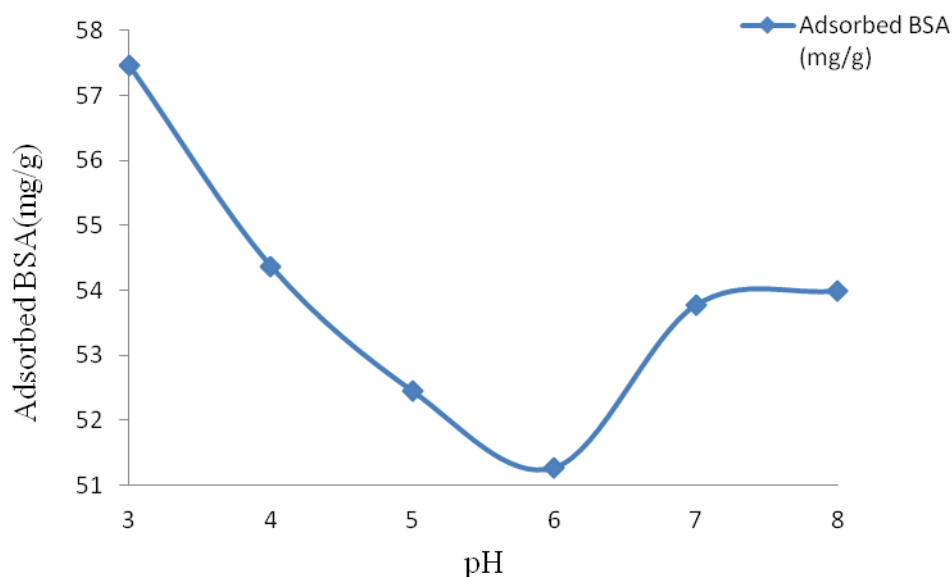


Figure 3.18 BSA adsorption capacity of PAMAM modified SPION in different pH; BSA concentration: 1.6 mg/mL, Incubation time: 3 h.

Table 3.12 pH effect in the range of 3.0-8.0 in the adsorbed amount of BSA.

pH	Adsorbed BSA (mg/g)
3.0	57.46
4.0	54.37
5.0	52.45
6.0	51.27
7.0	53.77
8.0	53.99

3.2.2.3 Effect of BSA Concentration on BSA Adsorption

The effects of BSA concentration, i.e., the amount of BSA adsorbed on the MCA functionalized PAMAM-SPIONs versus the initial concentration of BSA in the solution, were shown in Fig. 3.19. The adsorbed amount of BSA on the modified G5 was dependent on the equilibrium concentration of BSA. The amount of adsorbed BSA

continues to increase with increasing BSA concentration until it reaches a saturation of 2.0 mg/mL.

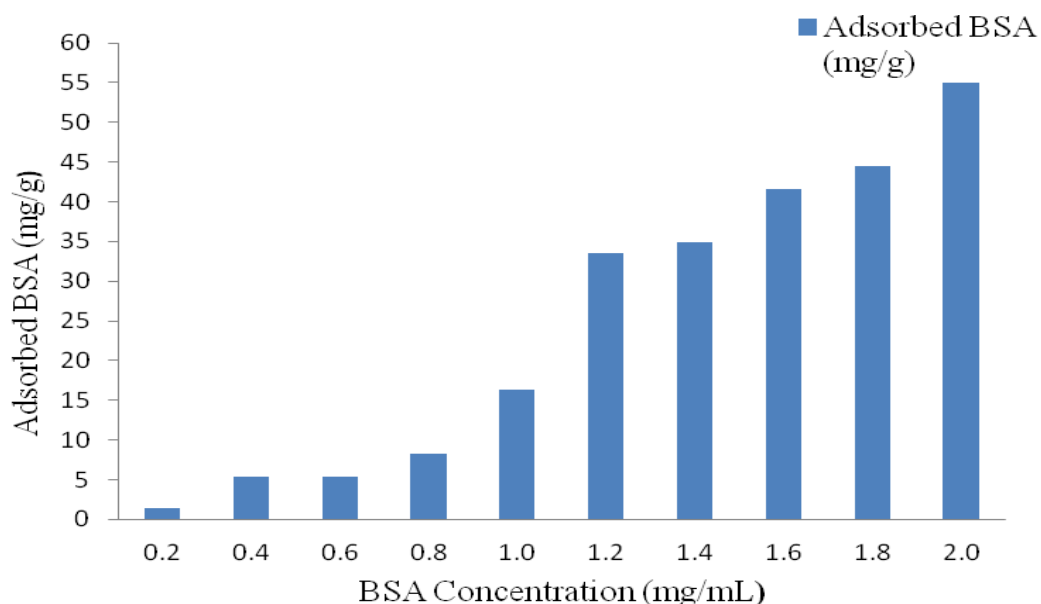


Figure 3.19 BSA adsorption capacity of PAMAM modified SPION in different BSA concentrations; Temperature: 25 °C, Incubation time: 3 h.

In the initial part of the adsorption curve, there is a steep slope which means there is a high affinity between BSA and incorporated modified G5 groups. The maximum adsorption capacity was obtained as 55.00 mg/g at 25 °C and pH 3.0 in acetate buffer.

A study conducted in 2013 by Denizli et al. [Denizli et al., 2013] showed that HSA adsorption first increased with increasing concentration of HSA and then reached a saturation value at an HSA concentration of 0.75 mg/mL.

Table 3.13 BSA concentration effect in the range of 0.2-2.0 mg/mL in the adsorbed amount of BSA.

BSA concentration	Adsorbed BSA (mg/g)
0.2	1.44
0.4	5.42
0.6	5.43
0.8	8.3
1.0	16.3
1.2	33.48
1.4	34.82
1.6	41.59
1.8	44.44
2.0	55.00

3.2.2.4. Effect of temperature on BSA Adsorption

The effect of temperature on the amount of BSA adsorbed on modified G5 was demonstrated in Figure 3.20 (from 25 to 75 °C). As seen from the figure, there is a decrease in the amount of adsorbed BSA with increasing temperature. The maximum adsorption capacity was 55.00 mg/g at 25 °C. According to Table 3.14, the amount of adsorbed BSA decreases from 55.00 mg/g to 36.32 mg/g (40%) as the temperature goes from 25 °C to 75 °C. This situation can be explained with the exothermic nature of the adsorption process. Another study by Denizli et al. [Denizli et al., 2013] considered the effect of temperature in the range of 4 °C - 45 °C on lysozyme adsorption and they found that the elevated temperature caused the decrease on the adsorption capacity of PHEMATrp-Cu (II) NPs. The maximum amount of adsorbed lysozyme was obtained at 4 °C in their work. Decreasing on adsorption capacity of lysozyme was 37% from 4 °C to 45 °C.

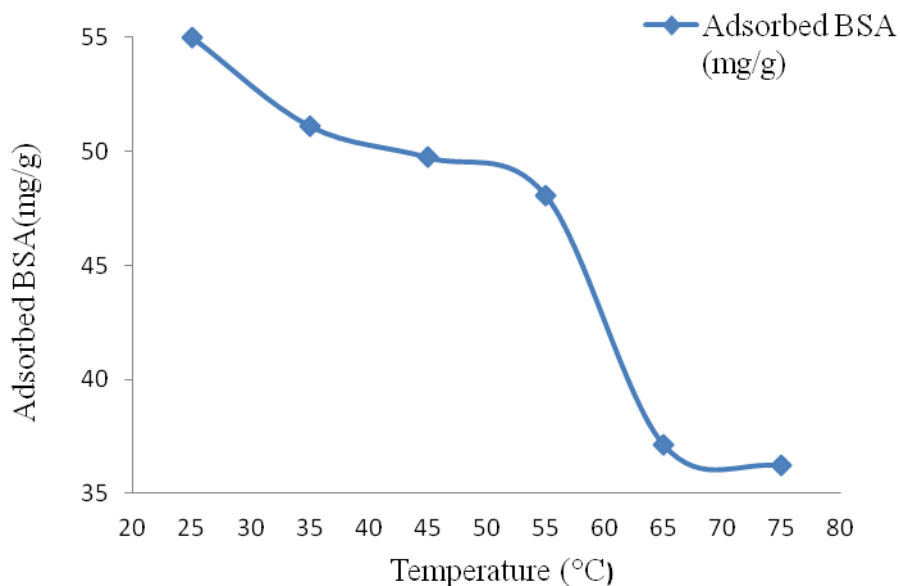


Figure 3.20 BSA adsorption capacity of PAMAM modified SPION in different temperature; BSA concentration: 2.0 mg/mL, Incubation time: 3 h.

Table 3.14 Temperature effect in the range of 25-75 °C in the adsorbed amount of BSA.

Temperature (°C)	Adsorbed BSA (mg/g)
25	55.00
35	51.11
45	49.74
55	48.81
65	37.16
75	36.32

3.2.2.5. Effect of Salt Concentration on BSA Adsorption

The effect of salt concentration on BSA adsorption is shown in Figure 3.21. The concentration of sodium chloride was taken between 0.05-0.25 mM. As it can be seen from the figure, the increased salt concentration causes a decrease in the adsorption capacity. The reason of the decrease of adsorption capacity can be arisen by repulsive

electrostatic interactions between the BSA molecule and the modified G5. In contrast, higher BSA adsorption capacities at low concentrations (50.27 mg/g in NaCl which has concentration of 0.05 mM) may be due to nonspecific adsorption.

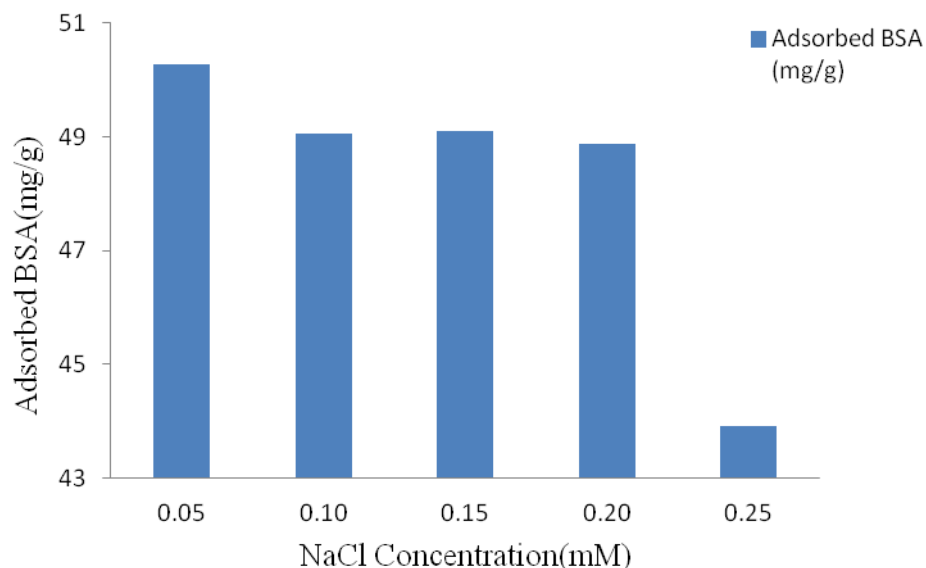


Figure 3.21 BSA adsorption capacity of PAMAM modified SPION in different NaCl concentrations; BSA concentration: 2.0 mg/mL, Incubation time: 3 h.

Denizli et al. [Denizli et al., 2009] reported that the high salt concentration showed high adsorption capacity on magnetic hydrophobic affinity nanobeads (HIC) but they stated that adsorption capacity of protein was affected from the salt type, and the type of protein. They used Na_2SO_4 as a salt source and lysozyme as protein.

Table 3.15 NaCl concentration effect in the range of 0.05-0.25 mM on adsorbed amount of BSA.

NaCl concentration (mM)	Adsorbed BSA (mg/g)
0.05	50.27
0.10	49.04
0.15	49.10
0.20	48.87
0.25	43.91

3.2.2.6 Effect of Different Metals on BSA Adsorption

Figure 3.22 shows the effect of different metals on BSA adsorption amount on the modified SPIONs. As seen from this figure, high adsorption capacity was achieved with the modified SPION-Cu²⁺ (55.00 mg/protein) due to the incorporation of PAMAM-MCA-Cu²⁺ chelating groups onto the dendrimer structure. In addition, the long chain PAMAM-MCA-Cu²⁺ complex acted as a spacer arm that could catch BSA easily. However, the lowest amount of adsorption was obtained from nickel chelation (37.75 mg/protein). The extreme decrease (approximately 30%) was significantly different with different metal ions species and this result clearly observed that different chelating metals, such as copper, zinc, cobalt, nickel can be used for the immobilization of proteins. Cu is the only metal ion that may retain with the support and protein via just one interaction, the other metal require a multi-point adsorption. Denizli and co-workers [Denizli et al., 2013] investigated that the effect of amino acid containing Cu (II) chelated NPs on lysozyme adsorption and they found that Cu (II) chelation had a big impact on the adsorption capacity of lysozyme.

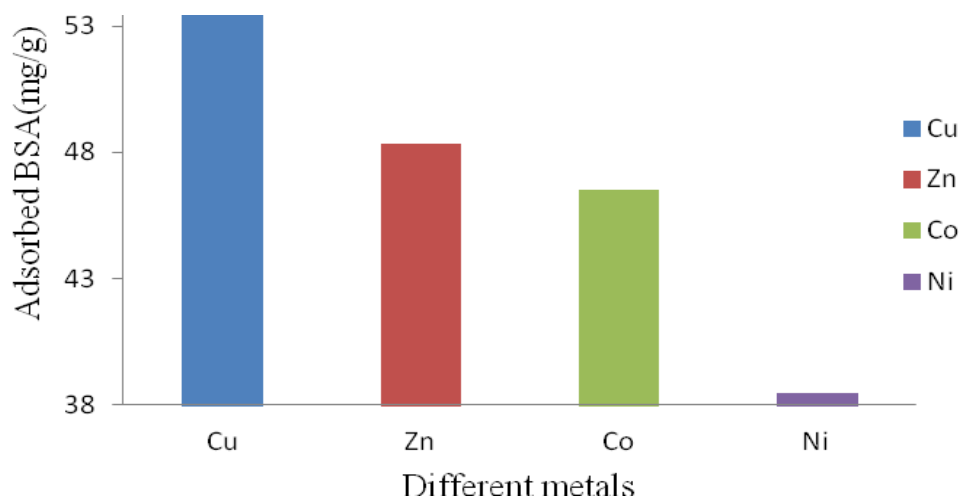


Figure 3.22 Effect of different metals on BSA adsorption capacity.

Table 3.16 Different metals effect on adsorbed amount of BSA.

Different Metals	Adsorbed BSA (mg/g)
Cu	55.00
Cr	48.36
Zn	46.54
Ni	38.49

3.2.2.7 Effect of Reusability on BSA Adsorption

In the magnetic separation processes, the reusability of support materials is important for enzyme immobilization. Thus, it is necessary to evaluate the regeneration efficiency of the support after inactivation of the adsorbed enzyme. Desorption of adsorbed BSA onto the modified SPIONs was done by using of 1.0 M NaCl solution. Figure 3.23 shows the reusability of modified SPIONs on immobilized BSA. To prove the reusability of the nanoparticles, the adsorption–desorption studies were iterated five times using the same nanoparticles. As it can be understood from Table 3.17, adsorption

capacity decreased from 61.13 mg/g to 50.56 mg/g for the same nanoparticles but this decrease is negligible and they can be used many times.

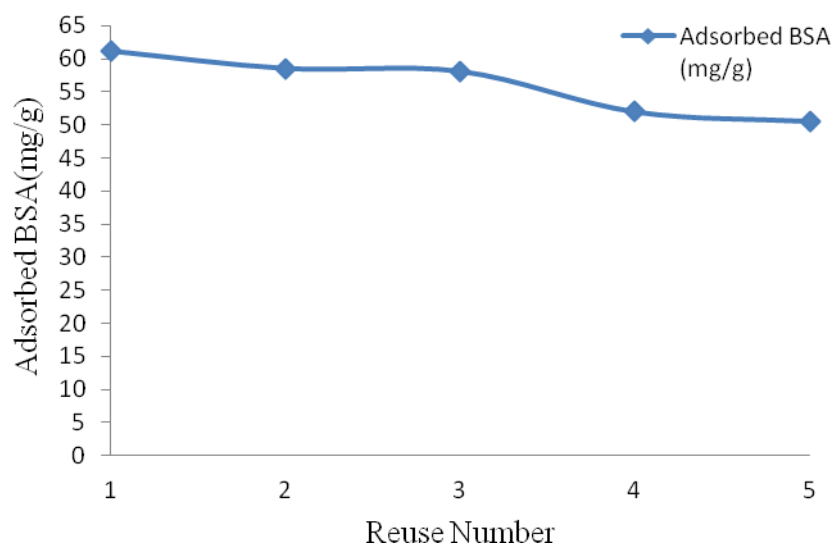


Figure 3.23 Effect of reusability of PAMAM modified SPION on BSA adsorption; BSA concentration: 2.0 mg/mL, Temperature: 25°C.

Table 3.17 Effect of reusability on adsorbed amount of BSA.

Reuse Number	Adsorbed BSA (mg/g)
1	61.13
2	58.53
3	58.08
4	51.99
5	50.56

Denizli et al. [Denizli et al., 2012] synthesized poly (hydroxyethyl methacrylate) based magnetic nanoparticles (mPHEMAH NPs) for DNA purification. They investigated that the effect of time, pH, concentration, temperature and reusability on

DNA adsorption. They repeated adsorption-desorption cycle six times and observed that mPHEMAH NPs can rebind DNA for several times without decreasing adsorption capacity of DNA substantially and it remained constant the end of the six cycle. This means that mPHEMAH NPs can be completely restored and reused.

CHAPTER 4

CONCLUSIONS

Nanoparticles which are used frequently in nanotechnology studies are being investigated. The size of the nanoparticles, the shape and surface functionality is also important. In addition to the content of nanoparticles, nanoparticles which have different surface functionality can be synthesized using various surfactants. The usage of different surfactants that are organic or inorganic give them additional functionality and they can be used many biological and biochemical applications.

In this study, the amount of adsorbed BSA on the modified superparamagnetic iron oxide nanoparticles (SPIONs) has been considered for various characteristics. Surface modification of SPIONs was achieved with amine and carboxylic groups. Synthesized SPIONs with the modification of polyamidoamine (PAMAM) dendrimer (SPION-G5-Cu²⁺) and carboxylic acid (SPION-G5-MCA-Cu²⁺) were used for BSA adsorption in a batch system. By using different experimental conditions, BSA adsorption behavior on the nanoparticles was investigated. Bovine serum albumin was used as a model protein and BSA was immobilized onto SPIONs at different pH, concentration, temperature, ionic strength, metal etc. Magnetic separation which is easy, rapid and effective for protein separation compared to other techniques thus preventing sample loss and providing better efficiency was chosen among the existing technologies. So, protein separation studies were performed magnetically with the help of functionalized magnetic nanoparticles which were magnetic supporting materials.

superparamagnetite nanoparticles revealed good properties as adsorptive beads and will therefore prove to be useful for applications in biotechnology. To conclude, the amine functional superparamagnetite nanoparticles revealed good properties as adsorptive beads and will therefore prove to be useful for applications in biotechnology.

In the second part of the study, PAMAM dendrimer modified SPIONs were functionalized with the carboxylic acid (MCA) to be used for immobilizing of BSA protein. After the optimization of experimental parameters, functional magnetic nanoparticles showed good properties as protein adsorption beads.

In summary, by using co-precipitation route, SPIONs which were functionalized with PAMAM and carboxylic acid were synthesized successfully. These nanobeads were used to immobilize BSA protein in a batch system. Protein separation was done by using these nanobeads which were functionalized SPIONs. The characterization results of functionalized SPIONs in the both studies are below:

- ✚ FT-IR result indicated that successful conjugation of SPION with amine and carboxylic acid.
- ✚ XRD result showed that functionalization of SPIONs with PAMAM dendrimer and carboxylic acid did not change characteristic pattern of SPIONs and the average crystallite size was found 9 ± 1 nm.
- ✚ TEM analysis proved that SPIONs have a mixture of single and polycrystalline character.
- ✚ TGA analysis presented that the increased weight loss of organic content dependent on increased generation. The maximum weight loss was determined as 41% in SPION-PAMAM-G5.
- ✚ VSM analysis exhibited that functionalized SPIONs were superparamagnetic and magnetization decreased with increased generation from 54 emu/g to 37.6 emu/g.

BSA adsorption on SPION-PAMAM and SPION-PAMAM-MCA was 48.08 mg/g for G5. While the maximum adsorption capacity for SPION-PAMAM was at pH 7.0 (52.84 mg/g), it was at pH 3.0 for SPION-PAMAM-MCA (57.46 mg/g). BSA adsorption results revealed that the amount of adsorbed BSA increased with increased BSA concentration. Equilibrium BSA concentration was 1.6 mg/mL for SPION-PAMAM and 2.0 mg/mL for SPION-PAMAM-MCA. The ionic strength of the solution

had also a major impact on the adsorption capacity. As the concentration of ions increased, the adsorption capacity of BSA decreased dramatically. The maximum BSA adsorption was obtained in 0.05 M of NaCl. The best performance for the BSA adsorption was observed when Cu ions were chelated (55.31 mg/g for SPION-PAMAM, 55.00 mg/g for SPION-PAMAM-MCA).

Functionalized SPIONs showed that reusability of these nanobeads were possible for many times and they did not decrease adsorption capacity of BSA significantly even after the end of the five adsorption-desorption cycle. The reusability behavior was also the proof of the usefulness of the magnetic protein separators for applications in fields related with biochemistry.

It was found that the surface carboxylic acid modification could significantly improve the BSA adsorption capacity of superparamagnetic nanoparticles (57.46 mg/g pH 3.0) which was higher than that of amino-modified superparamagnetic nanoparticles (52.84 mg/g at pH 7.0).

We expect that the results may have important implications in the binding effect of BSA onto nano-sized particles and should also contribute to the further application of using magnetic nanoparticle-based technologies to separate proteins from intact biological samples such as blood plasma and serum.

In conclusion, magnetic separation is an easy, simple, way to separate proteins due to quick processing time, reduced chemical need, and elimination of pretreatment. From the two types of investigated, which are SPION-PAMAM and SPION-PAMAM-MCA, functionalized SPIONs revealed good adsorption properties as a nano-support and will be useful in the protein separation technology.

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APPENDIX A

DECLARATION STATEMENT FOR THE ORIGINALITY OF THE THESIS, FURTHER STUDIES AND PUBLICATIONS FROM THESIS WORK

A.1 DECLARATION STATEMENT FOR THE ORIGINALITY OF THE THESIS

I hereby declare that this thesis comprises my original work. No material in this thesis has been previously published and written by another person, except where due reference is made in the text of the thesis. I further declare that this thesis contains no material which has been submitted for a degree or diploma or other qualifications at any other university.

Signature:

Date: June 09, 2015

A.2 PUBLICATIONS FROM THESIS WORK

Academic Journals

- Demir M., Şenel M., Baykal A., “Reversible Immobilization of BSA on Cu-chelated PAMAM Dendrimer Modified Iron Oxide Nanoparticles" Applied Surface Science, Vol.314, pp.697-703, 2014.
- Demir M., Şenel M., Baykal A., “Preparation and protein immobilization on Cu-Chelated carboxylic acid functional PAMAM dendrimer modified iron oxide nanoparticles " Material Research Bulletin, Under review.

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EDUCATION

Ph.D., Chemistry, Fatih University, Istanbul, Turkey, June 2015

Dissertation: “Polyamidoamine (PAMAM) functionalized magnetic nanoparticles for protein separation”

M.S., Chemistry, Fatih University, Istanbul, Turkey, February 2010

Thesis: “Synthesis and characterization of transition metals doped hexaferrites as microwave absorbers”

B.S., Agricultural Engineer, Trakya University, Tekirdağ, Turkey, June 1999

PROFESSIONAL EXPERIENCE

İNTERMAT AMBALAJ VE MATBAACILIK SAN. VE TİC. A.Ş. Istanbul
QUALITY ASSURANCE EXECUTIVE (2011 - 2014)

To ensure the continuity of the systems (ISO 22000:2005, AIB, BRC-IOP etc.) . To establish the QDMS (Quality Documentation Management System) for all systems. Tracing and checking all the works that is performed by the quality control personnels. Reporting to the general manager. To prepare the requirements for the special audits (Sedex, Disney etc.) To follow the customer complaint and managing customer relation.

FATİH UNIVERSITY DEPARTMENT OF CHEMISTRY Istanbul
PROJECT ASSISTANT (2007-2010 February)

Synthesis and characterization of nanoparticles and their applications

İSTANBUL PASTACILIK İMALAT VE PAZ. A.Ş. İstanbul
PRODUCTION and QUALITY ASSURANCE MANAGER (December 2005-May 2007)

The economic and technical criteria for raw materials purchased for the determination of eligibility, Production costs related to the work to be done, review and evaluation of customer complaints over, to provide necessary training to production personel (ISO 9001,HACCP etc.)

MELODİ ÇİKOLATA A.Ş. İstanbul
QUALITY ASSURANCE ENGINEER (August 2003-February 2004)

To ensure settling of the ISO 9000 Quality System in order to provide permanence of the system. Working in coordination with the other departments within the company and to achieve proper controls in order to realise production in hygienic and productive conditions. Making and examining microbiological analysis. Tracing and checking all the works that is performed by the laboratory assistants. Reporting to the factory manager

UNTEKS GIDA SAN. VE TİC. A.Ş. İstanbul
QUALITY ASSURANCE ENGINEER (November 1999-February 2003)

To make analysis first and last product .To ensure that the quality standards are applied properly. To ensure that documentation of quality assurance system is running properly. To check,evaluate and approve all the records that are carried out by the laboratory. Reporting to factory manager.

Aviko STARKING GIDA SAN. A.Ş. İstanbul
LABORATORY TECHNICIAN (September 1994- September 1995)
 (Before university)

AWARDS AND HONORS

- Ranked 1st among 33 graduates in Agricultural Engineering, Trakya University, June 1999
- Ranked 2nd among 331 students in the Trakya University Tekirdağ Agriculture Faculty, 1999

PUBLICATIONS

Academic Journals

- Demir M., Şenel M., Baykal A., “Reversible Immobilization of BSA on Cu-chelated PAMAM Dendrimer Modified Iron Oxide Nanoparticles" Applied Surface Science, Vol.314, pp.697-703, 2014.
- Demir M., Şenel M., Baykal A., "Preparation and protein immobilization on Cu-Chelated carboxylic acid functional PAMAM dendrimer modified iron oxide nanoparticles " Material Research Bulletin, Under review.
- Baykal A., Kavas H., Durmuş Z., Demir M., Kazan S., Topkaya R., "Sonochemical synthesis and characterization of Mn₃O₄ nanoparticles" Central European Journal of Chemistry, Vol.8, pp.633-638.
- Microwave Reflection and Transition properties of ZnO-NiO coated BaFe₁₂O₁₉ 10-15 GHz frequency, Current Applied Physics, Under review

AFFILIATIONS

- Union of Chambers of Turkish Engineers and Architects-UCTEA Chambers of Agricultural Engineers (UCTEA- CAE), member (1999 - Present)

TECHNICAL SKILLS

- Analytical and statistics programs: Origin
- Word processing and publication: Microsoft Word
- Spreadsheets and databases: Microsoft Excel, Access
- Operating systems: Microsoft Windows 3.1 through 8.1
- Presentation: Microsoft PowerPoint
- Email: Microsoft Outlook, Google Mail