OPTIMIZING CONDITIONS FOR PROTEIN ADSORPTION AND CONJUGATION ON TO MAGNETIC NANOPARTICLE

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OPTIMIZING CONDITIONS FOR PROTEIN ADSORPTION AND CONJUGATION ON TO MAGNETIC NANOPARTICLE

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ABSTRACT

Magnetic separation is a rapidly developing technology and mostly applied in the field of bioseparation. Usually, magnetic particles conjugated to specific ligands are employed to bind target molecules, to form complexes that can be separated by magnetic fields. Conjugation quality of particle and ligand is a crucial parameter governing capture efficiency of target molecules. In this study, bovine serum albumin (BSA) is used as a model for co-valent protein conjugation onto 50nm paramagnetic nanoparticles. The effects of ionic strength, pH, and activation time, among other factors, were investigated with regard to conjugation quality and bead capacity. An optimized protocol for conjugation of BSA onto 50nm paramagnetic particles was developed. In a further step, it was shown that the present protocol can be adapted to conjugation of anti-human CD14 monoclonal antibody to 50 nm paramagnetic nanoparticles, and that the conjugate can efficiently isolate CD14-positive mononuclear cells with more than 90% purity on a generic high gradient magnetic separation system. Insights derived from this study can be used for optimization of conjugation of other antibodies and proteins to magnetic nanoparticles.

KEY WORDS : MAGNETIC SEPARATION/MAGNETIC NANOPARTICLES/
PROTEIN CONJUGATION/CARBODIIMIDE METHOD

44 pages

การพัฒนาวิธีการจับโปรตีนบนผิวอนุภาคนาโน

OPTIMIZING CONDITIONS FOR PROTEIN ADSORPTION AND CONJUGATION ON TO MAGNETIC NANOPARTICLE

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บทคัดย่อ

แม่เหล็กแยกเป็นเทคโนโลยีที่พัฒนาอย่างรวดเร็วและนำไปประยุกต์ใช้ส่วนใหญ่อยู่ในเขตของการแยกเซลล์โดยปกติอนุภาคแม่เหล็กผันแกนด์ที่เฉพาะเจาะจงถูกจ้างมาเพื่อผูกโมเลกุลเป้าหมายที่จะสร้างคอมเพล็กซ์ที่สามารถแยกจากสนามแม่เหล็ก คุณภาพการผันคำกริยาของอนุภาคและแกนด์เป็นพารามิเตอร์ที่สำคัญการปกครองที่มีประสิทธิภาพการจับภาพของโมเลกุลเป้าหมาย ในการศึกษานี้ Bovine Serum Albumin (BSA) ถูกนำมาใช้เป็นแบบจำลองสำหรับการผันโปรตีนร่วม-พันธะบนอนุภาคนาโน paramagnetic 50nm ผลของความแข็งแรงของอิออน pH และเวลาการเปิดใช้งานท่ามกลางปัจจัยอื่น ๆ ที่ถูกตรวจสอบเกี่ยวกับการที่มีคุณภาพและความสามารถในการผันลูกปัดโปรโตคอลที่ดีที่สุดสำหรับการผันของ BSA บนอนุภาคparamagnetic 50nm ได้รับการพัฒนาในขั้นตอนต่อไปมันก็แสดงให้เห็นว่าโปรโตคอลปัจจุบันสามารถปรับให้เข้ากับการผันต่อต้านมนุษย์แอนติบอดี CD14 ถึง 50 นาโนเมตรและที่ผันได้อย่างมีประสิทธิภาพสามารถแยกเซลล์โมโนนิวเคลียร์ CD14 บวกที่มีความบริสุทธิ์มากกว่า 90% เมื่อ ทั่วไปลาดแม่เหล็กระบบแยกสูง ข้อมูลเชิงลึกที่ได้รับจากการศึกษาครั้งนี้สามารถนำมาใช้สำหรับการเพิ่มประสิทธิภาพของการผันแอนติบอดีอื่น ๆ และโปรตีนเพื่อนาโนแม่เหล็ก

44 หน้า

CONTENTS

LIST OF TABLES

LIST OF TABLES (cont.)

LIST OF TABLES (cont.)

Table **Page**

LIST OF FIGURES

CHAPTER I
INTRODUCTION

Nanomaterials are part of an industrial revolution to develop lightweight but strong materials for a wide variety of purposes. Similarly, nanoparticles refer to ultrafine particles which sizes are in the range of one to several hundred nanometers(nm), depending on the materials, fields and applications concerned. They include submicron particles and particles smaller than the longest wavelength of visible light of about 400 nm. In some applications, they are limited to particles smaller than 10–20 nm, where is their physical properties change significantly (Lin et al.,2002).

Magnetic separation is a recent developing technology and mostly applied in the field of bioseparation. The principle of this method is to utilize magnetic particles to bind the target molecules via ligand to form a complex that can be separated from the bulk solution by a magnetic field gradient. Its application includes enzyme immobilization (Dekker, 1989), cell sorting (Hancock and Kemshead, 1993), protein adsorption and purification (Abudiab and Beitle, 1998), nucleic acid detachment (Levison et al., 1998), and drug delivery (Khng et al., 1998) as well as diagnostics. Nanoparticles also have been introduced into human blood circulation, e.g. iron dextran complexes in treatment of iron deficiency, which render iron relatively nontoxic. Nanoparticles enclosed drugs, can be directed to a selective organ or tissue target using an external magnet (Avezzano et al., 1990), either directly or through antibodies which are bound to the magnetic nanoparticles. Compared to conventional separation, the advantages of magnetic separation are attributed to its speed, accuracy, and simplicity.

Adsorption and desorption behavior of bovine serum albumin (BSA) on surface-modified magnetic nanoparticles has been widely studied. In earlier studies it was shown that BSA can be covalently be bound to magnetic particles without losing its properties (Lin et al., 2002). The isoelectric point of BSA is at pH 4.5–5.0.This means that in a neutral solution, BSA is negatively charged, as a whole molecule (Hu and Su, 2003). BSA has ability to bind substances reversibly especially negative charged molecules. For that reason it is able to assume roles of transportation (Huang, Kim, and Dass, 2004).

In a previous study, Tzu-Hsien and Wang-Chien Lee, use trypsin and avidin as the model proteins in the formation of protein-nanoparticle conjugates. Magnetic nanoparticles prepared from an alkaline solution of ferrous and ferric ions covalently bind protein via the activation of functional groups by N-ethyl-N(3-diethylaminopropyl) carbodiimide (EDC). Higher concentration of added protein can yield a higher immobilization protein molecules on the particles. In contrast to EDC, the yield of protein immobilization via the activated cyanamide were relatively lower (Tzu-Hsien and Wang-Chien Lee, 2003).

In this study we aim to conjugation BSA on the magnetic nanoparticles by optimizing various conditions optimal for conjugation and validation the quality of the magnetic nanoparticles.

CHAPTER II
LITERATURE REVIEW

Magnetic nanoparticles

Magnetic nanoparticles are a class of particles with diameters of a few to several hundred nanometres (nm), possessing special magnetic characteristics. They normally consist of ferromagnetic cores containing iron, cobalt or nickel but also may include the rare earths metals. The most typically used particles contain magnetite (Fe_3O_4) or maghemite (Fe_2O_3), depending upon the size distribution of the nanoparticles,(E R Flyn and H C Bryant, 2005).

There are many type of nanoparticles such as, nanosized titanium particles, which have attracted extensiveresearch interest because of their diverse applications in, catalysis, energy conversion, and biomedical engineering, among others. Titanium colloids have been used as effective catalysts for the hydrogenation of titanium and zirconium sponses and as powerful activators for heterogeneous hydrogenation catalysts (Bonnemann, 1992,1994). Gold nanoparticles represent another frequently used particle used in research, positively-charged gold nanoparticles possess intrinsic peroxidaselike activity, and can catalyze oxidation of the peroxidase substrate 3,3,5,5 tetramethylbenzidine (TMB) by H2O2 to develop a blue color in aqueous solution, thus providing a simple approach to colorimetric detection of H2O2 and glucose (Yun Jv, Baoxin Li and Rui Cao, 2010).

The beads used in our experiments (fluidMAGARA, chemicell, Berlin, Germany) consist of a core of approximately 80%(v/v) magnetiteparticles, and had an outer diameter of 50 nm. Magnetite particles are embedded in a polysaccharide matrix, which can be modified to carry reactive groups, in our case, carboxylic groups. Due to amino groups in most of the proteins, binding of the proteins to the beads is possible via an activation step with carbodiimide. (Juliane Issle and Uwe Hartmann, 2007)

Different techniques for covalent attachment may be envisaged, depending on the principal functional groups located on the carrier surface. The most widely used methods of ligand coupling are the use of cyanogen bromide (A1-Ab- dulla et al., 1989; Dolinnaya et al., 1991) and periodate (Boorsma and Streefkerk, 1979; Tijssen and Kurstack, 1984) for hydroxyl groups, glutaraldehyde (Avrameas and Guilbert, 1972; Otto et al., 1973) and ethylene glycol diglycidyl ether (Sano et al., 1993) for amino groups, and carbodiimide for carboxylic groups (Molday et al., 1975; Shenoy et al., 1992).

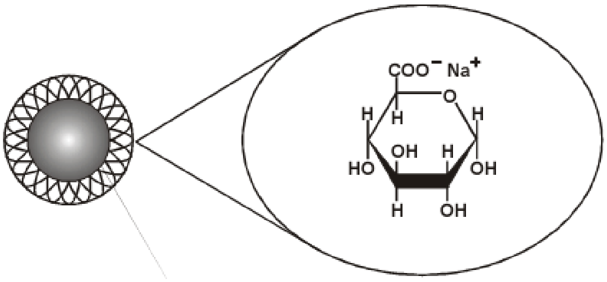


Figure 2.1 Functional Group of fluidMAG ARA,chemicell,Berlin,Germany (http://www.chemicell.com/products/nanoparticles/docs/PI_fluidMAG-ARA_4115.pdf)

Carbodiimide involves the activation of carboxylic acid groups to give NH-activated carboxylic acid groups which can react with free amino groups of the ligand polypeptide chains (Olde Damink et al., 1996). Moreover, glutaraldehyde and carbodiimide coupling may be done in one or two steps: in the one-step process, all the components of the reaction are present at the same time, while in the two-step method, the functional groups on the carrier surface (amino or carboxylic residues) are first activated by the appropriate reagent, the excess of which is removed before ligand attachment. **(**vermeersch and Remon, 1994**)**

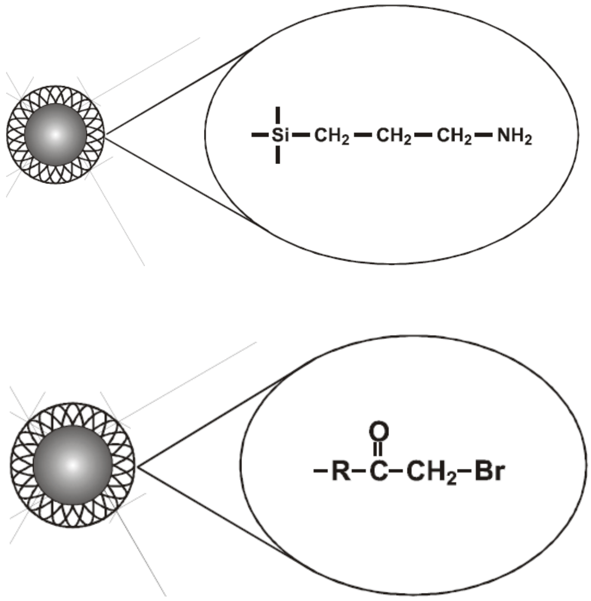


Figure 2.2 Different functional groups on magnetic nanoparticles.The first is amino group and second Bromoacetyl group (http://www.chemicell.com/products/Magnetic_Nanoparticle/Magnetic_Nanoparticles.html)

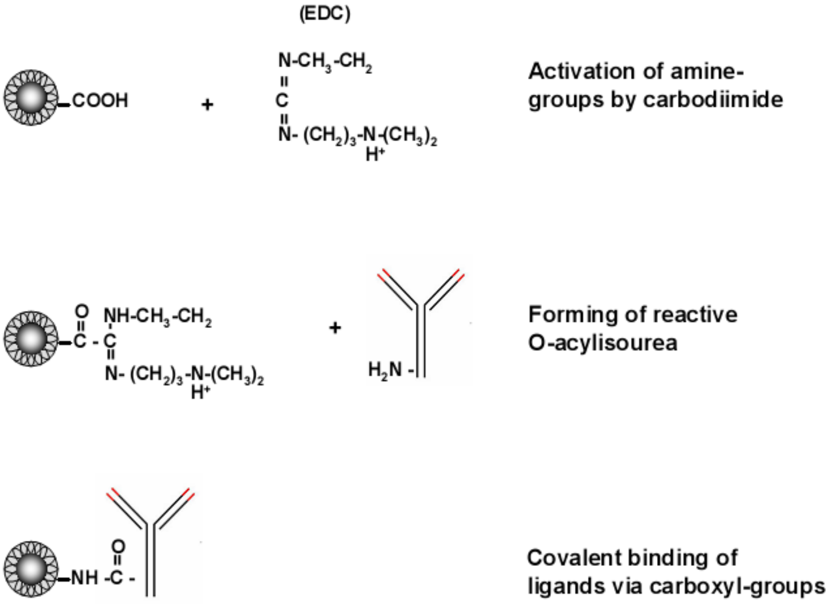


Figure 2.3 Covalent coupling Procedure on fluidMAG-ARA by carbodiimide method (http://www.chemicell.com/products/protocols/fluidara/index.html)

CHAPTER III

MATERIALS AND METHODS

3.1 Chemicals and reagents

3.1.1FluidMAG-ARA- nanoparticles are ferrofluids consisting of an aqueous dispersion of magnetic iron oxides with diameters 50 nm.The particles are covered with hydrophilic polymers which protect them against aggregation by foreign ions. The terminal functional groups isglucuronic acid which can be use as a weak cation exchanger and for covalent coupling of biomolecules (antibodies or proteins). (Chemicell, Germany)

3.2 Buffer for conjugation

3.2.2 0.1 M 2-(N-Morpholino)ethanesulfonic acid (MES) pH 4.7 and 6 (Merck,USA)

3.2.3 PBS pH 4.7 and 6 (Carlo Erba Reagent)

3.2.4Low ionic strength(LIS) pH 4.7 and 6 (Na_2HPO_4 0.072 g and KH_2PO_4 0.012 g, Carlo Erba Reagent add water 100 ml)

3.3 Carbodiimide reagent

3.3.1 1-cyclohexyl-3(2-morpholinoethyl) carbodiimidemetho ptoluensulfonate (CMC) (Sigma, USA, Cat No. C-1011)

3.3.21N-Hydroxysuccinimide (NHS)(Aldrich, USA, Cat No 130672-5G)

3.4 Bovine serum albumin (Usb , USA)

3.5Bradford Protein Assay (Bio-Rad Protein Assay, Bio Rad, Cat No 500-0006)

3.2Instruments

3.2.1 Magnetic separator

3.2.2 The automate plate reader (Wallac Vivtor^{2}V Model 1420 Multilabel HTS counter)

3.3 Methods

Conjugation was performed as specified by the company with modification.

3.3.1 Optimize PBS and MES buffers for conjugation

5 mg of fluidMAG-ARA particles washed with water three times by using a generic HGMS magnetic separator (X-Zell Biotech, Thailand).After washing the fluidMAG-ARA particles were resuspendedin 1 mlPBS pH 6 and 0.1 M MES pH 6 (the particles concentration will maintain at 5 mg/ml). The fluidMAG-ARA particles werechemically activated by adding 1-cyclohexyl-3(2-morpholinoethyl) carbodiimidemetho-p toluensulfonate (CMC) at 8 mg and N-Hydroxysuccinimide (NHS) at 4 mg. The agents were dissolved in 0.3 ml PBS pH 6 and MES at pH6.The solution containing the activating agents and the fluidMAG-ARA particles incubated for 30 minutes on the shaker.After washing with PBS pH 4.7, MES pH66 ml,the fluidMAG-ARA particles solution is concentrated to 1 ml.BSA 60 mg is used for immobilization and incubated 120 minutes on the shaker. The fluidMAG-ARA particles are washed once in PBS pH 4.7, MES pH6and supernatant was kept in 4 tube (1.5 ml each) to determine BSA adsorption. Bradford method was applied usingBio-Rad Protein Assay for determination of protein adsorption.The protein adsorption was determined indirectly from the protein amount remaining in the supernatant after conjugation process and measured by an automated plate reader.

3.3.2 Varied conditions for pH activation and conjugation buffers

5 mg of fluidMAG-ARA particles washed with water three times by using a generic HGMS magnetic separator (X-Zell Biotech, Thailand). After washing the fluidMAG-ARA particles were resuspended in PBS pH 6, PBS pH 4.7, MES pH 6,

PBS pH 6(the particles concentration will maintain at 5 mg/ml). The fluidMAG-ARA particles were chemically activated by adding 1-cyclohexyl-3(2-morpholinoethyl) carbodiimidemetho-p toluensulfonate (CMC) at 8 mg and N-Hydroxysuccinimide (NHS) at 4 mg in 0.3 ml PBS at 6, PBS pH 4.7, MES pH 6, PBS pH 6. The solution containing the activating agents and the fluidMAG-ARA particles incubated for 30 minutes on the shaker. After washing with MES pH 4.7, MES pH 4.7, MES pH 4.7, MES pH6 6 ml,the fluidMAG-ARA particles solution is concentrated to 1 ml. BSA 60 mg is used for immobilization and incubated 120 minutes on the shaker. The fluidMAG-ARA particles are washed once inMES pH 4.7, MES pH 4.7, MES pH 4.7, MES pH6 and supernatant was kept in 4 tube (1.5 ml each) to determine BSA adsorption. Bradford method was applied using Bio-Rad Protein Assay for determination of protein adsorption. The protein adsorption was determined indirectly from the protein amount remaining in the supernatant after conjugation process and measured by an automated plate reader.

3.3.3 Optimize pH for conjugation time

5 mg of fluidMAG-ARA particles washed with water three times by using a generic HGMS magnetic separator (X-Zell Biotech, Thailand). After washing the fluidMAG-ARA particles were resuspended in MESpH 6 (the particles concentration will maintain at 5 mg/ml). The fluidMAG-ARA particles were chemically activated by adding 1-cyclohexyl-3(2-morpholinoethyl) carbodiimidemetho-p toluensulfonate (CMC) at 8 mg and N-Hydroxysuccinimide (NHS) at 4 mg in 0.3 ml MES at pH 6. The solution containing the activating agents and the fluidMAG-ARA particles incubated for 30 minutes on the shaker. After washing with MES at pH 4.7, 6 and 7 6 ml,the fluidMAG-ARA particles solution is concentrated to 1 ml. BSA 60 mg is used for immobilization and incubated 120 minutes on the shaker. The fluidMAG-ARA particles are washed once in MES pH 4.7, 6, 7 and supernatant was kept in 4 tube (1.5 ml each) to determine BSA adsorption. Bradford method was applied using Bio-Rad Protein Assay for determination of protein adsorption. The protein adsorption was determined indirectly from the protein amount remaining in the supernatant after conjugation process and measured by an automated plate reader.

3.3.4 Optimize concentration of CMC and NHS for activation

5 mg of fluidMAG-ARA particles washed with water three times by using a generic HGMS magnetic separator (X-Zell Biotech, Thailand). After washing the fluidMAG-ARA particles were resuspended in MES pH 6 (the particles concentration will maintain at 5 mg/ml). The fluidMAG-ARA particles were chemically activated by adding 1-cyclohexyl-3(2-morpholinoethyl) carbodiimidemetho-p toluensulfonate (CMC) at 4, 8, 12 mg and N-Hydroxysuccinimide (NHS) at 2, 4, 6 mg in 0.3 ml MESpH 6. The solution containing the activating agents and the fluidMAG-ARA particles incubated for 30 minutes on the shaker. After washing with MES at pH 4.7 6 ml,the fluidMAG-ARA particles solution is concentrated to 1 ml. BSA 60 mg is used for immobilization and incubated 120 minutes on the shaker. The fluidMAG-ARA particles are washed once in MES pH 4.7 and keep supernatant in 4 tube (1.5 ml each) to determine BSA adsorption. Bradford method was applied using Bio-Rad Protein Assay for determination of protein adsorption. The protein adsorption was determined indirectly from the protein amount remaining in the supernatant after conjugation process and measured by an automated plate reader.

3.3.5 Optimize activation time

5 mg of fluidMAG-ARA particles washed with water three times by using a generic HGMS magnetic separator (X-Zell Biotech, Thailand). After washing the fluidMAG-ARA particles were resuspended in MES pH 6 (the particles concentration will maintain at 5 mg/ml). The fluidMAG-ARA particles were chemically activated by adding 1-cyclohexyl-3(2-morpholinoethyl) carbodiimidemetho-p toluensulfonate (CMC) at 8 mg and N-Hydroxysuccinimide (NHS) at 4 mg in 0.3 ml MES pH 6. The solution containing the activating agents and the fluidMAG-ARA particles incubated for 30, 45 and 60 minutes on the shaker. After washing with MES at pH 4.7 6 ml,the fluidMAG-ARA particles solution is concentrated to 1 ml. BSA 60 mg is used for immobilization and incubated 120 minutes on the shaker. The fluidMAG-ARA particles are washed once in MES pH 4.7 and keep supernatant in 4 tube (1.5 ml each) to determine BSA adsorption. Bradford method was applied using Bio-Rad Protein Assay for determination of protein adsorption. The protein adsorption was determined

indirectly from the protein amount remaining in the supernatant after conjugation process and measured by an automated plate reader.

3.3.6 Optimize conjugation time

5 mg of fluidMAG-ARA particles washed with water three times by using a generic HGMS magnetic separator (X-Zell Biotech, Thailand). After washing the fluidMAG-ARA particles were resuspended in MES pH 6 (the particles concentration will maintain at 5 mg/ml). The fluidMAG-ARA particles were chemically activated by adding 1-cyclohexyl-3(2-morpholinoethyl) carbodiimidemetho-p toluensulfonate (CMC) at 8 mg and N-Hydroxysuccinimide (NHS) at 4 mg in 0.3 ml MES pH 6. The solution containing the activating agents and the fluidMAG-ARA particles incubated for 30 minutes on the shaker. After washing with MES pH 4.7 6 ml. The fluidMAG-ARA particles solution is concentrated to 1 ml. BSA 60 mg is used for immobilization and incubated 60, 90 and 120 minutes on the shaker. After washing with MES at pH 4.7 6 ml, the fluidMAG-ARA particles solution is concentrated to 1 ml. BSA 60 mg is used for immobilization and incubated 120 minutes on the shaker. The fluidMAG-ARA particles are washed once in MES pH 4.7 and keep supernatant in 4 tube (1.5 ml each) to determine BSA adsorption. Bradford method was applied using Bio-Rad Protein Assay for determination of protein adsorption. The protein adsorption was determined indirectly from the protein amount remaining in the supernatant after conjugation process and measured by an automated plate reader.

3.3.7 Optimize buffers

5 mg of fluidMAG-ARA particles washed with water three times by using a generic HGMS magnetic separator (X-Zell Biotech, Thailand). After washing the fluidMAG-ARA particles were resuspended in MES pH 6, LIS pH 6(the particles concentration will maintain at 5 mg/ml). The fluidMAG-ARA particles were chemically activated by adding 1-cyclohexyl-3(2-morpholinoethyl) carbodiimidemetho-p toluensulfonate (CMC) at 8 mg and N-Hydroxysuccinimide (NHS) at 4 mg in 0.3 ml MES and LIS pH 6. The solution containing the activating agents and the fluidMAG-ARA particles incubated for 30 minutes on the shaker. After washing with MES and LIS pH 4.7 6 , the fluidMAG-ARA particles solution is

concentrated to 1 ml. BSA 60 mg is used for immobilization and incubated 120 minutes on the shaker. The fluidMAG-ARA particles are washed once in MES and LIS at pH 4.7 and keep supernatant in 4 tube (1.5 ml each) to determine BSA adsorption. Bradford method was applied using Bio-Rad Protein Assay for determination of protein adsorption. The protein adsorption was determined indirectly from the protein amount remaining in the supernatant after conjugation process and measured by an automated plate reader.

3.3.8 Protein adsorption determination

Bradford method was applied using Bio-Rad Protein Assay for the determination of the protein adsorption. In brief, protein adsorption was determined indirectly from the protein amount remaining in the supernatant after conjugation process and measured by an automated plate reader (Wallac Victor 2VModel1420 Multi label HTS counter). 20 µL of undiluted Bradford solution was added to 150 µL sample solution. Samples were loaded into 96 well plates with at least 3 repeats. The received data were compared with bovine γ-globulin protein standard. The optical density was measured at wavelength of 595 nm. The particles' protein-binding capacity was defined as the difference between initial protein concentration and depleted protein concentration of the supernatant per one mg of magnetic particles.

CHAPTER IV

RESULTS

Adsorption of BSA on magnetic nanoparticles at pH and salt concentrationsin the presence ofcarbodiimidewas carried out.The carbidiimide is used to activate a carboxyl group in one molecule and a free amino group in another molecule to form peptide bonds, or in other words, for protein adsorption.In this study,conditions were optimized for BSA adsorption and conjugation onto magnetic nanoparticles by examing the influence of different buffers and pH for activation, concentration of carbodiimide, activation time and conjugation time.

4.1 Selection ofbuffers for conjugation

The effect of MES and PBS buffers on BSA adsorption on BSA adsorption on magnetic nanoparticle is shown in table 4.1to 4.4 and is summarized in Figure 4.1. Percent BSA adsorption in MES is 30% and percent adsorption in PBS is 0%.It is shown that PBS cannot immobilize BSA on magnetic nanoparticlewhile MES shows favourableadsorption.

Table 4.1 Raw data standard curve: BSA concentration (µg/ml)

Absorbance(nm) / BSA (µg/ml)	30	15	7.5	3.75	0
test 1	0.480	0.368	0.326	0.282	0.226
test 2	0.466	0.368	0.319	0.282	0.227
test 3	0.466	0.369	0.322	0.275	0.217
average	0.471	0.368	0.322	0.280	0.223
standard deviation	0.01	0.00	0.00	0.00	0.01

Table 4.2 Raw data: Negative control, BSA passing the generic HGMS system without immobilization magnetic nanoparticles

Absorbance(nm) /Flow through volume	**frac 1**	**frac 2**	**frac 3**	**frac4**	**Total BSA(µg)**
test 1	0.519	0.542	0.296	0.281	
test 2	0.509	0.563	0.315	0.289	
test 3	0.494	0.531	0.308	0.29	
average	0.507	0.545	0.306	0.287	
standard deviation	0.013	0.016	0.010	0.005	
BSA(µg)per fraction(µg/ml)	33.01	37.74	7.94	5.49	
total BSA(µg)per fraction	**49.51**	**56.62**	**11.91**	**8.23**	**126.27**

Table 4.3 Raw data: Adsorption of BSA onto magnetic nanoparticles in PBS buffer

Absorbance(nm) /Flow throug volume	**frac 1**	**frac 2**	**frac 3**	**frac4**	**unbound(bound) BSA(µg)**
test 1	0.635	0.678	0.319	0.525	
test 2	0.613	0.679	0.317	0.25	
test 3	0.599	0.659	0.318	0.223	
average	0.616	0.672	0.318	0.333	
standard deviation	0.018	0.011	0.001	0.167	
BSA per fraction(µg/ml)	46.51	53.54	9.40	11.23	
total BSA(µg)per fraction	**69.77**	**80.31**	**14.09**	**16.84**	**181.01(0)**
			%absorption		**0**

Table 4.4 Raw data:Adsorption of BSA onto magnetic nanoparticles in MES buffer

Absorbance(nm) /Flow throug volume	**frac 1**	**frac 2**	**frac 3**	**frac4**	**unbound(bound) BSA(µg)**
test 1	0.419	0.447	0.283	0.247	
test 2	0.419	0.452	0.299	0.243	
test 3	0.417	0.494	0.297	0.249	
average	0.418	0.464	0.293	0.246	
standard deviation	0.001	0.026	0.009	0.003	
BSA per fraction(µg/ml)	21.91	27.64	6.28	0.46	
total BSA(µg)per fraction	**32.86**	**41.47**	**9.42**	**0.69**	**84.43(41.84)**
			%absorption		**33.13**

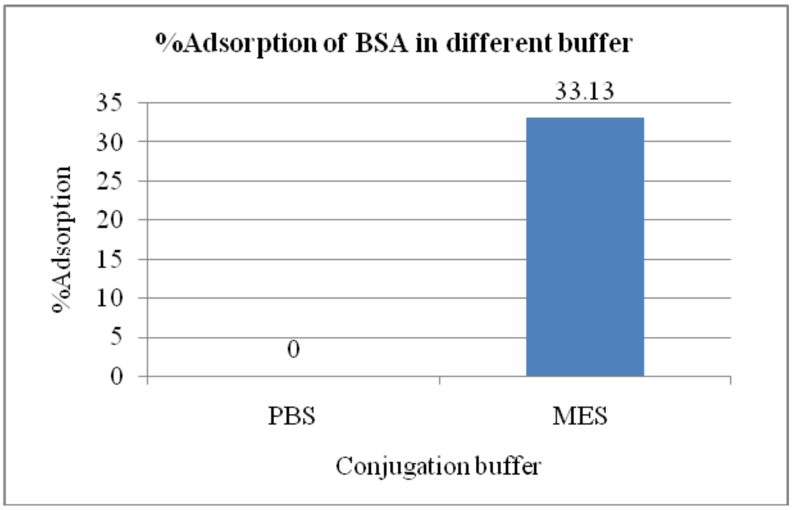


Figure 4.1 Comparisonof adsorption ofBSA in PBS and MES buffers

4.2 Optimize pH activation and conjugation buffer

The effect of MES and PBS buffers and pH during activation and conjugation for adsorption of BSA onto magnetic nanoparticles isshown from table 4.5 to 4.13 and is summarized in Table4.14.It is shown that conjugation in MES pH 4.7 can absorb BSA onto magnetic nanoparticle better than conjugation in MES pH 6.The activation buffer MES is better than PBS for BSA adsorbtion onto magnetic nanoparticle.The pH for activating 6 and 4.7 percent BSA show no difference.Magnetic nanoparticle are activated at pH 6 better than pH 4.7 and MES buffer is better than PBS buffer for BSA adsorption and conjugation.

Experiment I

Table 4.5 Raw data standard curve: BSA concentration (µg/ml)

Absorbance(nm) / BSA (µg/ml)	**30**	**15**	**7.5**	**3.85**	**0**
test 1	0.918	0.756	0.584	0.486	0.329
test 2	0.931	0.781	0.572	0.495	0.344
test 3	0.92	0.754	0.586	0.478	0.332
average	0.923	0.764	0.581	0.486	0.335
standard Deviation	0.01	0.02	0.01	0.01	0.01

Table4.6 Raw data: Negative control, BSA passing the generic HGMS system without immobilization magnetic nanoparticles

Absorbance(nm) /Flow through volume	frac1	frac2	frac3	frac4	Total BSA(μg)
test 1	0.718	0.808	0.403	0.348	
test 2	0.699	0.853	0.406	0.34	
test 3	0.664	0.804	0.420	0.369	
average	0.694	0.822	0.410	0.352	
standard deviation	0.03	0.03	0.01	0.01	
BSA(μg)per fraction(μg/ml)	15.03	21.38	0.95	-1.89	
total BSA(μg)per fraction	**22.55**	**32.07**	**1.43**	**-2.83**	**56.05**

Table 4.7 Raw data: Adsorption of BSA onto magnetic nanoparticles activated inPBS pH 6 and conjugated in MES pH 4.7

Absorbance(nm) /Flow through volume	frac1	frac2	frac3	frac4	unbound(bound) BSA(μg)
test 1	0.364	0.434	0.526	0.482	
test 2	0.355	0.435	0.529	0.481	
test 3	0.368	0.436	0.505	0.47	
average	0.362	0.435	0.520	0.478	
standard deviation	0.01	0.00	0.01	0.01	
BSA per fraction(μg/ml)	-1.39	2.21	6.42	4.33	
total BSA(μg)per fraction	**-2.09**	**3.32**	**9.64**	**6.49**	**19.44(36.61)**
			%absorption		**65.32**

Table 4.8 Raw data: Adsorption of BSA onto magnetic nanoparticles activated inPBS pH 4.7 and conjugated in MES pH 4.7

Absorbance(nm) /Flow through volume	frac1	frac2	frac3	frac4	unbound(bound) BSA(μg)
test 1	0.362	0.449	0.553	0.46	
test 2	0.354	0.427	0.517	0.46	
test 3	0.355	0.425	0.519	0.453	
average	0.357	0.434	0.530	0.458	
standard deviation	0.00	0.01	0.02	0.00	
BSA per fraction(μg/ml)	-1.66	2.14	6.90	3.33	
total BSA(μg)per fraction	**-2.48**	**3.22**	**10.36**	**5.00**	**18.57(37.48)**
			%absorption		**66.86**

Table 4.9 Raw data: Adsorption of BSA onto magnetic nanoparticles activated inMES pH 6 and conjugated in MES pH 4.7

Absorbance(nm) /Flow through volume	**frac1**	**frac2**	**frac3**	**frac4**	**unbound(bound) BSA(μg)**
test 1	0.385	0.435	0.5	0.41	
test 2	0.388	0.447	0.498	0.421	
test 3	0.387	0.444	0.487	0.435	
average	0.387	0.442	0.495	0.422	
standard deviation	0.00	0.01	0.01	0.01	
BSA per fraction(μg/ml)	-0.19	2.56	5.19	1.57	
total BSA(μg)per fraction	**-0.28**	**3.84**	**7.78**	**2.35**	**13.96(42.09)**
			%absorption		**75.09**

Table 4.10 Raw data: Adsorption of BSA onto magnetic nanoparticles activated in PBS pH 6 and conjugated in MES pH 6.

Absorbance(nm) /Flow through volume	**frac1**	**frac2**	**frac3**	**frac4**	**unbound(bound) BSA(μg)**
test 1	0.73	0.767	0.381	0.355	
test 2	0.701	0.749	0.39	0.359	
test 3	0.705	0.766	0.398	0.354	
average	0.712	0.761	0.390	0.356	
standard deviation	0.02	0.01	0.01	0.00	
BSA per fraction(μg/ml)	15.94	18.36	-0.04	-1.71	
total BSA(μg)per fraction	**23.92**	**27.53**	**-0.06**	**-2.56**	**51.45(4.60)**
			%absorption		**8.21**

Experiment II

Table 4.11 Raw data standard curve: BSA concentration (μg/ml)

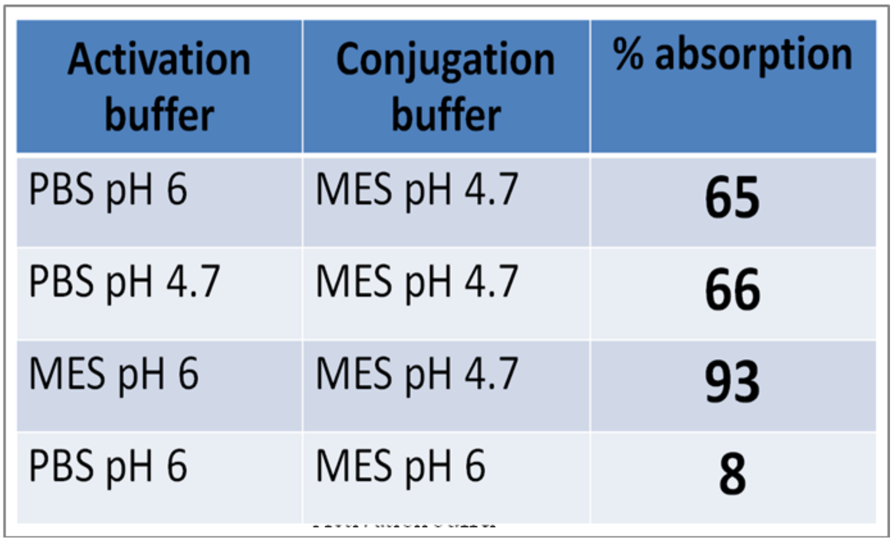
Absorbance(nm) / BSA (μg/ml)	**30**	**15**	**7.5**	**3.85**	**0**
test 1	1.015	0.789	0.651	0.565	0.365
test 2	0.838	0.707	0.633	0.521	0.407
test 3	0.927	0.780	0.643	0.556	0.421
average	0.927	0.759	0.642	0.547	0.398
standard deviation	0.09	0.04	0.01	0.02	0.03

Table 4.12 Raw data: Negative control, BSA passing the generic HGMS system without immobilization magnetic nanoparticles

Absorbance(nm) /Flow through volume	**frac1**	**frac2**	**frac3**	**frac4**	**Total BSA(µg)**
test 1	0.669	0.682	0.492	0.408	
test 2	0.693	0.815	0.535	0.405	
test 3	0.712	0.728	0.506	0.423	
average	0.691	0.742	0.511	0.412	
standard deviation	0.02	0.07	0.02	0.01	
BSA(µg)per fraction(µg/ml)	13.34	16.20	3.10	-2.53	
total BSA(µg)per fraction	**20.01**	**24.30**	**4.64**	**-3.79**	**44.72**

Table 4.13 Raw data: Adsorptionof BSA onto magnetic nanoparticles activated inMES pH 6 and conjugated in MES pH 4.7

Absorbance(nm) /Flow throug volume	**frac1**	**frac2**	**frac3**	**frac4**	**unbound(bound) BSA(µg)**
test 1	0.301	0.398	0.38	0.399	
test 2	0.337	0.38	0.44	0.449	
test 3	0.321	0.413	0.442	0.388	
average	0.320	0.397	0.421	0.412	
standard deviation	0.02	0.02	0.04	0.03	
BSA per fraction(µg/ml)	-7.77	-3.38	-2.04	-2.53	
total BSA(µg)per fraction	**-11.66**	**-5.07**	**-3.05**	**-3.79**	**0(44.72)**
			%absorption		**100 % ABS**



Activation buffer	Conjugation buffer	% absorption
PBS pH 6	MES pH 4.7	**65**
PBS pH 4.7	MES pH 4.7	**66**
MES pH 6	MES pH 4.7	**93**
PBS pH 6	MES pH 6	**8**

Table 4.14 Compare percent absorption of BSA with activate and conjugate in different pH and buffers

4.3 Optimize pH for conjugation

The effect of pH for conjugation in MES buffer on BSA adsorption on magnetic nanoparticle is shown in Figure4.3.BSA adsorption is 98% in the MES pH 4.7 butinsufficient in MES pH 6 and 7

Table 4.15 Raw data standard curve: BSA concentration (µg/ml)

Absorbance(nm) / BSA (µg/ml)	**30**	**15**	**7.5**	**3.85**	**0**
test 1	0.665	0.637	0.512	0.420	0.371
test 2	0.700	0.589	0.511	0.436	0.371
test 3	0.720	0.581	0.482	0.320	0.362
average	0.695	0.602	0.502	0.392	0.368
standard deviation	0.03	0.03	0.02	0.06	0.01

Table 4.16 Raw data: Negative control, BSA passing the generic HGMS system without immobilization magnetic nanoparticles

Absorbance(nm) /Flow through volume	frac1	frac2	frac3	frac4	Total BSA(µg)
test 1	0.654	0.478	0.428	0.37	
test 2	0.612	0.468	0.425	0.369	
test 3	0.689	0.484	0.458	0.374	
average	0.652	0.477	0.437	0.371	
standard deviation	0.04	0.01	0.02	0.00	
BSA(µg)per fraction(µg/ml)	22.76	8.36	5.09	-0.34	
total BSA(µg)per fraction	**34.14**	**12.54**	**7.64**	**-0.51**	**53.81**

Table 4.17 Raw data: BSA adsorptionduringconjugation in MES pH 4.7

Absorbance(nm) /Flow through volume	frac1	frac2	frac3	frac4	unbound(bound) BSA(µg)
test 1	0.354	0.387	0.355	0.305	
test 2	0.355	0.382	0.356	0.307	
test 3	0.348	0.382	0.342	0.303	
average	0.352	0.384	0.351	0.305	
standard deviation	0.00	0.00	0.00	0.00	
BSA per fraction(µg/ml)	-1.88	0.70	-1.99	-5.77	
total BSA(µg)per fraction	-2.81	1.06	-2.98	-8.66	1.06(52.76)
			%absorption		**98.04**

Table 4.18 Raw data: BSA adsorption during conjugation in MES pH 6

Absorbance(nm) /Flow through volume	frac1	frac2	frac3	frac4	unbound(bound) BSA(µg)
test 1	0.305	0.313	0.658	0.529	
test 2	0.307	0.267	0.651	0.575	
test 3	0.303	0.224	0.645	0.527	
average	0.305	0.268	0.651	0.544	
standard deviation	0.00	0.04	0.01	0.03	
BSA per fraction(µg/ml)	-5.77	-8.82	22.73	13.87	
total BSA(µg)per fraction	-8.66	-13.22	34.10	20.81	54.91(0)
			%absorption		0

Table 4.19 Raw data: BSA adsorption during conjugation in MES pH 7

Absorbance(nm) /Flow throug volume	**frac1**	**frac2**	**frac3**	**frac4**	**unbound(bound) BSA(μg)**
test 1	0.344	0.353	0.642	0.605	
test 2	0.336	0.349	0.616	0.562	
test 3	0.338	0.34	0.634	0.544	
average	0.339	0.347	0.631	0.570	
standard deviation	0.00	0.01	0.01	0.03	
BSA per fraction(μg/ml)	-2.95	-2.29	21.03	16.07	
total BSA(μg)per fraction	-4.42	-3.43	31.55	24.10	55.65(0)
			%absorption		**0**

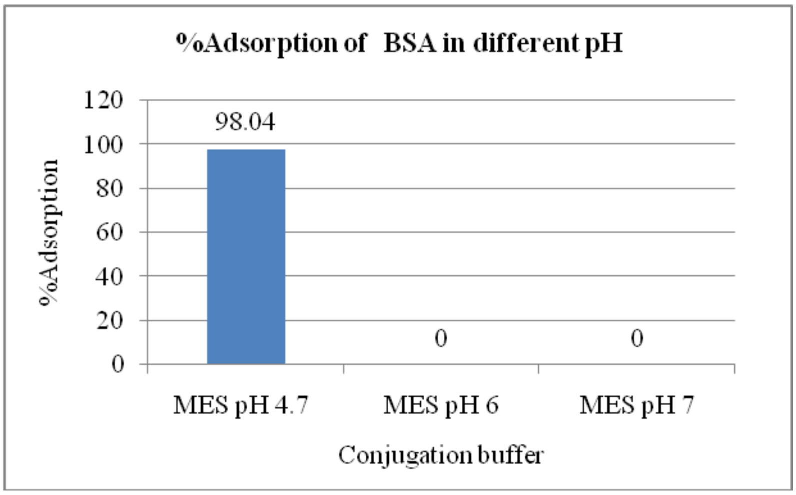


Figure 4.2 Comparison ofBSA adsorption in different pH

4.4 Optimization of concentration of CMC and NHS for activation

The effect of concentration of CMC and NHS on BSA adsorption onto magnetic nanoparticle in MES pH 6 for activation and MES pH 4.7 for conjugation is shown in Figure4.3.It is shown that 8mg CMC and 4 mg NHS and 12 mg CMC and 6 mg NHS can absorb BSA better than 4 mg CMC and 2 mg NHS.Percent adsorption of BSA onto magnetic nanoparticleswas between 8 mg CMC and 4 mg NHS and12 mg CMC and 6 mg are 100% absorption and 99%, it is not different.

Table 4.20 Raw data standard curve: BSA concentration (µg/ml)

Absorbance(nm) / BSA (µg/ml)	**30**	**15**	**7.5**	**3.85**	**0**
test 1	0.877	0.694	0.570	0.512	0.364
test 2	0.859	0.720	0.557	0.536	0.400
test 3	0.804	0.627	0.576	0.506	0.364
average	0.847	0.680	0.568	0.518	0.376
standard deviation	0.038	0.048	0.010	0.016	0.021

Table 4.21 Raw data: Negative control, BSA passing the generic HGMS system without immobilization magnetic nanoparticles

Absorbance(nm) /Flow through volume	**frac1**	**frac2**	**frac3**	**frac4**	**Total BSA(µg)**
test 1	0.697	0.718	0.391	0.362	
test 2	0.747	0.791	0.382	0.373	
test 3	0.744	0.709	0.377	0.345	
average	0.729	0.739	0.383	0.360	
standard deviation	0.03	0.04	0.01	0.01	
BSA(µg)per fraction(µg/ml)	19.86	20.51	-2.73	-4.25	
total BSA(µg)per fraction	29.79	30.77	-4.09	-6.37	60.56

Table 4.22 Raw data: CMC 4 mg and NHS 2 mg, activation 30 minutes

Absorbance(nm) /Flow through volume	**frac1**	**frac2**	**frac3**	**frac4**	**unbound(bound) BSA(µg)**
test 1	0.373	0.547	0.439	0.442	
test 2	0.378	0.626	0.612	0.533	
test 3	0.395	0.597	0.593	0.480	
average	0.382	0.590	0.548	0.485	
standard deviation	0.01	0.04	0.09	0.05	
BSA per fraction(µg/ml)	-2.81	10.77	8.02	3.91	
total BSA(µg)per fraction	-4.22	16.15	12.03	5.87	34.05(26.51)
			%absorption		**43.78**

Table 4.23 Raw data: CMC 8 mg and NHS 4 mg, activation 30 minutes

Absorbance(nm) /Flow through volume	**frac1**	**frac2**	**frac3**	**frac4**	**unbound(bound) BSA(µg)**
test 1	0.326	0.435	0.435	0.385	
test 2	0.355	0.441	0.435	0.44	
test 3	0.351	0.431	0.426	0.427	
average	0.344	0.436	0.432	0.417	
standard deviation	0.02	0.01	0.01	0.03	
BSA per fraction(µg/ml)	-5.29	0.69	0.45	-0.51	
total BSA(µg)per fraction	-7.94	1.03	0.68	-0.76	1.71(59.61)
			%absorption		**97.18**

Table 4.24 Raw data: CMC 12 mg and NHS 6 mg, activation 30 minutes

Absorbance(nm) /Flow throug volume	**frac1**	**frac2**	**frac3**	**frac4**	**unbound(bound) BSA(µg)**
test 1	0.372	0.435	0.473	0.43	
test 2	0.368	0.431	0.487	0.434	
test 3	0.355	0.429	0.49	0.435	
average	0.365	0.432	0.483	0.433	
standard deviation	0.01	0.00	0.01	0.00	
BSA per fraction(µg/ml)	-7.36	-3.03	0.33	-2.94	
total BSA(µg)per fraction	-11.05	-4.54	0.50	-4.41	0.50(57.58)
			%absorption		**99.14**

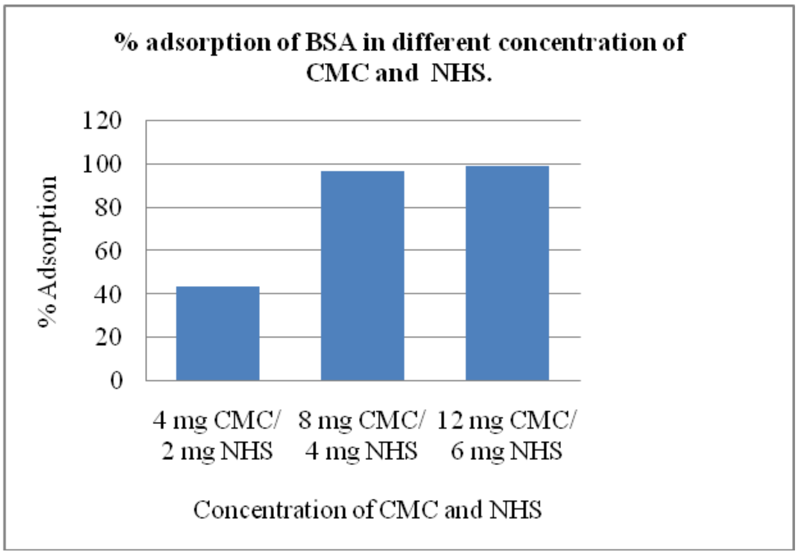


Figure4.3 Adsorption of BSA in different concentration of CMC and NHS

4.5 Optimization of activation time

The effect of activation time on BSA adsorption on magnetic nanoparticle in MES pH 6 with8mg CMC and 4 mg NHS for activation and MES pH 4.7 for conjugation is shown in Figure4.4.Percent BSA adsorption at 30 minutes than 45 and 60 minutes.The percent adsorption are 100%,91% and 92%

Table 4.25 Raw data standard curve: BSA concentration (µg/ml)

Absorbance(nm) / BSA (µg/ml)	**30**	**15**	**7.5**	**3.85**	**0**
test 1	0.895	0.649	0.611	0.52	0.42
test 2	0.939	0.78	0.614	0.596	0.417
test 3	0.917	0.746	0.608	0.568	0.487
average	0.917	0.725	0.611	0.561	0.441
standard deviation	0.02	0.07	0.00	0.04	0.04

Table 4.26 Raw data: Negative control, BSA passing the generic HGMS system without immobilization magnetic nanoparticles

Absorbance(nm) /Flow through volume	frac1	frac2	frac3	frac4	Total BSA(µg)
test 1	0.783	0.835	0.459	0.461	
test 2	0.732	0.848	0.455	0.484	
test 3	0.749	0.823	0.449	0.43	
average	0.755	0.835	0.454	0.458	
standard deviation	0.03	0.01	0.01	0.03	
BSA(µg)per fraction(µg/ml)	17.99	19.95	0.26	1.87	
total BSA(µg)per fraction	**26.98**	**29.92**	**0.38**	**2.81**	**60.09**

Table 4.27 Raw data: CMC 8 mg and NHS 4 mg, activation 30 minutes

Absorbance(nm) /Flow through volume	frac1	frac2	frac3	frac4	unbound(bound) BSA(µg)
test 1	0.4	0.402	0.463	0.347	
test 2	0.357	0.431	0.473	0.421	
test 3	0.387	0.399	0.445	0.436	
average	0.381	0.411	0.460	0.401	
standard deviation	0.02	0.02	0.01	0.05	
BSA per fraction(µg/ml)	-6.30	-4.39	-1.16	-5.00	
total BSA(µg)per fraction	-9.45	-6.59	-1.74	-7.50	0(60.09)
			%absorption		**100**

Table 4.28 Raw data: CMC 8 mg and NHS 4 mg, activation 45 minutes

Absorbance(nm) /Flow through volume	frac1	frac2	frac3	frac4	unbound(bound) BSA(µg)
test 1	0.360	0.503	0.529	0.481	
test 2	0.351	0.499	0.507	0.410	
test 3	0.361	0.518	0.480	0.411	
average	0.357	0.507	0.505	0.434	
standard deviation	0.01	0.01	0.02	0.04	
BSA per fraction(µg/ml)	-7.86	1.85	1.76	-2.88	
total BSA(µg)per fraction	-11.79	2.78	2.65	-4.31	5.42(54.67)
			%absorption		**90.97**

Table 4.29 Raw data: CMC 8 mg and NHS 4 mg, activation 60 minutes

Absorbance(nm) /Flow through volume	**frac1**	**frac2**	**frac3**	**frac4**	**unbound(bound) BSA(µg)**
test 1	0.428	0.525	0.434	0.435	
test 2	0.429	0.520	0.443	0.444	
test 3	0.454	0.531	0.423	0.455	
average	0.437	0.525	0.433	0.445	
standard deviation	0.01	0.01	0.01	0.01	
BSA per fraction(µg/ml)	-2.68	3.07	-2.92	-2.18	
total BSA(µg)per fraction	-4.02	4.60	-4.38	-3.27	4.60(55.49)
			%absorption		**92.34**

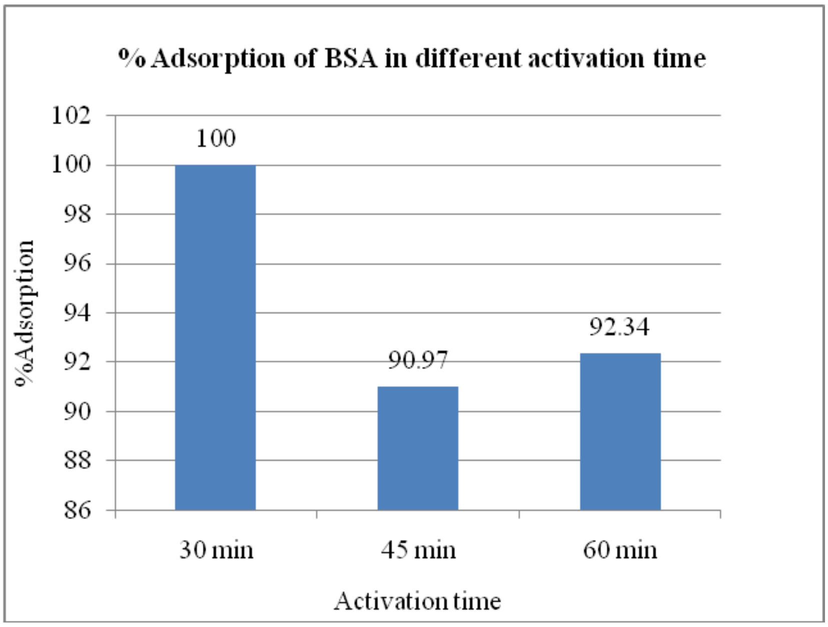


Figure 4.4 Comparison of BSAadsorption depending on activation time

4.6 Optimization of conjugation time

The effect of activation time on BSA adsorption onto magnetic nanoparticles in MES pH 6 with 8mg CMC and 4 mg NHS, 30 minutes activation and MES pH 4.7 was examined. Result are shown in Figure4.5.Percent BSA adsorption at difference conjugation time 60,90 and 120 minutes are 94%,89% and 91%.Percent BSA adsoption at 60 minutes is better than 90 and 120 minutes.

First experiment

Table 4.30 Raw data standard curve: BSA concentration (µg/ml)

Absorbance(nm) / BSA (µg/ml)	30	15	7.5	3.75	0
test 1	0.811	0.551	0.577	0.467	0.332
test 2	0.763	0.613	0.567	0.439	0.327
test 3	0.704	0.66	0.587	0.414	0.331
Average	0.759	0.608	0.577	0.440	0.330
standard deviation	0.05	0.05	0.01	0.03	0.00

Table 4.31 Raw data: Negative control, BSA passing the generic HGMS system without immobilization magnetic nanoparticles

Absorbance(nm) /Flow through volume	frac1	frac2	frac3	frac4	Total BSA(µg)
test 1	0.569	0.647	0.337	0.278	
test 2	0.556	0.602	0.332	0.278	
test 3	0.566	0.632	0.335	0.279	
Average	0.564	0.627	0.335	0.278	
standard deviation	0.01	0.02	0.00	0.00	
BSA(µg)per fraction(µg/ml)	12.66	12.89	0.29	-1.76	
total BSA(µg)per fraction	18.99	19.33	0.44	-2.64	32.12

Table 4.32 Raw data: Percent adsorption when conjugate 60 minutes

Absorbance(nm) /Flow through volume	**frac 1**	**frac 2**	**frac 3**	**frac4**	**unbound(bound) BSA(μg)**
test 1	0.304	0.391	0.406	0.357	
test 2	0.308	0.397	0.398	0.356	
test 3	0.294	0.375	0.395	0.353	
average	0.302	0.388	0.400	0.355	
standard deviation	0.01	0.01	0.01	0.00	
BSA per fraction(μg/ml)	-5.10	0.72	1.53	-1.48	
total BSA(μg)per fraction	-7.65	1.07	2.30	-2.22	3.37(33.75)
			%absorption		**90.92**

Table 4.33 Raw data: Percent adsorption when conjugate 90 minutes

Absorbance(nm) /Flow through volume	**frac1**	**frac2**	**frac3**	**frac4**	**unbound(bound) BSA(μg)**
test 1	0.297	0.398	0.418	0.357	
test 2	0.305	0.397	0.410	0.357	
test 3	0.284	0.395	0.399	0.351	
average	0.295	0.397	0.409	0.355	
standard deviation	0.01	0.00	0.01	0.00	
BSA per fraction(μg/ml)	-5.55	1.33	2.16	-1.50	
total BSA(μg)per fraction	-8.33	1.99	3.25	-2.25	5.24(30.88)
			%absorption		**85.49**

Table 4.34 Raw data: Percent adsorption when conjugate 120 minutes

Absorbance(nm) /Flow throug volume	**frac 1**	**frac 2**	**frac 3**	**frac4**	**unbound(bound) BSA(μg)**
test 1	0.511	0.420	0.407	0.333	
test 2	0.277	0.422	0.392	0.368	
test 3	0.306	0.403	0.403	0.360	
average	0.365	0.415	0.401	0.354	
standard deviation	0.13	0.01	0.01	0.02	
BSA per fraction(μg/ml)	-0.85	2.57	1.60	-1.59	
total BSA(μg)per fraction	-1.27	3.86	2.40	-2.39	6.25(29.86)
			%absorption		**82.69**

Second experiment

Table 4.35 Raw data standard curve: BSA concentration (µg/ml)

Absorbance(nm) / BSA (µg/ml)	30	15	7.5	3.75	0
test 1	0.695	0.577	0.471	0.401	0.320
test 2	0.698	0.577	0.456	0.411	0.315
test 3	0.701	0.551	0.459	0.404	0.315
average	0.698	0.568	0.462	0.405	0.317
standard deviation	0.00	0.02	0.01	0.01	0.00

Table 4.36 Raw data: Negative control, BSA passing the generic HGMS system without immobilization magnetic nanoparticles

Absorbance(nm) /Flow through volume	frac1	frac2	frac3	frac4	Total BSA(µg)
test 1	0.649	0.627	0.362	0.330	
test 2	0.619	0.609	0.364	0.335	
test 3	0.630	0.592	0.358	0.334	
Average	0.633	0.609	0.361	0.333	
standard deviation	0.01	0.02	0.00	0.00	
BSA(µg)per fraction(µg/ml)	22.48	16.88	2.06	0.69	
total BSA(µg)per fraction	**33.72**	**25.33**	**3.09**	**1.04**	**63.17**

Table 4.37 Raw data: Percent adsorption when conjugate 60 minutes

Absorbance(nm) /Flow throug volume	frac 1	frac 2	frac 3	frac4	unbound(bound) BSA(µg)
test 1	0.278	0.333	0.338	0.334	
test 2	0.305	0.337	0.370	0.359	
test 3	0.303	0.341	0.376	0.357	
Average	0.295	0.337	0.361	0.350	
standard deviation	0.02	0.00	0.02	0.01	
BSA per fraction(µg/ml)	-4.09	-0.81	1.11	0.22	
total BSA(µg)per fraction	-6.13	-1.21	1.67	0.33	1.99(61.18)
			%absorption		**96.04**

Table 4.38 Raw data: Percent adsorption when conjugate 90 minutes

Absorbance(nm) /Flow throug volume	**frac 1**	**frac 2**	**frac 3**	**frac4**	**unbound(bound) BSA(µg)**
test 1	0.297	0.376	0.394	0.366	
test 2	0.295	0.368	0.393	0.363	
test 3	0.299	0.367	0.393	0.302	
average	0.297	0.370	0.393	0.344	
standard deviation	0.00	0.00	0.00	0.04	
BSA per fraction(µg/ml)	-3.96	1.82	3.63	-0.28	
total BSA(µg)per fraction	-5.94	2.73	5.45	-0.42	8.18(55.00)
			%absorption		**87.05**

Table 4.39 Raw data: Percent adsorption when conjugate 120 minutes

Absorbance(nm) /Flow throug volume	**frac 1**	**frac 2**	**frac 3**	**frac4**	**unbound(bound) BSA(µg)**
test 1	0.302	0.420	0.405	0.365	
test 2	0.304	0.417	0.387	0.350	
test 3	0.304	0.396	0.040	0.354	
average	0.303	0.411	0.277	0.356	
standard deviation	0.00	0.01	0.21	0.01	
BSA per fraction(µg/ml)	-3.46	5.02	-5.52	0.72	
total BSA(µg)per fraction	-5.19	7.53	-8.28	1.07	6.25(29.86)
			%absorption		**82.69**

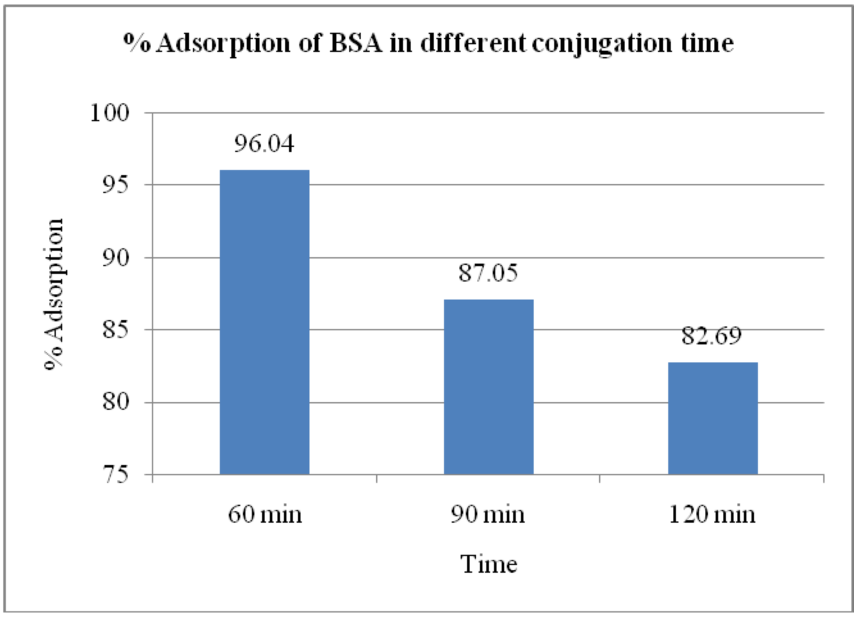


Figure 4.5 Comparison ofBSA adsorption depending on in different conjugation time

4.7 Optimization of buffers

The effect of MES and LIS buffer on BSA adsorption on magnetic nanoparticle in MES pH 6 with 8mg CMC and 4 mg NHS, 30 minutes activation and 60 minutes conjugation in MES pH 4.6was examined. Results are shown in Figure4.7. Percent adsorption on of MES is 100% and percent adsorption of LIS is 76%.MES buffer is better than LIS.

First experiment

Table 4.40 Raw data: Negative control, BSA passing the generic HGMS system without immobilization magnetic nanoparticles

Absorbance(nm) /Flow through volume	**frac1**	**frac2**	**frac3**	**frac4**	**Total BSA(µg)**
test 1	0.642	0.716	0.367	0.368	
test 2	0.651	0.686	0.364	0.360	
test 3	0.651	0.720	0.387	0.376	
average	0.648	0.707	0.373	0.368	
BSA(µg)per fraction(µg/ml)	14.43	17.54	-0.03	-0.28	
total BSA(µg)per fraction	**21.64**	**26.31**	**-0.05**	**-0.42**	**47.95**

Table 4.41 Raw data standard curve: BSA concentrationin LIS (µg/ml)

Absorbance(nm) / BSA (µg/ml)	**30**	**15**	**7.5**	**3.75**	**0**
test 1	0.667	0.536	0.437	0.371	0.340
test 2	0.668	0.565	0.438	0.372	0.318
test 3	0.683	0.528	0.448	0.358	0.305
average	0.673	0.543	0.441	0.367	0.321
standard deviation	0.01	0.02	0.01	0.01	0.02

Table 4.42 Raw data: Percent adsorption in LIS buffer(first exp)

Absorbance(nm) /Flow through volume	**frac1**	**frac2**	**frac3**	**frac4**	**unbound(bound) BSA(µg)**
test 1	0.307	0.316	0.304	0.306	
test 2	0.311	0.325	0.317	0.312	
test 3	0.305	0.306	0.283	0.304	
average	0.308	0.316	0.301	0.307	
standard deviation	0.00	0.01	0.01	0.00	
BSA per fraction(µg/ml)	-2.11	-1.45	-2.63	-2.14	
total BSA(µg)per fraction	-3.16	-2.17	-3.95	-3.21	0(47.95)
			%absorption		**100**

Table 4.43 Raw data standard curve: BSA concentrationin MES (µg/ml)

Absorbance(nm) / BSA (µg/ml)	**30**	**15**	**7.5**	**3.75**	**0**
test 1	0.713	0.551	0.430	0.379	0.313
test 2	0.697	0.541	0.448	0.371	0.306
test 3	0.701	0.523	0.432	0.370	0.292
average	0.704	0.538	0.437	0.373	0.304
standard deviation	0.01	0.01	0.01	0.00	0.01

Table 4.44 Raw data: Percent adsorption in MES buffer(first exp)

Absorbance(nm) /Flow through volume	**frac1**	**frac2**	**frac3**	**frac4**	**unbound(bound) BSA(µg)**
test 1	0.317	0.299	0.305	0.303	
test 2	0.313	0.299	0.294	0.298	
test 3	0.303	0.289	0.291	0.287	
average	0.311	0.296	0.297	0.296	
standard deviation	0.00	0.00	0.01	0.00	
BSA per fraction(µg/ml)	-0.85	-2.01	-1.93	-1.98	
total BSA(µg)per fraction	-1.28	-3.01	-2.90	-2.98	0(47.95)
			%absorption		**100**

Second Experiment

Table 4.45 Raw data: Percent adsorption in LIS buffer(µg/ml)

Absorbance(nm) / BSA (µg/ml)	**30**	**15**	**7.5**	**3.75**	**0**
test 1	0.906	0.711	0.545	0.457	0.370
test 2	0.885	0.673	0.540	0.444	0.364
test 3	0.763	0.652	0.533	0.425	0.350
Average	0.851	0.679	0.539	0.442	0.361
standard deviation	0.08	0.03	0.01	0.02	0.01

Table 4.46 Raw data: Percent adsorption in LIS buffer(Second exp)

Absorbance(nm) /Flow through volume	**frac1**	**frac2**	**frac3**	**frac4**	**unbound(bound) BSA(µg)**
test 1	0.461	0.5	0.381	0.375	
test 2	0.468	0.458	0.357	0.359	
test 3	0.494	0.464	0.372	0.378	
average	0.474	0.474	0.37	0.371	
standard deviation	0.02	0.02	0.01	0.01	
BSA per fraction(µg/ml)	5.24	5.22	-1.02	-0.98	
total BSA(µg)per fraction	7.86	7.83	-1.54	-1.48	15.68(32.27)
			%absorption		**67.30**

Third experiment

Table 4.47 Raw data: Percent adsorption in LIS buffer(Third exp.)

Absorbance(nm) /Flow through volume	**frac1**	**frac2**	**frac3**	**frac4**	**unbound(bound) BSA(µg)**
test 1	0.515	0.481	0.379	0.354	
test 2	0.510	0.462	0.356	0.372	
test 3	0.505	0.473	0.359	0.357	
average	0.510	0.472	0.365	0.361	
standard deviation	0.01	0.01	0.01	0.01	
BSA per fraction(µg/ml)	7.38	5.10	-1.34	-1.56	
total BSA(µg)per fraction	11.07	7.65	-2.02	-2.35	18.71(29.24)
			%absorption		**60.98**

Table 4.48 Raw data standard curve: BSA concentrationin MES (µg/ml)

Absorbance(nm) / BSA (µg/ml)	**30**	**15**	**7.5**	**3.75**	**0**
test 1	0.903	0.684	0.556	0.447	0.351
test 2	0.959	0.704	0.554	0.459	0.344
test 3	0.895	0.664	0.531	0.426	0.336
average	0.919	0.684	0.547	0.444	0.344
standard deviation	0.03	0.02	0.01	0.02	0.01

Table 4.49 Raw data: Percent adsorption in MES buffer(Second exp.).

Absorbance(nm) /Flow throug volume	**frac 1**	**frac 2**	**frac 3**	**frac4**	**unbound(bound) BSA(µg)**
test 1	0.352	0.339	0.328	0.316	
test 2	0.366	0.340	0.354	0.331	
test 3	0.362	0.350	0.351	0.325	
average	0.360	0.343	0.344	0.324	
standard deviation	0.01	0.01	0.01	0.01	
BSA per fraction(µg/ml)	-0.70	-1.59	-1.52	-2.59	
total BSA(µg)per fraction	-1.05	-2.39	-2.28	-3.88	0(47.95)
			%absorption		**100**

Table 4.50 Raw data: Percent adsorption in MES buffer(Third exp.)

Absorbance(nm) /Flow throug volume	**frac 1**	**frac 2**	**frac 3**	**frac4**	**unbound(bound) BSA(µg)**
test 1	0.365	0.344	0.355	0.321	
test 2	0.351	0.360	0.337	0.325	
test 3	0.372	0.328	0.372	0.322	
average	0.363	0.344	0.355	0.323	
standard deviation	0.01	0.02	0.02	0.00	
BSA per fraction(µg/ml)	-0.56	-1.54	-0.98	-2.66	
total BSA(µg)per fraction	-0.84	-2.31	-1.47	-3.99	0(47.95)
			%absorption		**100**

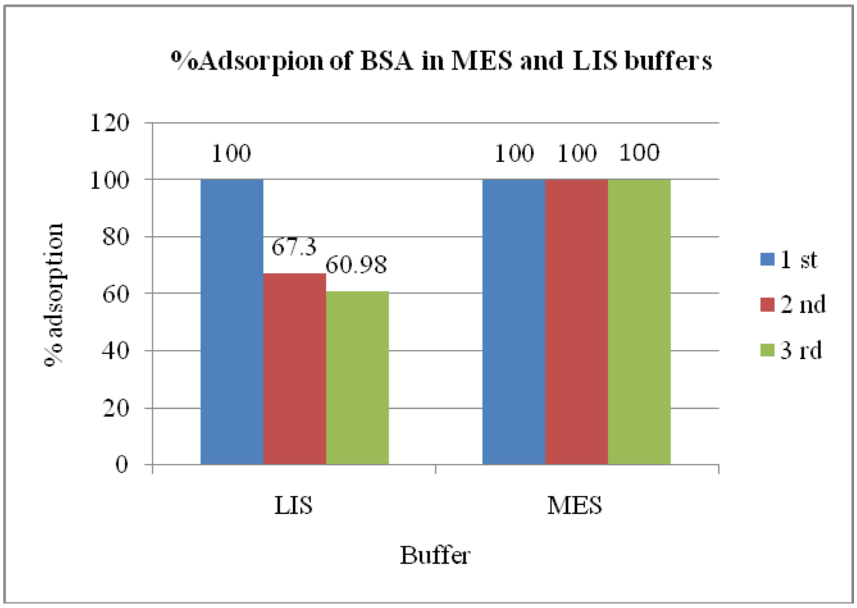


Figure 4.6 Comparison of BSA adsorption in MES and LIS buffers

CHAPTER V
DISCUSSION

5.1 Selection buffers for conjugation

Comparison of the effect of MES and PBS buffers onto BSA adsorption on magnetic nanoparticlesshowed that MES was a better conjugationbuffer than PBS. MES buffer is a non ionic buffer that showed to be more appropriatefor binding of BSA to nanoparticles. It has been described previously thationic strength negatively affects binding of nanoparticlesand BSA. The effect of salt concentration and charge in the buffer, can block nanoparticles surface by forming ion shield leading to reduction of protein adsorption.(Z.G.Peng et.)

5.2 Optimized pH of the activation buffer

Both MES and PBS are activation buffers for the magnetic nanoparticles. Their effects alter depending on the range of pH. Changing functional group on the nanoparticles can alter immobilization of the BSA. Result showed that MES at pH 6 provides bindingwith BSA better than PBS buffer. Acidic pH was optimal for the interaction between nanoparticle and protein.

5.3 Optimized pH for conjugation processes

MES pH 4.7 is the best condition for conjugation of BSA withnanoparticles. The maximum adsorption of BSA occurred at pH 4.64, which is close to isoeletric point of BSA, pI = 4.7. At pH 4.7, nanoparticles had positive charge while BSA had zero net surface charge which was appropriate form to bind with nanoparticles. At pH higher than 4.7, both nanoparticles and BSA carrypositive charge. This causes repulsionand inhibits adsorption of BSA onto nanoparticles.

5.4 Optimized concentration of CMC and NHS for activation

The chemicell protocol recommends a working concentration of CMC of 10 mg. This study varied concentration of CMC at lower and higher concentration to validate the effect of concentration on immobilization of BSA onto nanoparticles. The results showed that the best concentration of CMC was 8 mg and for NHS was 4 mg,which were lower than the recommended concentration.

5.5 Optimized activation time of activation process

The best duration for activation ofnanopaticles was 30 minute. The study showed that increased duration had no possitive effect on absorption of BSA ontonaoparticles.

5.6 Optimized conjugation time of BSA conjugation process

Chemicellprotocol recommends 120 minutes for conjugate BSA onto nanoparticles. In this study we showed that at 60 minutes BSA could absorb on to nanoparticles greater than at 90 or120 minutes. This result reduced the time of the process.

5.7 Optimized buffers

When compared the effect of MES and LIS buffers on BSA adsorption on magnetic nanoparticles, the result showed MES wasa better conjugation buffer than LIS.This could be that the MES buffer is non ionic solution which appropriate for the binding reaction. MES reagent isquite expensive, thereforefurtherinvestigation to find alternative buffers to replace it is warranted.

CHAPTER VI
CONCLUSION

The best condition to activate and conjugate BSA on to nanoparticles, were established by using MES at pH 6 with 8 mg of CMC and 4 mg of NHS and under activation time of 30 minutes. After activation then change buffer to MES at pH 4.7 for conjugation and added BSA to conjugate for 60 minutes. This protocol was modified from Chemicell protocol which was easy to conjugate BSA and other protein onto nanoparticles. These nanoparticles could be used to purify target cells by magnetic separation method. These nanoparticles can replace commercial kit and reduce cost.

REFFERENCES

Abudiab, T., and Beitle, R. R., Jr. (1998). Preparation of magnetic immobilized metal affinity separation media and its use in the isolation of proteins. *J Chromatogr A***795**(2)**,** 211-7.

AI-Abdulla, I.H., Mellor, G.W., Childerstone, M.S., Sidki, A.M. and Smith, D.S., Comparison of three differentactivation methods for coupling antibodies to magnetisablecellulose particles. *J. Immunol*. Methods, 122 (1989) 253- 258.

Avrameas, S. and Guilbert, B., Enzyme-immunoassay for measurement of antigens using peroxidase conjugates. *Bioehimie*, 54 (1972) 837-842.Avezzano, E. A., Fleischer, D. E., Merida, M. A., and Anderson, D. L. (1990). Giant fibrovascular polyps of the esophagus. *Am J Gastroenterol***85**(3)**,** 299-302.

Bonnemann,H.;Brijoux,W.;Brinkmann,R.;Dinjus,E.;Fretzen,R.;Joussen,T.;Korall,B.*J. Mol.Catal*. 1992, 74, 323.

Bonnemann,H.;Brijoux,W.;Brinkmann,R.;Fretzen,R.;Joussen,T.;Koppler,R.;Korall,B.; Neiteler,P.;Richter,J. *J.Mol.Catal*.1994, 86, 129.

Boorsma, D.M. and Streefkerk, J.G., Periodate or glutaraldehyde for preparing peroxidase conjugates?. *J. Immunol. Methods*, 30 (1979) 245-255.

Dekker, R. F. (1989). Immobilization of a lactase onto a magnetic support by covalent attachment to polyethyleneimine-glutaraldehyde-activated magnetite. *Appl Biochem Biotechnol***22**(3)**,** 289-310.

E R Flyn and H C Bryant (2005). A biomagnetic system for in vivo cancer imaging *Phys Med Biol* (50)6, 1273-1293

Hancock, J. P., and Kemshead, J. T. (1993). A rapid and highly selective approach to cell separations using an immunomagnetic colloid. *J Immunol Methods***164**(1)**,** 51-60.

Hu, T., and Su, Z. (2003). A solid phase adsorption method for preparation of bovine serum albumin-bovine hemoglobin conjugate. *J Biotechnol***100**(3)**,** 267-75.

Huang, B. X., Kim, H. Y., and Dass, C. (2004). Probing three-dimensional structure of bovine serum albumin by chemical cross-linking and mass spectrometry. *J Am Soc Mass Spectrom***15**(8)**,** 1237-47.

Juliane Issle and Uwe Hartmann. 2007. Scanning foece microscopy on spatially and temporally varying magnetic substrates for cell cultivation. Journal of physics:conference series 61,487-491.

Khng, H. P., Cunliffe, D., Davies, S., Turner, N. A., and Vulfson, E. N. (1998). The synthesis of sub-micron magnetic particles and their use for preparative purification of proteins. *Biotechnol Bioeng***60**(4)**,** 419-24.

Levison, P. R., Badger, S. E., Dennis, J., Hathi, P., Davies, M. J., Bruce, I. J., and Schimkat, D. (1998). Recent developments of magnetic beads for use in nucleic acid purification. *J Chromatogr A***816**(1)**,** 107-11.

Li, J. J., Muralikrishnan, S., Ng, C. T., Yung, L. Y., and Bay, B. H. Nanoparticle-induced pulmonary toxicity. *Exp Biol Med (Maywood)***235**(9)**,** 1025-33.

Lin, J., Qiu, S., Lewis, K., and Klibanov, A. M. (2002). Bactericidal properties of flatsurfaces and nanoparticles derivatized with alkylated polyethylenimines. *Biotechnol Prog***18**(5)**,** 1082-6.

Molday, R.S., Dreyer, W.J., Rembaum, A. and Yen, S.P.S., New Immunolatex spheres: visual markers of antigens on lymphocytes for scanning electron microscopy. *J. Cell. Biol*., 64, (1975) 75-88.

Olde Damink, L.H.H., Dijkstra, P.J., van Luyn, M.J.A., van Wachem, P.B., Nieuwenhuis, P. and Feijen, J., In vitro degradation of dermal sheep collagen cross-linked using a water-soluble carbodiimide. Biomaterials, 17 (1996) 679 -684.

Sano, S., Kato, K. and Ikada, Y., Introduction of functional groups onto the surface of polyethylene for protein immo-bilization. *Biomaterials*, 14 (1993) 817 - 822.

Tzu-Hsien and Wang-Chien Lee (2003), Immobilization of Proteins on Magnetic Nanoparticles.Biotechnology and Bioprocess Engineering, 8: 263-267

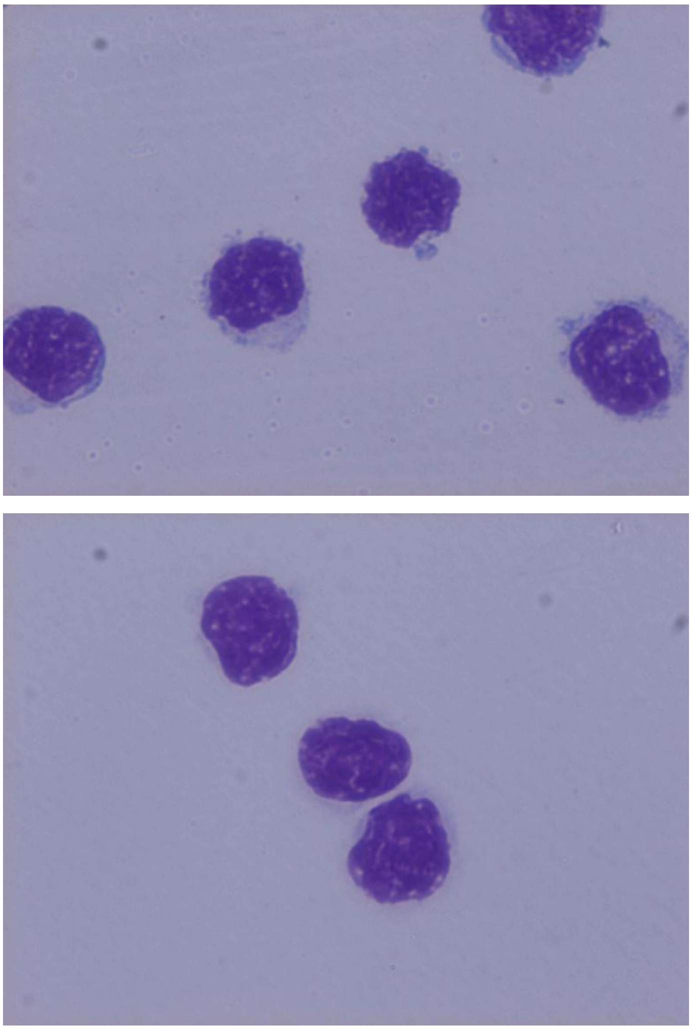
Vermeersch, H. and Remon, J.P., Immunogenicity of poly-D- lysine, a potential polymeric drug carrier. *J. Controlled Release*, 32 (1994) 225-229.

Yun Jv, Baoxin Li and Rui Cao (2010),Positively-charged gold nanoparticles as peroxidiase mimic and their application in hydrogen peroxide and glucose detection ,*Chem. Commun.*, 46, 8017-8019

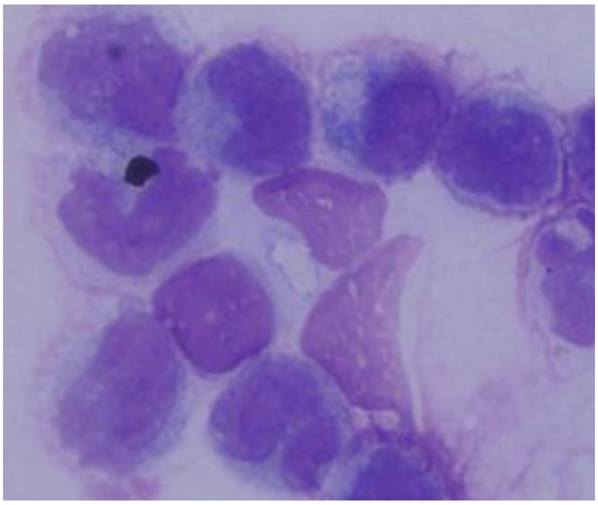
APPENDIX

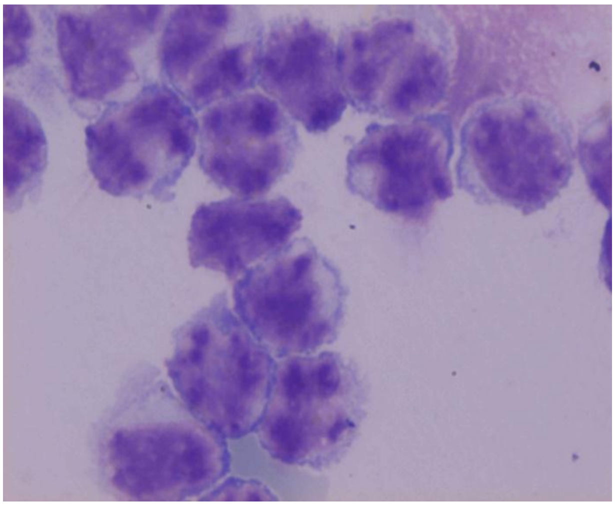
An optimized protocol for conjugation of BSA onto 50nm paramagnetic particles is presented. In a further step, it is shown that the present protocol can be adapted to conjugation of anti-human CD14 and CD3 monoclonal antibody to 50 nm paramagnetic nanoparticles, and that the conjugate can efficiently isolate CD14-positive mononuclear cells up to more than 80% purity on a generic high gradient magnetic separation system. Insights derived from this study can be used for optimization of conjugation of other antibodies and proteins to magnetic nanoparticles.

CD3 positive cells with 50 nm HGMS beads (photo taken at 100X)



1- Isolation of CD14 positive cells with 50 nm HGMS beads (photo taken at 100X)





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