Synthesis of Uniform Sized Iron-oxide Nanoparticles with PEG modification for Enhancement of Protein Immobilization

Pei-Ru Chen, Yun-Ju Chuang

Abstract— In this research we propose the utilization of iron oxide nanoparticles for the enhancement of protein binding efficiency to SAMs functionalized surface. Uniform sized iron oxide nanoparticles with diameter of 5 and 10 nm have been successfully fabricated by using micelle method in solvent phase, and the surfaces were modified by PEG-COOH functional group for dispersion in water and conjugation with protein. Anti-rabbit IgG-cy3 was successfully conjugated on the nanoparticle surfaces after the activation of PEG-COOH group. Binding efficiency between iron oxide-protein conjugation and self-assembly molecules (SAM) by external magnetic field with different AC frequencies was tested and demonstrated a 374% enhancement.

Index Terms— Iron oxide, Nanoparticles, PEG, Self-assembly molecules.

1 Introduction

Nanosized particles have physical and chemical properties of neither the atom nor the bulk counterparts [1], for example the supermagnetic property in magnetic nano particles. Many of them have been widely employed to biochemistry applications [2][3]. Among them, Fe_3O_4 nanoparticles are very promising in the applications as protein binding reporters or medical image enhancement agents. However, to Synthesis Fe_3O_4 nanoparticle by wet chemical co-precipitation method can not well control nanoparticle sizes [4]. Therefore, micelle method was developed in a solvent phase to restrict nanoparticle growth for nanoparticle size control and resolve aggregation issue during nanoparticle formation [5]. In this research, we further modified the hydrophobic surface of nanoparticle with hydrophilic functional groups, including COOH, on the nanoparticle surface [6] [7] for their well dispersion in water phase. Therefore, antibodies and antigens can be immobilized on the particle surfaces after the activation of COOH functional group [8] in solutions. The enhancement of binding efficiency between magnetic -nanoparticle conjugated proteins and SAMs (Self Assembled Monlayers) functionalized surface is carried out by agitation with external magnetic field during protein-surface interaction.

2 Experiment

2.1 Synthesis of Iron Oxide Nanoparticles

The synthesis of uniform iron oxide nanoparticles was carried out by the micelle method in solvent base [5]. The mixture of 2 mM Fe(acac)3, 10 mM 1,2-hexadecandiol, 6 mM oleic acid, 6 mM oleylamine, and 20 mL of benzyl ether were heated at 110 ℃ for 1 h, with N2 flow. was raised to 205 ℃ with a heating rate of 8 ℃ /min and kept at 205 ℃ for 30min to prevent abrupt boiling. The mixture was then heated to the reflux temperature of 297 ℃, and kept refluxing for 30min for the formation of iron oxide nanoparticles. The solution mixture was cooled to the room temperature, and the nanoparticles were precipitated out by adding ethanol and DI-water.5nm nanoparticles were obtained and dissolved in hexane.

For the fabrication of iron-oxide nanoparticles with diameter of 8 nm, the reflux process was extended to 2 hours. Different approach to obtain larger nanoproticles can be accomplished by repeat the whole process and each of them can enlarge the particle size by 2.5 nm. Maximum nanoparticle size of 10nm can be obtained by utilizing this cycling process.

2.2 PEG-COOH Functional Group Modification

PE-PEG-COOH is used to modify Fe3O4 surface to obtain hydrophilic property for dispersion in water phase. 20mg Fe3O4 and 10mg PE-PEG-COOH 2000 were dissolved in 5ml chloroform and heated at 60℃ for 10 min. Then the solvent was evacuated out by vacuum for overnight. Afetr that, 60℃ DI-water was then added to the product and the surface modified nanoparticles were slowly dissolved in water. Finally, followed by centrifugation and purification, Fe3O4 nanoparticles were obtained in water phase.

2.3 Antibody Conjugation

The surface modified Fe3O4 nanoparticles were prepared in 100mL PBS buffer solution with the addition of 20mM EDC and 5mM Sulfo-NHS for 1 hour for functional group activation. This solution was centrifuged and washed by PBS buffer for three times and then 0.1mg/ml anti-rabbit IgG-cy3 was added and incubate 1 hr for protein conjugation with nanoparticles. Protein-conjugated nanoparticles were centrifuged and washed three times by PBS buffer and dispersed in PBS buffer solution. The nanoparticle synthesis and surface modification processes are shown in Fig. 1.

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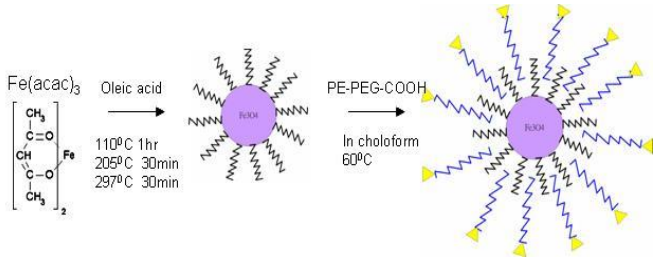

Fig. 1. The process flow for Fe_3O_4 nanoparticle fabrication and surface functional group modification.

2.4 Affinity Test of Antibody on Fe3O4 Nanoparticle

A solution of 20ul(0.002mg/ml) Fe_3O_4-antibody conjugate was dropped on a glass slide, and then 5500 Gauss magnetic field was applied to attract the Fe_3O_4-antibody conjugate for 1 min. After PBS buffer solution washing and drying process, the fluorescence intensity of the attracted nanoparticles was detected for the determination of conjugation efficiency.

2.5 Magnetic Induced Binding Efficiency Enhancement between Iron Oxide-protein Conjugation and Self-assembly Molecules

Glass slide was immersed in 0.5% APTS (3-amino - propyltrimethoxysilane) alcohol solution for 1.5 hours for SAMs self-assembly, and annealed at 80°C for 2 hours. Then 20ul (0.002mg/ml) Fe_3O_4-antibody conjugate was directly dropped on the SAMs coated glass slides. Two modes of agitation were employed, including the utilization of a permanent magnet of 100 Gauss underneath the drop for nanoparticle concentration on the surface, and an electrical magnet field upto 200 Gauss arranged on the top for inducing an alternating magnetic filed for the enhancement of particle bumping frequency onto the surface, as shown in Fig. 2.

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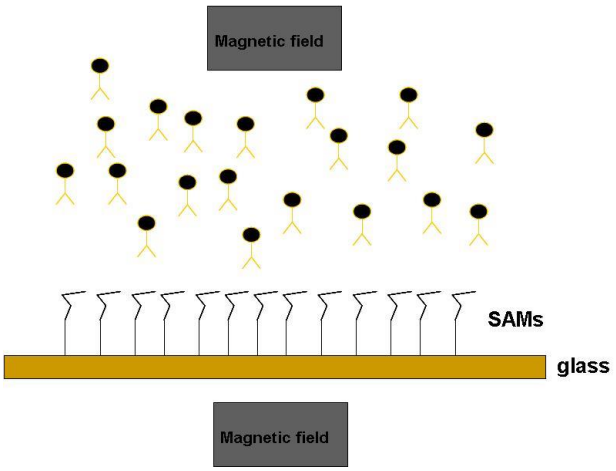

Fig. 2. Binding efficiency enhancement between Fe_3O_4-nanoparticles-protein conjugate and self-assembly monolayers (SAMs) induced by the agitation of external magnetic field with different AC frequencies.

3 Results

Synthesized Fe3O4 nanoparticles under two different approaches are observed by TEM and the images are shown in Fig. 3. From these TEM images we can see the particle size of the reflux process is less uniform than that of the repeating process. The average particles size is 7.52±1.26 nm in diameter by the reflux process after 2 hours enlargement process, while it is 10.08±0.438 nm for the repeating process.

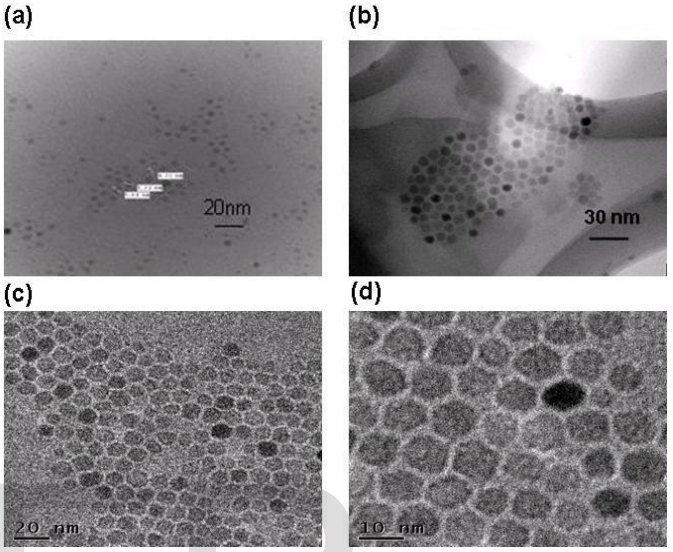

Fig. 3. The TEM image of Fe_3O_4 nanoparticles (a)5nm Fe_3O_4 nanoparticles (b) 8nm Fe_3O_4 nanoparticle and the reflux process was extended to 2 hours (c)(d) 10nm Fe_3O_4 and this approach to obtain larger nanoproticles can be accomplished by repeat the whole process.

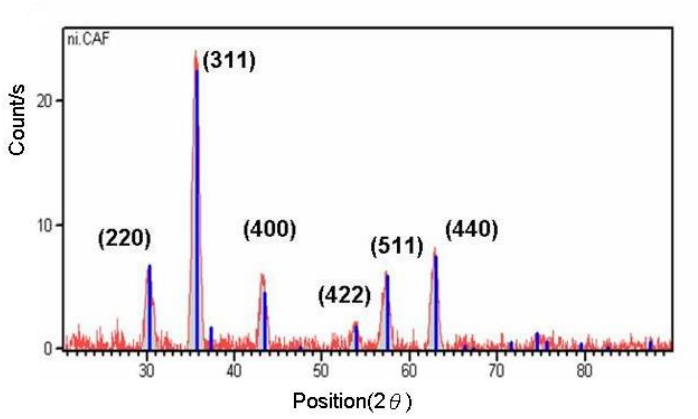

Fig.4. The XRD pattern of magnetic nanoparticles.

The XRD pattern of the magnetic nanoparticles was carried out to check the chemical composition of the Fe3O4 nanoparticles. Fig. 4 shows the characteristic peaks of XRD pattern of the nanoparticles. Those peaks demonstrated the diffraction angles of 30.1°, 35.48°, 43.36°, 57.2° and 62.82°, respectively, representing the diffraction surfaces of the nanoparticle crystal are in the positions of peak (220), peak (311), peak (400), peak

(422) and peak (511), an indication of iron oxide material.As demonstrated in this document, the numbering for sections upper case Arabic numerals, then upper case Arabic numerals, separated by periods. Initial paragraphs after the section title are not indented. Only the initial, introductory paragraph has a drop cap.

Magnetic measurements of the Fe_3O_4 nanoparticles indicate that the iron oxide particles are superparamagnetic at room temperature, as shown in Fig. 5, meaning that the thermal energy can overcome the anisotropy energy barrier of a single particle, and the net magnetization of the particle assemblies in the absence of an external field is zero. Under a large external field, the magnetization of the particles aligns with the field direction and reaches its saturation value (saturation magnetization, *ós*). For example, *ós* for 10 nm Fe_3O_4 nanoparticles is 51.1 emu/g.

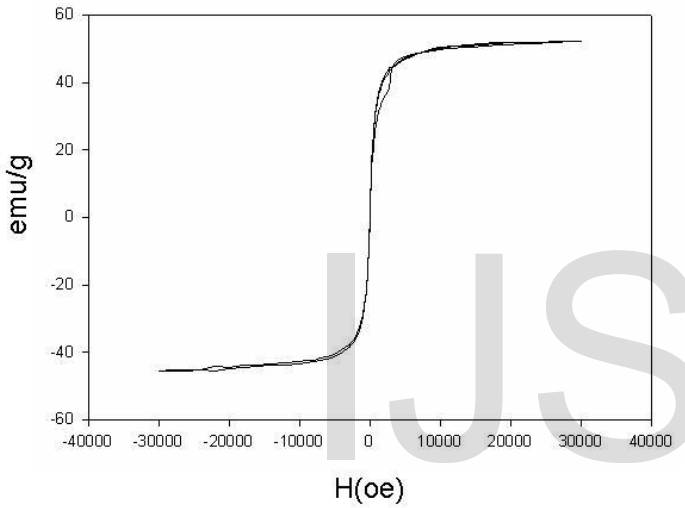

Fig.5. Hysteresis loops of the 10nm Fe_3O_4 nanoparticles measured by SQUID at 300K.

X-ray photoelectron spectra of the Fe_3O_4 nanoparticles are shown in Fig. 6. The peaks of the C_{1s}, O_{2p}, N_{1s}, and Fe_{2p} observed in Fig. 6a collectively indicate the organic coating on the surface of the Fe_3O_4 nanoparticles. Detail of the XPS (Fig. 6b) shows binding energies of 709.1 and 722.5 eV, corresponding to $Fe_{2p}{}^{3/2}$ and $Fe_{2p}{}^{1/2}$, respectively, in good agreement with the values reported for Fe_3O_4 in the handbook.

Then TGA (Thermal Gravimetric Analysis) was used to depict the coating material of the Fe_3O_4 nanoparticles. As Shown in Fig. 7, the first weight loss position representing the damage of the hydrophobic layer of oleic acid while the second one reveals the damage of the hydrophilic layer of PEG molecule. This provides the evidence of the existence of double layers coated on Fe_3O_4 nanoparticles.

Two kinds of PEG were employed, including methyl-PEO12-NHS Ester (MW=685) and PE-PEG-COOH 2000(MW=2000). Compared to iron oxide nanoparticles modified with PEG of different molecular weight in water phase, the PEG with larger molecular weight disperses better in water phase because it has longer hydrophilic pole.

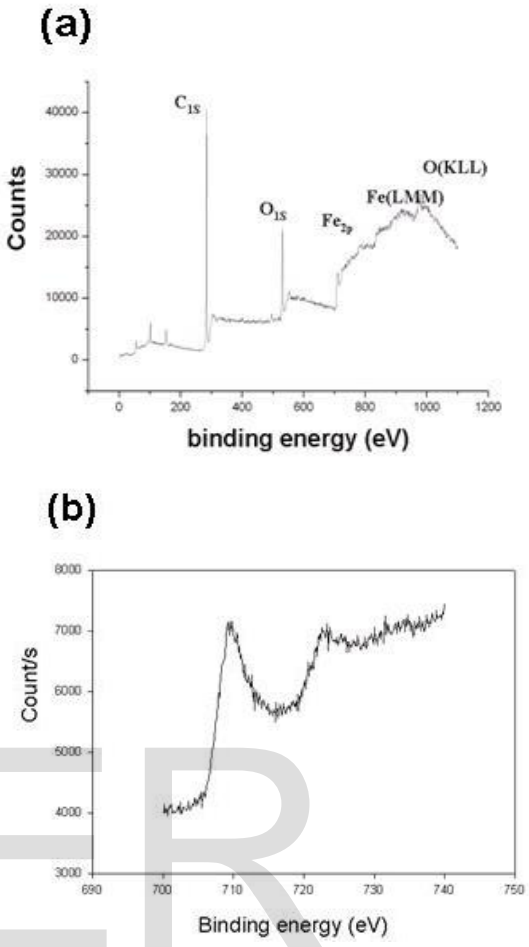

Fig.6 XPS of the synthesized magnetite nanoparticles, revealing the iron state at Fe 2p1/2 and Fe 2p3/2.

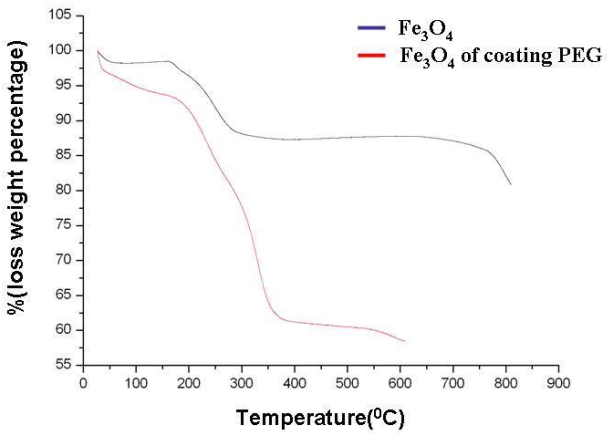

Fig. 7. The TGA pattern shows the hydrophobic layer of oleic acid and hydrophilic layer of PEG molecule.

Magnetic nanoparticles conjugated with antibody and Fluorescence was observed by the fluorescence scanner. Fig. 8

showed the images before and after the UV 532nm wavelength excitation on the nanoparticles by the fluorescence scanner. We must prove the antibodies and Fe_3O_4 nanoparticles were really conjugated together. So, we used a magnet to apply an external magnetic field. We can ensure that the antibodies exist around the magnetic nanoparticles. First the magnetic particles are dissolved in Di-water and drop on the slide glass. We can see the black particle aggregate by 5500 Gauss magnetic field before the UV excitation. After UV excitation the antibody (anti-rabbit-IgG-cy3) was emitted green fluorescence and aggregated in the same position. It attested to the antibody be conjugated with Fe_3O_4 nanoparticles.

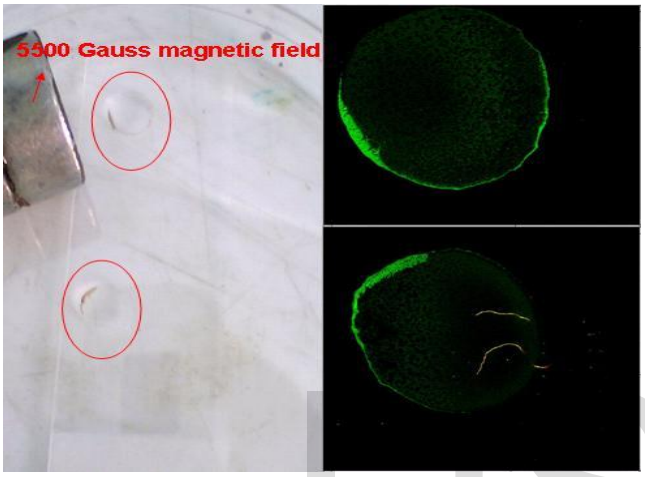

Fig. 8. Affinity test of antibody binding to Fe_3O_4 nanoparticles.

Then we wanted to enhance Binding efficiency between iron oxide-protein conjugation and self-assembly molecules (SAM). First, a magnetic field of 5500 Gauss was applied to attract the drip for 1 min to concentrate the antibody, then we used two electromagnets to attract the top and below of drip and changed the applied voltage frequency of the top electromagnet. It made the iron oxide-protein bob in the drip. The reason was increasing collision frequency, Fg.10 shows chart of changing voltage frequency of the top electromagnet between binding efficiency. We can see the parameter 750Hz has the best binding efficiency between iron oxide-protein conjugation and SAM (APTS). It can promote binding efficiency to 374 % (operated form/control form*100%). We guess the applied voltage frequency be to near the nature frequency of iron oxide-protein in PBS buffer. In this way, we used BSA (bovine serum albumin) to break functional group activation of iron oxide. It avoided the wrong binding between iron oxide and SAM (APTS).

We also conducted experiments to compare the binding efficiency with and without iron oxide nanoparticles under different agitation time with 750Hz external magnetic field and the results was shown in Fig.11. The experimental results showed the fluoresce intensity of agitating simple 30min approach no Fe_3O_4 form. It proved the available method to shorten the time between antibody (anti-rabbit-IgG-cy3) and self-assembly molecules.

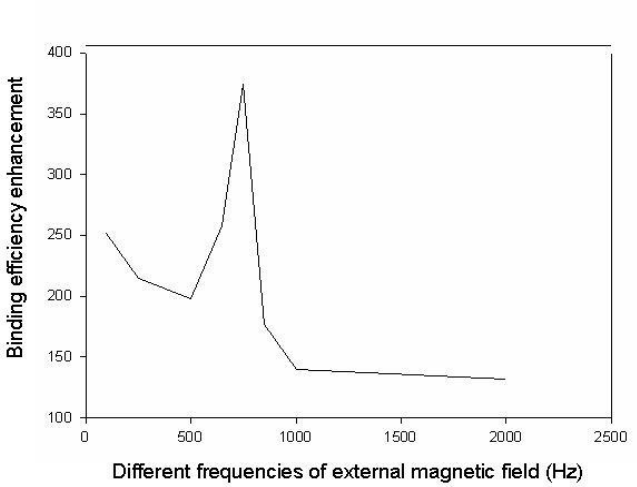

Fig. 9. Binding efficiency enhancement of iron oxide–protein to SAMs by the agitation of external magnetic field at different frequencies.

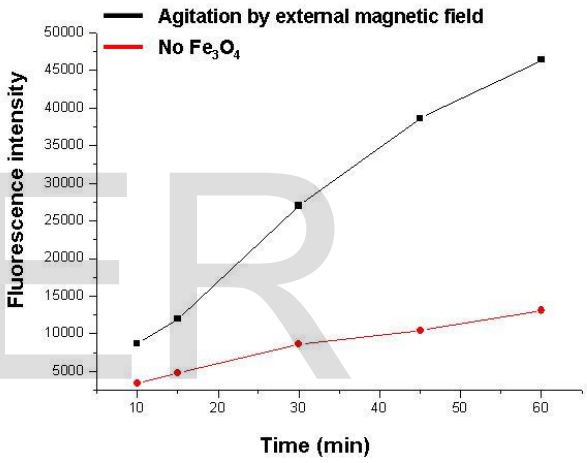

Fig. 11. Compare to agitate time by external magnetic field.

4 Conclusions

Iron oxide nanoparticles size distribution is uniform with a less than 10% variation. the surfaces were modified by PEG-COOH functional group for dispersion in water and conjugation with antibody. Binding efficiency between iron oxide-protein conjugation and self-assembly molecules (SAM) by external magnetic field with different voltage frequency was tested and demonstrated a 374% enhancement. The fluoresce intensity of agitating simple 30min approach no Fe_3O_4 form.

References

[1] T. Hanemann, D. V. Szabo, "Polymer-Nanoparticle Composites: From Synthesis to Modern Applications," *Materials*, vol. 3, no. 6, pp. 3468-3517, 2010.

[2] S. Bucak , D. A. Jones, P. E. Laibinis, T. A. Hatton, "Protein Separations Using Colloidal Magnetic Nanoparticles," *Biotechnology Progress*, vol. 19, no. 2, pp. 477-484, 2008.

[3] N. Nagatani, R. Tanaka, T. Yuhi, T. Endo, "Gold nanoparticle-based novel enhancement method for the development of highly sensitive immunochromatographic test strips," *Science and Technology of Ad-*

vanced Materials, vol. 7, o. 3, pp. 270-275, 2006.

[4] D. K. Kim, Y. Zhang, W. Volt, K. V. Rao, M. Muhammed, "Synthesis and characterization of surfactant-coated superparamagnetic monodispersed iron oxide nanoparticles," *Magnetic Materials*, vol. 225, no.1-2, pp. 30-36, 2001.

[5] S. Sun, H. Aeng, " Size-Controlled Synthesis of Magnetite Nanoparticles," *Journal of The American Chemical Society,* vol. 124, no. 28, pp. 8204-8205, 2002.

[6] S. Zalipsky, "Functionalized Poly(ethylene glycols) for Preparation of Biologically Relevant Conjugates," *Bioconjugate Chemistry*, vol. 6, no.2, pp. 150-165, 1995.

[7] M. J. Roberts, M. D. Bentley, J. M. Harris, " Chemistry for peptide and protein PEGylation, "*Advanced Drug Delivery Reviews*, vol. 54, no. 4, pp. 459-476, 2002.

[8] L. Norbs, F. Buchegger, R. Gurny, E. Allemann, "Poly(lactic acid) nanoparticles labeled with biologically active Neutravidin™ for active targeting," *European Journal of Pharmaceutics and Biopharmaceutics*, vol. 58, no. 3, pp. 483-490, 2004.