

Preparation and Characterization of Chitosan-coated Fe₃O₄ Nanoparticles using Ex-Situ Co-Precipitation Method and Tripolyphosphate/Sulphate as Dual Crosslinkers

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Abstract. The unique properties of nanomaterial provide great opportunities to develop in several fields. Several types of nanoparticles have been proven beneficial for biomedical and therapeutic agent development. Particularly for clinical use, nanoparticles must be biocompatible and non-toxic. Iron oxide nanoparticles consist of either magnetite (Fe₃O₄) or maghemite (γ -Fe₂O₃) was eligible to use for in vivo application including targeting drug delivery. Due to their distinct properties, these nanoparticles could be directed to the specific target under external magnetic field. However, nanoparticles have a tendency to form agglomeration. Therefore, surface modification was required to reduce the agglomeration. In this study, nanoparticles of Fe₃O₄ were produced and coated by biomaterial (chitosan) using ex-situ co-precipitation method. Nanoparticles of Fe₃O₄ were synthesized by adding ammonia water into iron ferric and ferrous solution. Synthesis process of Fe₃O₄ was conducted prior to adding chitosan. Chitosan was then cross-linked by a combination of tripolyphosphate/sulphate. The different composition ratio and crosslinking time provide the different physical and magnetic characteristics of nanoparticles. Particle and crystallite size was determined by using Scanning Electron Microscopy (SEM) and X-Ray Diffraction (XRD) respectively, whereas magnetic characteristic was determined by Electron Spin Resonance (ESR). The results showed that the ratio enhancement between chitosan: Fe₃O₄ increase the particle size, while decreased the crystallite size. Morphology and particle size were influenced by the ratio of crosslinkers. It was found that the higher tripolyphosphate content was contributed to the small size and more spherical morphology. In addition, the influence of crosslinking time toward crystallite size was determined by altering stirring time. The longer duration of crosslinking time, provide the larger crystallite size of chitosan-Fe₃O₄. There was an interesting correlation between particle size and amount of Fe₃O₄ phase with ESR curve.

1.Introduction

Nanoparticles become an interesting topic to further investigate due to their unique characteristics. During the last decade, several studies show that nanoparticles are capable to be employed at cellular and molecular level. Nanoparticles are developed on numerous application fields. Nanoparticles can be synthesized by various methods which include microemulsion, thermal decomposition, hydrothermal synthesis, sonochemical, and coprecipitation. Coprecipitation method is mostly used due to low cost, high yield product, not requiring hazardous solvent and high temperature. Nanoparticles can be synthesized from either organic (polymer) or inorganic (metal) matter [1-3].

Magnetic nanoparticles are excellent for targeted drug delivery system. This material could be pointed to the specific target under control of the external magnetic field. However, for the biomedical



purpose, nanoparticles must be biocompatible, biodegradable, and less toxic. Among several magnetic nanoparticles, only Fe_3O_4 and Fe_2O_3 possessing characteristics in vivo application [3].

Magnetic nanoparticles have less stability and tend to form agglomeration due to its small particle size and a large surface to volume ratio. It was also easily oxidized in air. Therefore, surface modification is needed to stabilize magnetic nanoparticle and avoid oxidation processes. Moreover, the surface modifier might offer additional functional group capable to attach another molecule such as protein and drug [2].

Chitosan is a product of deacetylated chitin which has biocompatible and biodegradable characteristics. Therefore, it can be used along with magnetic (Fe_3O_4) nanoparticle in drug delivery system. Chitosan has good stability in the neutral state, however less stable under pH 6.5. Chitosan nanoparticle can be manufactured through additional of crosslinker [4,5]. Crosslinker has also contributed to sustaining chitosan stability in acid condition. The pH of a tumor cell is about 5.5, without crosslinker agent chitosan will degraded. Therefore, drug release will rapidly occur. The existence of crosslinking agent that attached on chitosan- Fe_3O_4 nanoparticle structure utilized to inhibit chitosan degradation as well as controlling drug release from [6,7].

Tripolyphosphate (TPP) is a type of harmless anionic crosslinker. TPP can interact by inter- and intramolecular with chitosan to provide better spherical morphology. According to the previous report by Shu and Zhu (2002), the combination of dual crosslinker consisting of TPP/sulphate and TPP/citrate can give smaller particle size with spherical morphology [8]. Thereby as mentioned above, in this study nanoparticle chitosan- Fe_3O_4 will be conducted by using a combination of both crosslinker agents, TPP, and sulphate, for drug delivery system candidate.

Physical properties and magnetic characteristic of nanoparticles could be affected by various factors. Those factors include the composition of materials, crosslinker agents, pH, the existence of oxygen, temperature, and duration of reaction [8,9]. In this research, some parameters affecting particle size, crystallite size, and magnetic characteristic of nanoparticles were observed. Among the different parameters studied are a chitosan- Fe_3O_4 ratio, TPP: sulphate ratio, and crosslinking time. Nanoparticles are then characterized by using X-Ray Diffraction (XRD), Scanning Electron Microscopy-Energy Dispersive X-Ray Spectroscopy (SEM-EDX), and Electron Spin Resonance (ESR).

2. Material and Method

2.1 Chemicals

Chemicals consisting of ferric chloride ($\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$, 99%) , ammonia (NH_3 , 25% (v/v) in H_2O), sodium sulphate (Na_2SO_4 , $\geq 99\%$), and acetic acid glacial (CH_3COOH , 100% were purchased from Merck, Indonesia); ferrous chloride ($\text{FeCl}_2 \cdot 4\text{H}_2\text{O}$, 98%), chitosan (LMW, 75-85 % deacetylated) and sodium tripolyphosphate ($\text{Na}_5\text{P}_3\text{O}_{10}$, 85%) were purchased from Sigma-Aldrich, Singapore.

2.2 Synthesis of magnetic iron oxide nanoparticles

Magnetite iron oxide (Fe_3O_4) nanoparticles without chitosan coating (bare Fe_3O_4) were synthesized by the co-precipitation of Fe(II) and Fe(III) salts, 1.72 g and 4.70 g respectively in 50 ml deionized water. Ammonia solution (3M) was added to the iron salts solution through syringe pump (10 mL h^{-1}) under mechanical stirring and the pH of the solution reached 10. The black precipitant was formed from this process. The process ends by washing with de-ionized water to remove any alkali metals until neutral state ($\text{pH} \pm 7$). Samples then dried by freeze drying process before being characterized.

2.3 Synthesis of chitosan-coated magnetic nanoparticles

Magnetic nanoparticles of Fe_3O_4 coated by chitosan were synthesized using ex-situ co-precipitation method. In this term, Fe_3O_4 were synthesized prior to surface coating by chitosan layer. The first step is a synthesis of the Fe_3O_4 nanoparticle by mixing of $\text{FeCl}_2 \cdot 4\text{H}_2\text{O}$ (1.72 g) and $\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$ (4.70 g) salts in 50 mL deionized water and followed by adding a drop of Ammonia solution (3 M) through syringe pump (10 mL h^{-1}). The pH of the solution was adjusted to 10. During this process, the mixture solution was stirred by a magnetic stirrer. The black precipitant obtained in this process was indicated as Fe_3O_4 nanoparticles. Before coated by chitosan, Fe_3O_4 nanoparticles were washed several times

with de-ionized water to remove any alkali metals, and separated by magnetic decantation. Surface modification of Fe_3O_4 nanoparticles was carried out by adding the different amount of 1% chitosan solution (10 mL, 20 mL, and 30 mL) into 10 mL of water suspended Fe_3O_4 nanoparticles to form ferrogel solution. The mixture was stirred by a magnetic stirrer for 30 minutes. Afterwards, 10 mL of dual crosslinkers consisting of TPP and sulphate (with volume ratio TPP: sulphate 3:1) was added to the solution of chitosan- Fe_3O_4 through a syringe pump (10 mL h^{-1}) under mechanical stirring for 1 h. The yielded solution was then stirred for additional 1 h. The colloidal chitosan-coated magnetic Fe_3O_4 nanoparticles were extensively washed with de-ionized water and separated by magnetic decantation for several times. Samples were then dried by freeze drying process.

The Nanoparticles were prepared according to the procedure above. Nevertheless, instead of adding crosslinkers, 10 mL of dual crosslinker TPP: sulphate was added with different ratio 1:1, 3:1 and 5:1 into ferrogel solution with constant ratio composition of chitosan: Fe_3O_4 1:1. This different composition ratio of crosslinker was intended to understand the effect of crosslinker toward physical properties and magnetic characteristic of nanoparticles of chitosan- Fe_3O_4 . The same procedure for preparing nanoparticles was also followed. But, instead of stirring after the addition of cross-linked, nanoparticles with a constant composition ratio of chitosan: Fe_3O_4 1:1 and ratio composition of TPP: sulphate 3:1 was stirred further for 1 h. Another batch was prepared and stirred for 2 and 3 hours to determine the effect of crosslinking time toward final properties of nanoparticles.

2.4 Characterization

Morphological characterization and particle size determination of dried nanoparticles chitosan-coated Fe_3O_4 and bare Fe_3O_4 were performed by Scanning Electron Microscope-Energy Dispersive X-Ray Spectroscopy (Hitachi High-Tech TM3000; Hitachi High-Technologies Corporation, Tokyo, Japan). Crystallite size of nanoparticles was determined by X-Ray Diffractometer (Philips type X'pert MPD; from PANalytical B.V., Eindhoven, Netherlands) at room temperature using $\text{Cu K}\alpha$ radiation ($\lambda = 1.5406 \text{ \AA}$) with the 2θ range of $15 - 65^\circ$. Crystallite size of nanoparticles was achieved through calculation using modified Debye-Scherrer Equation as given in Equation 1 [10],

$$\ln\beta = \ln \frac{K\lambda}{L \cdot \cos\theta} = \ln \frac{K\lambda}{L} + \ln \frac{1}{\cos\theta} \quad (1)$$

Where β is full-width at half-maximum diffraction peak (FWHM), K is Scherrer constant related to crystallite shape, λ is x-ray wavelength (nm), $\cos \theta$ is Bragg angle (rad), and L is crystallite size (nm). Crystallite size was obtained by plotting $\ln \beta$ against $\ln (1/\cos\theta)$.

Furthermore, the iron phase abundance was calculated by material analysis using diffraction (MAUD) software. Magnetic properties were observed by Electron Spin Resonance (Leybold Heraeus; Leybold, Huerth, Germany) at room temperature with electric current 0.3 A and frequency were 33.7-76.5 MHz, respectively. Magnetic properties were confirmed by calculating the slope of ESR curve. The higher slope of the curve indicates higher magnetism of the nanoparticles.

3. Results and Discussion

3.1 Formation steps of Fe_3O_4 nanoparticles

In this research, nanoparticles based on biomaterial of chitosan- Fe_3O_4 were synthesized as a candidate of drug delivery system by ex-situ co-precipitation method. Unsoy, *et.al*, (2014) described that coprecipitation method is a very advantageous method to synthesize nanoparticles for biomedical application due to the simple and mild synthesis conditions [6]. In addition, nanoparticles that are produced by coprecipitation have higher biocompatibility and biodegradability. Physical properties and magnetic characteristics of nanoparticles were influenced by several factors such as composition ratio and crosslinking time.

During synthesis of Fe_3O_4 , the color of the iron solution changed slowly from light brown, dark brown, and finally to black by adding base solution continuously. The formation of black precipitant was indicating the formation of the Fe_3O_4 nanoparticle. The color appearance changing because of the

change of phase compound that formed during synthesis processes [11]. The possible reaction for the formation of the Fe_3O_4 nanoparticle can be written as follows [3]:



3.2 Crystallite size, iron phase abundance, morphology, and magnetic properties of nanoparticles

Dried powder of bare Fe_3O_4 and chitosan- Fe_3O_4 were then characterized by using X-Ray Diffractometer. **Figure 1.** shown XRD patterns of bare Fe_3O_4 and chitosan- Fe_3O_4 with various composition ratio and crosslinking time. Characteristic peaks were observed in the XRD pattern at 2θ of 9.6° , 30.1° , 35.5° , 43.1° , 54.5° , 57.6° and 63.6° corresponding to the diffractions of 220° , 311° , 400° , 422° , 511° and 440° crystal faces of Fe_3O_4 spinel structure (ICSD #26410). Peak broadening was indicating small size of nanoparticles. The crystallite size of nanoparticles was evaluated from the XRD data using modified Debye-Scherrer equation. Crystallite size of bare Fe_3O_4 and chitosan- Fe_3O_4 nanoparticles in different composition ratio and crosslinking time were presented in **Table 1**.

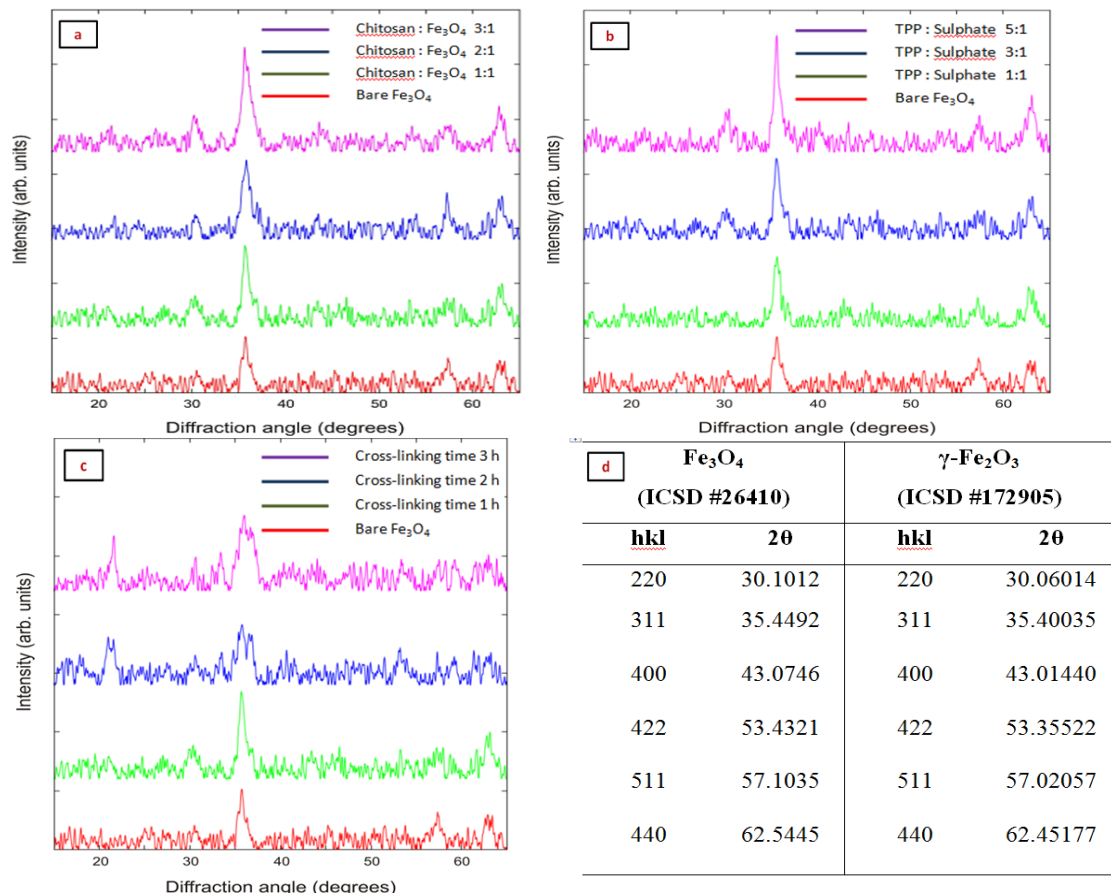


Figure 1. X-ray diffraction patterns of both bare Fe_3O_4 and chitosan- Fe_3O_4 nanoparticles with various composition ratio (a, b) and cross-linking time (c), miller indices and 2θ position of magnetite (Fe_3O_4) and maghemite ($\gamma\text{-Fe}_2\text{O}_3$) according to ICSD data.

The crystallite size of nanoparticle decreases as composition ratio consists of chitosan: Fe_3O_4 and TPP: sulphate ratio increases. However, crystallite size increases as crosslinking time increases. XRD patterns of chitosan- Fe_3O_4 in various crosslinking time (2 and 3 h) show a different pattern to the

others since revealing a peak at 2θ 21.56⁰. It was identified as the peak of chitosan with orthorhombic structure [12].

Table 1. Determination of crystallite size and particle size of nanoparticles.

Parameter	L ^a (nm)	Particle Size ^b (nm)
Bare Fe₃O₄	11.90	72.3-162
Ratio of Chitosan: Fe₃O₄		
1:1	6.20	253-482
2:1	4.27	456-793
3:1	3.78	583-882
Ratio of TPP: Sulphate		
1:1	7.79	368-593
2:1	6.20	253-482
3:1	5.34	202-273
Crosslinking Time (h)		
1	6.20	253-482
2	8.75	283-320
3	9.44	142-226

^a Calculated by Modified Debye-Scherrer equation (1)

^b Measured by SEM

The XRD data were also used for determining the iron content of each sample. The percentage of iron phase abundance of the chitosan-Fe₃O₄ nanoparticles were exhibited in **Table 2**. As shown in **Figure 2**, the samples contained not only a Fe₃O₄ phase but also γ -Fe₂O₃. The percentage of Fe₃O₄ on the bare Fe₃O₄ sample is less than chitosan-Fe₃O₄. Crystal of γ -Fe₂O₃ is arranged with a Fe³⁺ ion in octahedral and tetrahedral sites. Crystal of Fe₃O₄ arranged by Fe³⁺ ion and Fe²⁺ ion in octahedral sites as well as a Fe³⁺ ion in tetrahedral sites. Whereas, the Crystal structure of γ -Fe₂O₃ arranged by a Fe³⁺ ion in octahedral and tetrahedral sites. Crystal structure of Fe₃O₄ and γ -Fe₂O₃ were depicted in **Figure 2**.

Table 2. Percentage of iron phase abundance on various samples.

Reaction Parameter	Magnetite (%)	Maghemite (%)
Bare Fe ₃ O ₄	87.98	12.02
Chitosan:Fe ₃ O ₄ (1:1)	93.29	6.71
Chitosan:Fe ₃ O ₄ (2:1)	92.73	7.27
Chitosan:Fe ₃ O ₄ (3:1)	89.35	10.65
TPP:Sulphate (1:1)	89.88	10.12
TPP:Sulphate (3:1)	93.29	6.71
TPP:Sulphate (5:1)	96.3	3.7
Crosslinking Time 1 h	93.29	6.71
Crosslinking Time 2 h	94.52	5.48
Crosslinking Time 3 h	97.12	2.88

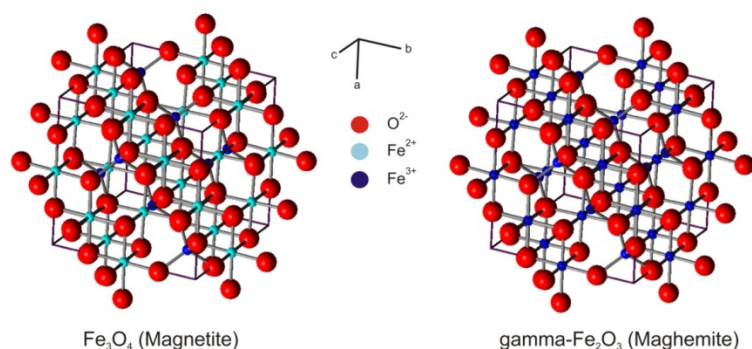


Figure 2. Model of crystal structure of magnetite (Fe_3O_4) and maghemite ($\gamma\text{-Fe}_2\text{O}_3$).

The composition ratio of crosslinker plays an important role during synthesis because it can be affecting physical properties of synthesis products. In this work, chitosan molecules were crosslinked by different volume of tripolyphosphate and sulphate as crosslinking agents. Particle size and morphology of synthesis products of bare Fe_3O_4 and chitosan- Fe_3O_4 nanoparticles in different crosslinker volume ratio have been observed by SEM-EDX and presented in **Figure 3**. According to SEM images, it was known that the particles are forming clusters. Clustering could occur during purification of Fe_3O_4 before chitosan coated. Surface morphology of chitosan-coated Fe_3O_4 is almost spherical that compared with bare Fe_3O_4 (without chitosan). Particles size determined by SEM-EDX were listed in **Table 1**. The particle size of chitosan- Fe_3O_4 decreased with the increasing of TPP: sulphate ratio.

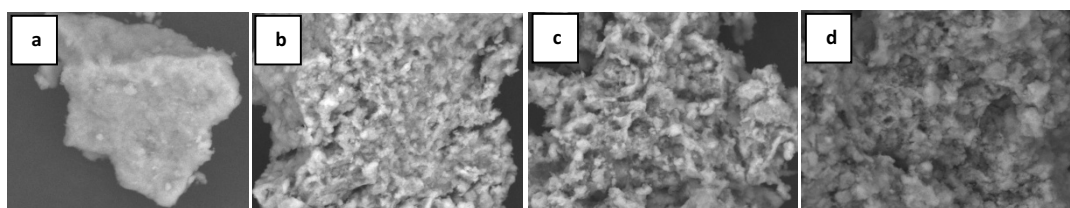


Figure 3. SEM images of nanoparticles (a) bare Fe_3O_4 , and chitosan- Fe_3O_4 with various crosslinker ratio (b) 1:1, (c) 3:1, (d) 5:1 with 3000 times magnification.

Comparing between EDX spectra of bare Fe_3O_4 and chitosan coated Fe_3O_4 samples are presented in **Figure 4**. The peaks around 0.8, 6.3, and 6.8 keV are related to the binding energies of Fe [2]. The spectrum of bare Fe_3O_4 contained two peaks, which were assigned to Fe, and O. However, chitosan- Fe_3O_4 contained six peaks that assigned not only for Fe and O but also C, N, P, and S. The peak of C and N shows the existence chitosan layer on the surface of the iron oxide. Furthermore, the peak of P and S exhibits the existence of crosslinker agents.

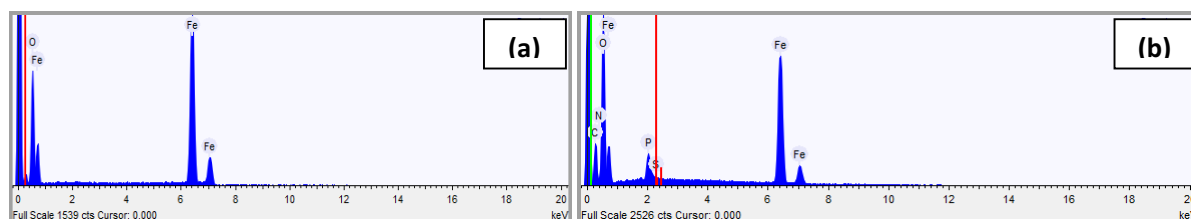


Figure 4. EDX spectra of (a) bare Fe_3O_4 , (b) chitosan- Fe_3O_4 with constant crosslinker ratio 3:1, Chitosan: Fe_3O_4 1:1, and 1 h crosslinking time.

The composition ratio of chitosan and Fe_3O_4 was influenced the magnetic characteristic of nanoparticles. Magnetic characteristic was determined by using electron spin resonance. The resonance frequency of each sample with different chitosan: Fe_3O_4 ratio was evaluated in the same frequency region (33.7-76.5 MHz). In contrast, ESR signal of bare Fe_3O_4 was obtained at 30.6 MHz. The differentiation of ESR curve profile can be observed by calculating the slope of each curve. The results show that the slope of ESR curve decreases as chitosan: Fe_3O_4 ratio increases. The slope of each ESR curve on various samples with different chitosan: Fe_3O_4 ratio was presented in **Figure 5(a)**, while the nanoparticle responses under the influence of external magnetic field were shown in **Figure 5(b)**.

The slope of ESR curve was increasing with the increase of Fe_3O_4 content on various samples. Samples have active response toward electromagnetic radiation waves. Considering the slope of ESR curves, asymmetrical Zeeman effects may be occurred caused by the changed of the energy system. Whole energy system will increase or decrease depends on the amount of Fe_3O_4 on various samples. The sharper slope of ESR curve was affected by the higher Fe_3O_4 content on various the samples. It was indicating the increasing energy of the overall system.

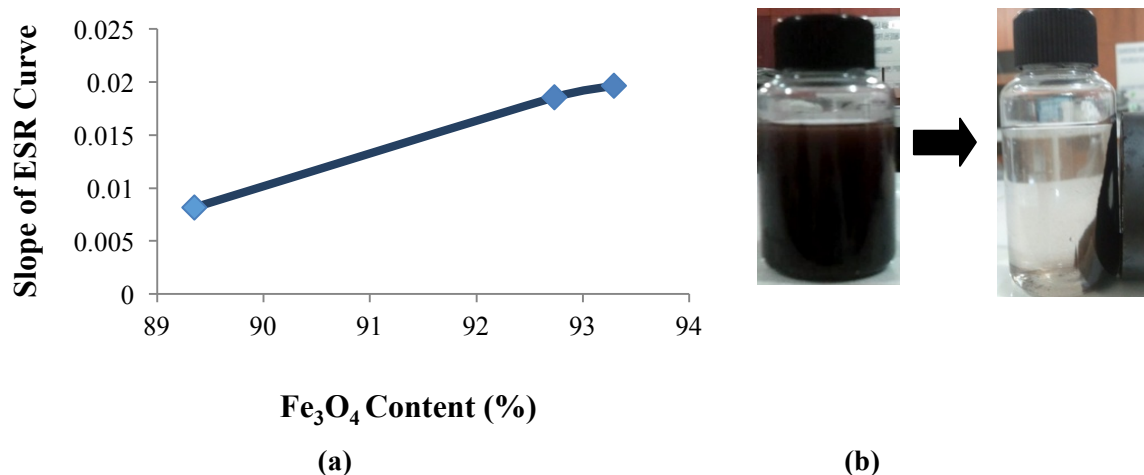


Figure 5. (a) Correlation curve between the slope of ESR curve toward the percentage of Fe_3O_4 in chitosan- Fe_3O_4 samples with various chitosan: Fe_3O_4 ratio, (b) magnetic properties test of the chitosan- Fe_3O_4 nanoparticle.

According to the result that given by SEM, XRD and ESR, there was an interesting correlation between particle size, the percentage of Fe_3O_4 , and slope of ESR curve for nanoparticles produced by different composition ratio and crosslinking time. The smaller particle size would provide the higher percentage of Fe_3O_4 and the higher slope of ESR curve.

4. Conclusion

In this work, magnetic nanoparticles (Fe_3O_4) have been prepared by ex-situ co-precipitation method at room temperature. Composition ratio and crosslinking time were affecting physical properties and magnetic characteristic of nanoparticles product. The higher ratio of chitosan and Fe_3O_4 were produced materials with small crystallite size and low Fe_3O_4 content. Furthermore, the volume ratio of crosslinker was also affecting the particle size and morphology of the material. The smallest size of nanoparticles with spherical shape would be established in a higher ratio of TPP: sulphate. There was an interesting correlation between particle size, the composition of Fe_3O_4 , and slope of ESR curve. The smaller particle size will lead to the increase of Fe_3O_4 content as well as increase the value of ESR slope.

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