

## CHAPTER III EXPERIMENTAL

### 3.1 Materials

Acetic acid was purchased from Univar, Australia. Ammonium hydroxide (NH<sub>4</sub>OH), dimethylformamide (DMF), dimethylsulfoxide-d<sub>6</sub> (DMSO-d<sub>6</sub>), ferric chloride hexahydrate (FeCl<sub>3</sub>·6H<sub>2</sub>O), ferrous chloride tetrahydrate (FeCl<sub>2</sub>·4H<sub>2</sub>O), potassium carbonate (K<sub>2</sub>CO<sub>3</sub>), and triethylamine were bought from Merck, Germany. Low molecular weight chitosan (LCS, Mw=15000 g/mol, 90% degree of deacetylation) was a gift from Chitin Research Center, Chulalongkorn University, Thailand. (3-Chloropropyl)-trimethoxysilane, oleic acid, phthalic anhydride, and sodium hydride (NaH) were purchased from Sigma-Aldrich, Inc., USA. Sodium azide (NaN<sub>3</sub>) and hydrazine were bought from Fluka Chemika, Switzerland. Propargyl bromide was purchased from Tokyo Kasei Kogyo Co., Ltd., Japan. Sodium hydroxide (NaOH) and potassium hydroxide (KOH) were purchased from Carlo Erba, Italy. Acetone, dichloroethane, ethanol, hexane, tetrahydrofuran, and toluene were bought from RCI Labscan, Thailand. All of chemicals were used as obtained without further purification.

### 3.2 Instruments and Equipment

Structural analysis were carried out by using an Equinox 5 Bruker Fourier transform infrared spectrometer (FTIR) based on KBr and ZnSe methods, and an Avance 500 Bruker Biospin Nuclear magnetite spectroscope (NMR). The phase of iron oxide was recorded by a D/DMAX 2200 Rigaku wide angle X-ray diffractometer (WAXD) based on 5-90° 2 $\theta$  with a scanning rate of 2.5° 2 $\theta$  /min under 0.05° 2 $\theta$ /scan. Thermal properties of product were analyzed on a Perkin Elmer Pyris Diamond thermogravimetric/ differential thermal analyzer (TG/DTA) with a N<sub>2</sub> flow and a heating rate of 10 °C/min in range of 80-950°C. Hydrodynamic radius and zeta potential of the products in various pH and solvent conditions were identified on

a Malvern Zeta Sizer Nano Series with a detection angle of  $173^\circ$ , dynamic light scattering (DLS). Dispersability image and individual size were recorded on a 100 kV H-7650 Hitachi transmission electron microscope (TEM) by using copper grid and carbon grid. Magnetism of the particles were evaluated by vibrating sample magnetometer (VSM) at Khonkaen University. Absorption of plasmid DNA (pDNA) colloidal solution of *E.coli* was elucidated by a UV-vis spectrophotometer at Mahidol University.

### 3.3 Methodology

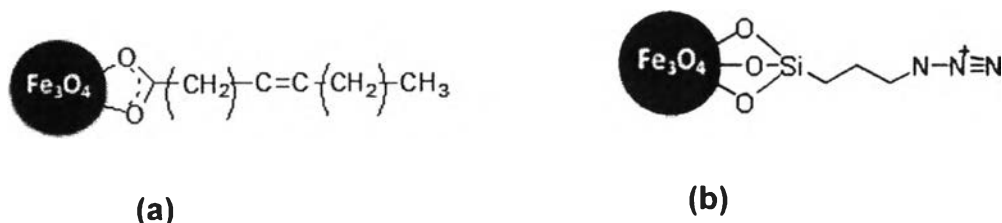
#### 3.3.1 Synthesis of Azide-magnetite Nanoparticles

Magnetite particles were coated with oleic acid as reported by Liu *et al.*, 2010. Briefly,  $\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$  and  $\text{FeCl}_2 \cdot 4\text{H}_2\text{O}$  were dissolved in DI water in the ratio of 2:1 under nitrogen atmosphere, followed by adding a small amount of oleic acid into the solution under stirring. After 30 mins stirring, a 25%  $\text{NH}_4\text{OH}$  solution was added dropwise within 15 mins to the mixture. The suspension was heated at  $85^\circ\text{C}$  for 1 h and the temperature was reduced to room temperature. Superfluous ammonium oleate in the black ferro-fluid was removed by dialyzing against DI water before freeze drying to obtain black particles of oleic-magnetite as shown in Figure 3.1 (a).

The as-synthesized oleic-magnetite nanoparticles were reacted with an excess amount of (3-chloropropyl)-trimethoxysilane containing a small amount of acetic acid as a catalyst at  $60^\circ\text{C}$  for 72 h. The particles were washed with tetrahydrofuran and acetone. In each step the particles were separated by a high power magnet. The particles were dried under vacuum at room temperature. In this step, the co-precipitation of  $\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$  and  $\text{FeCl}_2 \cdot 4\text{H}_2\text{O}$  reaction was also carried out without oleic acid as a comparative study.

The as-synthesized chloro-magnetite nanoparticles were reacted with an excess amount of  $\text{NaN}_3$  in DMF at  $80^\circ\text{C}$  for 72 h (Figure 3.1 (b)). The nanoparticles were washed with DMF, ethanol, and acetone and dried under vacuum

at room temperature. In this step, the reactions in hexane, toluene, and dichloroethane were also carried out as comparative studies.

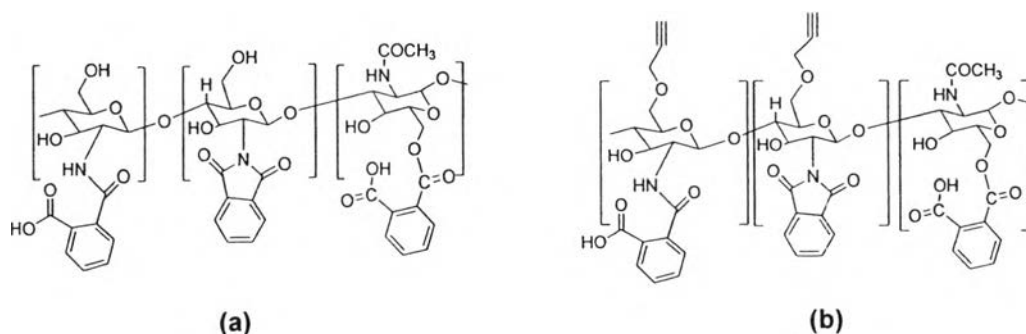


**Figure 3.1** Chemical structures of (a) oleic-magnetite particles and (b) azide magnetite particles.

### 3.3.2 Synthesis of Alkyne-phthaloylchitosan

LCS was reacted with phthalic anhydride (5 mol equivalent to pyranose ring) in DMF for 6 h at 120°C under vacuum and continuously stirred at 60°C under nitrogen atmosphere overnight (Yoksan, *et al.*, 2003) (Figure 3.2 (a)). The clear solution obtained was precipitated in cold water, washed in DI water, dialyzed for 5 days, and dried with freeze dry to obtain yellow powder product of phthaloylchitosan.

The phthaloylchitosan obtained was reacted with an excess propargyl bromide and a small amount of triethylamine at room temperature. Similarly, the reaction was carried out at 60, 90, and 120°C. The reaction was also carried out with a small amount of KOH, NaOH, and NaH as a catalyst at 60, 90, and 120°C, except NaH at 0°C. The solution was precipitated in cold water, dialyzed for 5 days, and freeze dried to obtain yellowish powder of alkyne-phthaloylchitosan (Figure 3.2 (b)).



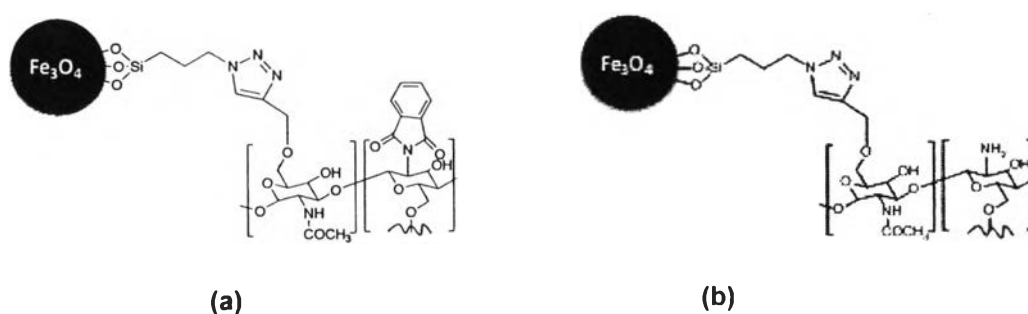
**Figure 3.2** Chemical structures of (a) phthaloylchitosan and (b) alkyne-phthaloylchitosan.

### 3.3.3 Conjugation of Magnetite Nanoparticles with Chitosan via “Click”

#### Reaction

Azide magnetite nanoparticles were reacted with alkyne phthaloylchitosan in DMF at room temperature for 72 h to obtain phthaloylchitosan-magnetite nanoparticles (Figure 3.3 (a)). In similar, the reactions at 50, 70, and 90°C were also carried out. The product obtained was separated by a high power magnet, and washed with DMF, toluene, and acetone many times. The powder was dried under high vacuum at room temperature.

The as-synthesized phthaloylchitosan-magnetite nanoparticles were deprotected phthalamido group reacting with excess hydrazine solution at 80°C overnight to obtain chitosan-magnetite as shown in Figure 3.3 (b). The chitosan-magnetite obtained was purified by a high power magnet, followed by washing with ethanol and acetone several times, and drying under high vacuum at room temperature.



**Figure 3.3** Chemical structures of (a) phthaloylchitosan-magnetite and (b)chitosan-magnetite nanoparticles.

The chitosan-magnetite nanoparticles was calculated for the percent of triazole group generated from the “click” reaction by using the following equation

$$\text{Percent of relative of triazole} = \frac{I_B - I_A}{I_B} \times 100$$

whereas,  $I_B$  and  $I_A$  are integrations of FTIR spectra at  $N_3$  functional group position ( $2098\text{ nm}^{-1}$ ) belonging to azide position before and after the reaction of azide magnetite nanoparticles with alkyne phthaloylchitosan.

#### 3.3.4 Direct Conjugation of Chitosan and Magnetite Nanoparticles

Magnetite nanoparticles obtained from the co-precipitation of  $Fe_2Cl_4 \cdot 4H_2O$  and  $Fe_3Cl_6 \cdot 6H_2O$  in the ratio of 1:2 in 25%  $NH_4OH$  was coated with LCS (0.5 wt%) adding 2% acetic acid solution with vigorous stirring and ultrasonication for 20 min. Twenty-five percent of  $NH_4OH$  was added to the mixture. The product obtained was washed with water and vacuum dried.

#### 3.3.5 DNA Separation Study

The *E. coli* in TE buffer solution was quantitatively analyzed based on absorptions at 260 and  $280\text{ nm}^{-1}$  observed by UV-vis spectrometer. The chitosan-magnetite (0.5 mg) was added in the solution of *E. coli*, followed by incubating for 5 mins. The supernatant was collected and the UV absorption at 260 and  $280\text{ nm}^{-1}$  were traced. In this step, other samples such as chitosan-magnetite via direct synthesis and Dynabeads<sup>®</sup> were also studied.