We presented synthesis and physical characterization of iron oxide magnetic nanoparticles (Fe₃O₄) for biomedical applications in the size range of 10-30 nm. Magnetic nanoparticles were synthesized by the coprecipitation method, and the particles’ size was controlled by two different injection methods of sodium hydroxide (NaOH). The synthesized magnetic nanoparticles were then modified by using series of linkers including tetraethyl orthosilicate (TEOS), 3-aminopropyltriethoxysilane (APTES), and glutaraldehyde (GA) to generate the structure of Fe₃O₄/SiO₂/NH₂/CHO, which can be used for immobilization of protein A. Additionally, we used transmission electron microscopy (TEM), X-ray powder diffraction (XRD), vibrating-sample magnetometry (VSM), and Fourier-transform infrared spectroscopy (FTIR), for characterization of properties and structure of the nanoparticles. An immobilization of protein A on magnetic nanoparticles was studied with a UV-Vis spectrum (UV-Vis) and fluorescence electron microscopy and Bradford method. Results showed that an XRD spectrum with a peak at (311) corresponded to the standard peak of magnetic nanoparticles. In addition, the magnetic nanoparticles with \( d \geq 30 \) nm have higher saturation magnetizations in comparison with the smaller ones with \( d \leq 10 \) nm. However, the smaller magnetic nanoparticles offered higher efficiency for binding of protein A, due to the high surface/volume ratio. These particles with functional groups on their surface are promising candidates for biomedical applications, e.g., drug delivery, controlled drug release, or disease diagnosis in point-of-care test.
1. Introduction

Nowadays, there are various sorts of virus and bacterial pathogens that cause disease *in vivo* and human death [1–3]. Infectious elements cause various cancers or tumors are basically required to be detected to prevent the infection and the transmission. The available detection techniques include the traditional methods of treating diseases such as enzyme-linked immunosorbent assay (ELISA) based on antigen-antibody interaction [4–6] and polymerase chain reaction (PCR) which amplified DNA molecules through temperature-dependent cyclic steps [7–10]. According to improved research direction in nanomedicine, they require integration of detection and diagnosis as well as therapy with modern molecular imaging and living-cell detection.

In the past few decades, nanotechnology has been considered as an important advancement in science and technology. It is related to the production of materials at the nanometer scale. There has been a large research interest in nanoscale materials that is due to their unique properties such as high surface area, large number of binding sites on their surface, high surface reactivity, and strong absorption activity [11–14]. Those characteristics offer novel application in biomedicine. Nowadays, technologies have been developed for detection and treatment of various diseases such as nanodispensing systems using carbon nanotubes (CNTs) for chemotherapy [15, 16], drug delivery system using silica nanoparticles (SiO2) with attached fluorescent dye molecules [17–20], using silver (Ag) nanoparticles for antibacterial property against clinically isolated multidrug-resistant microorganisms [21, 22], and biosensor utilizing gold (Au) nanoparticles for DNA detection [23, 24]. However, those methods have inherent drawbacks due to the lack of carrier properties. For example, those particles cannot be controlled to deliver drug into the target organisms.

In recent years, a great deal of efforts has been focused on the development of nanocarriers for diagnosis and treatment of diseases. One of the nanocarriers is magnetic nanoparticles under control of an external magnetic field. Magnetic nanoparticles are superparamagnetic materials with high saturation magnetization, nontoxic, and highly biocompatible which have great potential for biomedical applications such as efficient bioseparation, sensitive biosensing, and specific drug delivery as well as magnetic resonance imaging (MRI) contrast enhancement [25, 26]. Various sizes of superparamagnetic nanoparticles offer potential application in biomedicine with various elements including cell (around 100 μm), proteins (around 10 nm), and virus (around 100 nm) [27–29]. However, the change in size of magnetic nanoparticles is associated with the change in saturation moment, which is an important property to demonstrate the resonant responsibility to an external magnetic field of the particles. Additionally, the promising biomedical applications of magnetic nanoparticles basically require the particles to monodisperse. So the magnetic nanoparticles are required to be covered by nonmagnetic materials to prevent oxidation and agglomeration. Consequently, each nanoparticle has identical chemical and physical properties for controlled bioelimination and biodistribution [30–34].

Recently, extensive researches have shown that protein A is one of the promising biochemical linkers because of its ability to bind with immunoglobulins, fibrinogen, and C-reactive protein (CRP) [35]. This property led us to attempt to detect target proteins for disease diagnosis.

In this work, we demonstrated superparamagnetic nanoparticles for immunoassay with its modified surface (Fe3O4/SiO2/NH2/CHO) for immobilization of protein A. The heterogeneous layers were coated for a wide dynamic range of detection and high specificity of the particle surface. We coated silica layer (SiO2) on the magnetic nanoparticle surface for a subsequent self-assembled monolayer of aminopropyltriethoxysilane (APTES) and glutaraldehyde (GA) for subsequent absorption of protein A, which could capture target molecules specifically. We characterized successive immobilization of each layer of SiO2, NH2, CHO, and protein A in a quantitative manner. The absorption efficiency of protein A is 82.35% and 52.94% for the size of the synthesized magnetic nanoparticles of 10 nm and 30 nm, respectively. This led us to believe that the synthesized magnetic nanoparticles could find potential applications for disease diagnosis and disease treatment.

2. Materials and Methods

2.1. Agents. Iron(II) chloride tetrahydrate (FeCl2·4H2O) (≥99%), iron(III) chloride hexahydrate (FeCl3·6H2O) (≥99%), ammonium hydroxide (NH4·H2O) (≥99%), sodium hydroxide (NaOH) (≥98%), ethanol (C2H5·OH) (99%), and phosphate-buffered saline (PBS, pH 7.4) were purchased from Merck. Glutaraldehyde (GA), 3-aminopropyltriethoxysilane (APTES), biotin (C10H16N2O3S) (≥99%), biotin-fluorescence isothiocyanate (biotin-FITC, C33H32N4O8S), and tetraethyl orthosilicate (TEOS, Si(OC2H5)4) (≥99%) were bought from Sigma-Aldrich.

2.2. Fabrication of Magnetic Nanoparticles. Magnetic nanoparticles were synthesized based on the coprecipitation method, and its size could be controlled by change in pH of solutions, types of base, ionic concentration, mole concentration ratio between Fe2+ and Fe3+, and reaction temperature [36, 37]. The optimized amount between Fe2+ and Fe3+ was a mole concentration ratio of 1:2 [36]. In this work, we used 4.3 g of FeCl3·6H2O mixed with 1.6 g of FeCl2·4H2O and then diluted with 120 mL of distilled water (DI). After that, this solution was separated into two parts and stored under nitrogen gas condition. Additionally, two parts of mixing solution were vigorously stirred with a speed of 800 cycles/min at a temperature of 80°C within 15 min. In this work, we controlled the size of magnetic nanoparticles by change in injection methods of sodium hydroxide solution [37]. 2 mL of 2 M sodium hydroxide was immediately injected in the first mixing solution while a similar amount of sodium hydroxide was dropped into the second one. Both parts were continuously stirred within 45 min and then cooled down to room temperature. This synthesized nanoparticles (Fe3O4) were washed by DI water (three times at least) via magnetic decantation and then dried in vacuum at 40°C.
2.3. Immobilization of Protein A on Synthesized Nanoparticles. The surface of Fe3O4 nanoparticles was then modified with a functional group for immobilization of protein A. For biomedical application, Fe3O4 nanoparticles should be monodisperse by covering of nonmagnetic materials [38]. As shown in Figure 1, Fe3O4 nanoparticles were covered by the silica layer (SiO2) based on the Stober method [37]. In this case, 200 mg of Fe3O4 nanoparticles was diluted into 50 mL of mixing solution including ethanol and DI water with a volume ratio of 3:2. After that, TEOS at 2 mL and NH3(l) were subsequently injected to the suspension and stirred at a speed of 100 cycles/minute for 24 h with temperature of 40°C under nitrogen gas. Then, this solution was stirred by using an ultrasonic machine for 30 min. Finally, we obtained modified magnetic nanoparticles with a core-shell structure of Fe3O4/SiO2 as shown in Figure 1. The silica-coated particles were then washed with DI water to remove the excess solution while the magnetic nanoparticles were retained via magnetic decantation. The silica-coated magnetic nanoparticles were dried in vacuum at 40°C.

To form an amine group on the particle surface, Fe3O4/SiO2 nanoparticles were diluted with DI water and mechanically stirred at the speed of 100 cycles/minute at room temperature to make dispersion of the nanoparticles. Then, 1 mL of 25% glutaraldehyde (GA) was injected into the mentioned suspension and continuously stirred for 24 h to generate the structure of Fe3O4/SiO2/NH2/CHO as shown in Figure 1. The impurities on obtained nanoparticles were removed by washing with PBS three times.

For immobilization of protein A on magnetic nanoparticles, 100 mg of Fe3O4/SiO2/NH2/CHO nanoparticles was mixed with 0.5 mg of protein A [40]. Then, the mixture was diluted into 15 mL of PBS for 12 h at room temperature. Then, we used a magnetic bar to collect the magnetic nanoparticles with conjugation of protein A. The nonspecific bonding of protein A was eliminated by washing with PBS three times.

3. Results and Discussion

Figure 2 shows the XRD spectra of the synthesized superparamagnetic nanoparticles using the coprecipitation method. In this method, we controlled the size of particles by change in the injection method of the base. Figure 2(a) corresponds to the patterns of magnetic nanoparticles synthesized with...
immediate injection of sodium hydroxide whereas Figure 2(b) shows the patterns of the synthesized nanoparticles by a droplet of sodium hydroxide.

As shown in Figure 2, the crystalline nature of magnetic nanoparticles exhibits six distinct peaks at (220), (311), (400), (422), (511), and (440), which are consistent with the expected composition of Fe₃O₄. The position and relative intensity of diffraction peaks for both patterns (Figures 2(a) and 2(b)) are also matched with the database in JCPDS file (No. 01-075-1373) for bulk Fe₂O₄. These results indicated that the synthesized particles were mainly consisted of magnetite Fe₃O₄. Additionally, we also estimated the average size of the synthesized magnetic nanoparticles using the Debye-Scherrer equation below [41].

\[
D_p = \frac{k\lambda}{\chi \cos \theta},
\]

where \(D_p\) is the average crystalline size of the synthesized Fe₃O₄, \(k\) is the Scherrer constant (0.8-1.39), \(\lambda\) is the wavelength of radiation; \(\chi\) is the full width at half maximum (FWHM), and \(\theta\) is the position of the maximum diffraction. We used the diffraction peak at (311) for estimation because of the high signal-to-noise ratio. The size of Fe₃O₄ particles was estimated around 10 nm and 30 nm corresponding to the synthesis with immediate injection of NaOH and a droplet of NaOH, respectively. The difference in the size of Fe₃O₄ nanoparticles (Fe₃O₄NPs) was due to the balance between the speed of sprout production and speed of crystal growth. When the speed of sprout production is faster than that of crystal growth, the particles will have the smaller size. This case was corresponding to immediate injection of NaOH into an aqueous mixture of ferric and ferrous salts which resulted in a smaller size (around 10 nm) of nanoparticles (Figure 2(a)). In the other case, the size of particles was large (around 30 nm) due to the droplet of NaOH making reduction in the speed of sprout production.

To double-check the size of the synthesized magnetic nanoparticles by XRD patterns, we conducted a TEM analysis of Fe₃O₄ nanoparticles. Figures 3(a) and 3(c) show the TEM images of the synthesized magnetic nanoparticles with two different injection methods of NaOH as we mentioned above. It was also worth noting that the similar sizes of Fe₃O₄NPs were observed in comparison with the XRD analysis using the Debye-Scherrer equation. Moreover, it was clearly shown that Fe₃O₄NPs were monodisperse and has a spherical shape, leading to offer advanced applications in biomedicine.

Additionally, Fe₃O₄NPs were then coated with a thin silica layer (2-3 nm) using the Stober method as shown in Figures 3(c) and 3(d). Note that there are a lot of factors that affect silica-coated Fe₃O₄NP size, e.g., temperature, pH, type of alcohol, amount of TEOS added, and amount of catalyst used during the process. In this work, we used ammonia to promote the condensation of TEOS. The outer silica surface of silica-coated Fe₃O₄NPs is desirable not only because it prevents agglomeration and oxidation of Fe₃O₄NPs but also because it has extensive hydroxide groups on the surface, which can be functionalized to generate various chemical linkers on its surface. Additionally, the silica layer can also offer better protection against toxicity in biological applications.

The major hysteresis loop of uncoated and SiO₂-coated Fe₃O₄NPs (primary Fe₃O₄NPs sizes of 10 nm and 30 nm) was measured by the VSM at room temperature. As shown in Figure 4, the coercivities of each sample are 63 emu/g for uncoated Fe₃O₄NPs (10 nm), 48 emu/g for SiO₂-coated Fe₃O₄NPs (10 nm), 85 emu/g for uncoated Fe₃O₄NPs (30 nm), and 76 emu/g for SiO₂-coated Fe₃O₄NPs (10 nm). Results showed that the coercivity of the SiO₂-coated Fe₃O₄NPs is lower than that of the uncoated ones and the smaller nanoparticles' size has less magnetization in comparison with the larger ones. It is well-known that the magnetic behavior of magnetite nanoparticles depends on their dipole-dipole interactions, which is strongly affected by the distance between particles. This means that the change in interparticle interactions can be used to control an agglomeration. In this work, the SiO₂ layer surrounding the Fe₃O₄NPs acts as an insulating layer to lock electron transfer, causes an increase in distance between the nanoparticles, and also prevents their agglomeration. Thus, the SiO₂-coated Fe₃O₄NPs shows a decrease in coercivity. Furthermore, magnetization of the synthesized nanoparticles is larger than that of magnetic nanoparticles as reported in the literature [42] and is comparable with the results obtained in the literatures [43, 44].

For surface functionalization of the synthesized magnetic nanoparticles, APTES was used to generate an amine group on the SiO₂ layer forming the structure of Fe₃O₄/SiO₂/NH₂. As shown in Figure 5, FTIR results displayed the spectra of Fe₃O₄, Fe₃O₄/SiO₂, and Fe₃O₄/SiO₂/NH₂ structures, which reveal the distinct peaks in the range of 3384 cm⁻¹, 1627 cm⁻¹, 1402 cm⁻¹, 1095 cm⁻¹, 956 cm⁻¹, 801 cm⁻¹, 572 cm⁻¹, and 471 cm⁻¹. The peaks at 572 cm⁻¹ and 471 cm⁻¹ represented the vibration of Fe-O as shown in Figure 5(a, b, and c), and those peaks also were specific ones for the Fe₃O₄ structure [39].
However, there was not a peak at 632 cm\(^{-1}\), leading us to believe that there was not an Fe\(_2\)O\(_3\) phase presented in the synthesized magnetic nanoparticles (Fe\(_3\)O\(_4\)) [45]. Moreover, the peak at 471 cm\(^{-1}\) was also identified as Si-O-Si bending vibrations [46], leading to enhance the intensity of this peak as shown in Figure 5(b and c). The peaks at 1095 cm\(^{-1}\) and 801 cm\(^{-1}\) also corresponded to the stretching vibrations of Si-O-Si, while the peak at 956 cm\(^{-1}\) was referred as the stretching vibrations of Si-OH [46]. This thus indicated that TEOS has covered the Fe\(_3\)O\(_4\)NPs to form an Fe\(_3\)O\(_4\)/SiO\(_2\) structure. The bands at 3384 cm\(^{-1}\), 1627 cm\(^{-1}\), and 1402 cm\(^{-1}\) were found at the stretching vibrations of OH, bending vibrations of H-O-H, and bending vibrations of OH as shown in Figure 5(a, b and c), respectively [46]. In addition, the peaks at 3384 cm\(^{-1}\) and 1627 cm\(^{-1}\) were also referred as stretching vibrations of N-H and bending vibrations of amide-II [39] that caused an increase in intensity of those peaks as shown in Figure 5(c), indicating that the amine group (NH\(_2\)) has been existing on the surface of Fe\(_3\)O\(_4\)/SiO\(_2\) to generate the structure of Fe\(_3\)O\(_4\)/SiO\(_2\)/NH\(_2\). The similar spectra of FTIR
was found for the case of the synthesized Fe$_3$O$_4$NPs with a size of 10 nm.

For biological applications, GA was then added to the functionalized nanoparticles (Fe$_3$O$_4$/SiO$_2$/NH$_2$) to form the structure of Fe$_3$O$_4$/SiO$_2$/NH$_2$/CHO. As we mentioned above, protein A with an amount of 0.5 mg was mixed with the functionalized magnetic nanoparticles (Fe$_3$O$_4$/SiO$_2$/NH$_2$/CHO) with both sizes (10 nm and 30 nm). In this case, we expected that the smaller Fe$_3$O$_4$NPs offer higher binding efficiency than the bigger ones. It was due to the fact that the smaller nanoparticles have a large surface/volume ratio [27]. This could be supported by the UV-Vis measurement of the absorption of protein A. Figure 6 shows the absorption spectrum of protein A for three cases including before immobilization on Fe$_3$O$_4$NPs (line a), after immobilization on Fe$_3$O$_4$NPs of 30 nm (line b), and after immobilization on Fe$_3$O$_4$NPs of 10 nm (line c). As expected, the absorbance at the peak of 280 nm, which was the standard peak for protein [47], decreased from 0.08 to 0.03 with the size of Fe$_3$O$_4$NPs changing from 30 nm to 10 nm, respectively. The binding efficiency (BE) can be calculated by the following equation:

$$\text{BE} \%= \frac{C_{\text{bd}}}{C_{\text{tot}}},$$

where $C_{\text{bd}}$ is the concentration of protein A binding to Fe$_3$O$_4$NPs and $C_{\text{tot}}$ is the initial concentration of protein A. Based on this measurement, we properly estimated the binding efficiency of protein A, which is 82% and 53% for size of the synthesized magnetic nanoparticles of 10 nm and 30 nm, respectively. To double-check the immobilization of protein on Fe$_3$O$_4$NPs, we conducted measurement using a fluorescent microscope. After immobilization of protein A on Fe$_3$O$_4$NPs, we added biotin-FITC on the solution. In this case, protein A can react with biotin-FITC through protein-protein interaction. As shown in Figure 7, the green color with a wavelength of 485 nm was emitted by FITC under excitation of the pump light. This indicated that biotin-FITC has been conjugated with protein A on the structure of Fe$_3$O$_4$/SiO$_2$/NH$_2$/CHO/protein A, leading to believe that protein A has been successfully immobilized.

The use of the synthesized magnetic nanoparticles may offer several benefits for drug delivery and detection of target proteins, as mentioned below. First, the silica layer can eliminate the toxicity of Fe$_3$O$_4$NPs when they are introduced into the human body for treatment. Additionally, the silica layer can also act as an insulating layer to control electron tunneling between particles, which may be important in charge transfer or magnetooptics. Second, surface modification via functional group immobilization is being pursued with great interest, since it can provide unique opportunities to engineer the interface of solid substrates while retaining particles’ basic geometry. Moreover, the structure of Fe$_3$O$_4$/SiO$_2$/NH$_2$/CHO can be conjugated with various proteins for specific target detection such as immunoglobulin G (IgG), which can specifically capture fibrinogen—a specific biomarker for heart disease and cardiovascular disease. Finally, the synthesized magnetic nanoparticles (10 nm) with magnetization of 85 emu/g can be used for drug delivery and cancer treatment by generation of heat under application of external magnetic field.

### 4. Conclusions

We presented the immobilization of protein A on the synthesized magnetic nanoparticles. The size of magnetic nanoparticles was controlled by the injection method of sodium hydroxide, leading to obtain the smaller size (10 nm) by immediate introduction of NaOH, due to the fact that the speed of sprout production was faster than the speed of crystal growth. The synthesized Fe$_3$O$_4$NPs with high magnetization of 85 emu/g offer benefits for drug delivery and cancer treatment. An immobilization of protein A on the functional
Fe$_3$O$_4$NPs (Fe$_3$O$_4$/SiO$_2$/NH$_2$/CHO) with high efficiency of 82% presented may find potential applications for fibrinogen-based diagnosis of strokes, heart disease, cardiovascular disease, and Alzheimer’s disease.

Data Availability

The data used to support the findings of this study are available from the corresponding author upon request.

Conflicts of Interest

The authors report no conflicts of interest in this work.

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