Morphology study of SPIONs coated apoferritin using small-angle neutron/X-ray scattering and transmission electron microscopy

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Abstract. The morphology structure of superparamagnetic iron nanoparticle (FeO/γ-Fe₂O₃) or SPIONs coated by apoferritin was studied to understand the ability of apoferritin in coating iron oxide. The system was synthesized in a solution to approach the human body's physiological condition. Small-angle neutron and x-ray scattering (SANS and SAXS) techniques were conducted to reveal the nanostructure of the nanoparticle. The fractal structure was observed by SANS for the SPIONs itself, with the diameter of the building sphere was 2.8 nm. Meanwhile, the SAXS data suited a model of the polydisperse sphere, which assumed as the existence of coated and non-coated SPIONs apoferritin and free apoferritin itself. Transmission Electron Microscopy (TEM) data confirmed the assumption and concluded that apoferritin unable to cover all the iron oxide.

1. Introduction
The safety of the drug, administrated into the human body, is still the central issue in cancer diagnostic and therapy. The side effect of a drug, affecting to non-cancerous tissue, is the main problem and reduce the possibility of cancer survival after chemotherapy. Therefore, new drugs based on nanoparticles become interesting research to enhance the efficacy of chemotherapy in recent years [1], [2]. One of those is superparamagnetic iron oxide nanoparticles (SPIONs) that are recognized as a promising material for cancer diagnostic and therapy. SPIONs are utilized as cancer diagnostic as a contrast agent and applied as a cancer therapy by hyperthermia [3]–[5]. However, SPIONs have a critical limitation due to its colloidal stability, opsonization by the circulating immunoglobulins, and the possibility of its physical properties loss [6]. This limitation can be overcome by coating the SPIONs with some biocompatible and nontoxic material such as protein and polymer [4], [7]. Furthermore, the coating material can be functionalized for targeting the drug to increase the efficacy and accuracy of the drug delivery to the target cell or organs[8]–[10].

Apoferriatin is a complex core-shell protein, consist of 24 subunits, which role as iron storage protein and regulating the iron content of cells in a mammalian body. Apoferriatin can be disassembly into subunits at low pH and reassembly if their surrounding is backward to neutral pH[11]. Due to these unique properties of apoferriatin, many researchers are interested in using it as cargo for drug delivery devices with various metals such as SPIONs. In the previous work, we have synthesized
SPIONs and coated them with apoferritin. This work aims to reveal the nanostructure morphology of SPIONs coated apoferritin and evaluate apoferritinability in coating SPIONs.

2. Materials and Methods

The synthesis of the SPIONs as magnetite or maghemite (Fe,0/γ-Fe,0) were prepared by coprecipitation of ferric and ferrous salts with 0.5 stoichiometry based on Massart method [12]. The SPION-Apoferritin sample then characterizes with SANS, SAXS, and TEM.

2.1. Synthesis of SPIONs coated apoferritin

The synthesis of SPIONs in detail described in other reports [13]. FeCl·4H, FeCl·6H, NH, NaOH, HCl, and HNO were purchased from Merck. Equeen spleen apoferitin was purchased from Sigma Aldrich. All chemical reagents were used without further purification.

Solids of FeCl·4H and FeCl·6H were dissolved in 0.05 M hydrochloric acid. The solution was then poured into a 1.5M ammonia solution. Nitric acid 1M was then added, followed by centrifugation of the solution to form a stable SPIONs colloid.

The apoferritin (concentration of 5 mg/mL) was slowly and gently added with 0.1 M hydrochloric acid until reaching pH 2 to make the apoferritin disassembly into smaller units. The acidic apoferritin solution was mixed with the SPIONs colloid. It then stirred for 30 minutes. The mixture was then adjusted up to pH 7 by adding 0.1 M sodium hydroxide. This step will allow the apoferritin subunit to get assembly into the 24-mer protein and coat the SPIONs nanoparticles.

To get a monodisperse system of SPIONs coated apoferritin, the solution was centrifuged with Amicon centrifugal filter unit 50 kDa.

2.2. SANS Experiment

The SANS experiment was conducted using SN2, PSTBM-BATAN, Indonesia[14]. The SPIONs coated apoferritin at pH 7 was placed in a quartz cell with an inner depth of 2mm. The SPIONs coated apoferritin at pH 7 was dialyze in D2O buffer for 24 hours. This step was purposed to reduce the incoherent background from H2O. The SANS experiment was conducted in two configuration settings. The first set was in a position detector of 2m with a collimation length of 4m for 8 hours. The detector position was moved farther to 6m with a collimation length of 8m for 12 hours. The sample was run with a thermal neutron (λ=3.9Å) for both configurations.

2.3. SAXS Experiment

The SAXS experiment was conducted using beamline 3, SLRI, Thailand[15]. The SPIONs coated apoferritin at pH 7 was placed in a capton windows cell. The SAXS experiment was set in two detector positions, i.e., 800mm and 4300mm. The sample was run for 300 seconds for both detector positions.

2.4. TEM analysis

The TEM analysis of SPIONs was done using TEM brand JEOL type 1400 in the Department of Chemistry, Gadjah Mada University, Indonesia. There are two methods applied for SPIONs sample measurement, with and without staining. For staining, it was used uranyl acetate that is common and knows staining material for biology[16]. Uranyl acetate will give negative staining for biological material. Pure apoferritin was observed with the staining method as control data.

2.5. Data analysis

SANS data were corrected by GRASP data reduction software from ILL, France [17], while SAXS data was corrected by SAXSit software from SLRI, Thailand [18]. This step was aimed to eliminate the scattering background from the scattering data from the sample. Further analysis needs to combine the data from two detector positions. This was done using Igor SANS data analysis from NIST, USA [19]. Igor SANS data analysis was also used to calculate and simulate the data.
3. Result and Discussion

The analysis of the SANS scattering profile of SPIONs coated apoferritin (figure 1) shows the fractal structure exists in the system. The fractal has block diameter and correlation length of 2.8 and 8.9 nm, respectively. The fractal dimension of the system is 2.7, which indicates that the agglomeration of the SPIONs is growth in partly three dimensions (x, y, z-axis direction). Therefore, the agglomeration of SPIONs particles will be formed an imperfect sphere. All those parameters are based on the fractal system modeled by Texeira [20]. The schematic figure of the fractal structure of the SPIONs based on SANS data is modeled in figure 2.

![Figure 1. SANS profile for the SPIONs-Apoferitin system](image1)

![Figure 2. The schematic model of SPIONs growth as fractal](image2)

The SAXS scattering data (figure 3) shows a different profile from SANS. It is fitted with a polydisperse sphere model, with the median particle size is 4.6 Å. The polydispersity of the system is represented by the value of sigma, which is 0.56. This value indicated that the system is high polydisperse. The polydispersity profile of the SPIONs coated apoferitin system is showed in Figure 4.

The different models, fitted to SANS and SAXS, indicate that these two techniques have a different way to see the particle in the system. Neutron, in SANS, sees the particles based on their scattering length density. The fractal structure, fitted SANS scattering profile, indicates that SANS only sees the SPIONs and doesn't see the apoferritin. It can happen due to the dialysis process during sample preparation before the SANS experiment. The dialysis from H2O based buffer to D2O based barrier makes the buffer in the system become H2O-D2O mixed. Scattering length density of protein will be identical with a solvent containing 42% D2O [21].
Meanwhile, in the SAXS experiment, an x-ray will see the difference of the electron density between the particles (apoferitin and SPIONs) and the solvent. Therefore, SAXS scattering data is fitted with a polydisperse model because of the existence of various sized particles. The polydisperse sphere model, which is suited to the SAXS scattering data, show that there are many particles with different size exist in the system. In other words, SAXS see all nanoparticle in the system.
Furthermore, it can be assumed that some of the free apoferritin and free SPIONs exist in the system. The schematic model of this system based on SAXS data are shown in figure 5.

This assumption is then confirmed with TEM data shown in figure 6. When the observation of the SPIONs coated apoferritin system was done using TEM without staining, the apoferritin did not see by the TEM camera. This observation method only captures the SPIONs (figure 6A). The apoferritin then is seen by the TEM camera when the staining method was applied. The uranyl acetate as a staining compound bound to the apoferritin as organic and enhances the electron density of the apoferritin. Consequently, all particles, i.e., free SPIONs, free apoferritin, and SPIONs coated apoferritin, were all captured by TEM camera (figure 6C).

![Figure 6](image)

**Figure 6.** TEM data (A) SPIONs-Apoferritin without staining, (B) pure apoferritin, and (C) SPIONs-Apoferritin with staining. The colorful arrows indicate the three types of particles in the system; red arrows refer to free apoferritin, green arrows refer to SPIONs coated apoferritin, yellow arrow refers to non-coated SPIONs.

The non-coated SPIONs, the coated SPIONs, and the free apoferritin are all existed in the system. It is showed that apoferritin could reassembly to its core-shell structure at neutral pH after disassembly at low pH in the SPIONs synthesis process. However, some of the apoferritin tends to reassembly as themselves rather than interact and coat SPIONs. This behavior of apoferritin has not been well explained yet. The agglomeration of SPIONs during its synthesis process might be the cause of apoferritin to unable coat SPIONs.

## 4. Conclusion

The SPIONs synthesized with the coprecipitation method have gained a small size. However, the data from SANS, SAXS, and TEM showed the existence of non-coated SPIONs, coated SPIONs, and free apoferritin in the system. The agglomeration between SPIONs that made them grew in a different size made apoferritin unable to coat them all. The utilization of apoferritin properties to reassembly at neutral pH seemed to be promising methods to coat SPIONs as the uniformity of the SPIONs could be reached.

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## References

[1] A. Rodzinski *et al.*, “Targeted and controlled anticancer drug delivery and release with magnetoelectric nanoparticles,” *Sci. Rep.*, vol. 6, no. January, pp. 1–14, 2016.

[2] L. Mohammed, H. G. Gomaa, D. Ragab, and J. Zhu, “Magnetic nanoparticles for
environmental and biomedical applications: A review,” Particuology, vol. 30, pp. 1–14, 2017.

[3] U. Sakulkhu, M. Mahmoudi, L. Maurizi, J. Salaklang, and H. Hofmann, "Protein corona composition of superparamagnetic iron oxide nanoparticles with various physico-chemical properties and coatings," Sci. Rep., vol. 4, pp. 1–9, 2014.

[4] R. A. Revia and M. Zhang, “Magnetite nanoparticles for cancer diagnosis, treatment, and treatment monitoring: Recent advances,” Mater. Today, vol. 19, no. 3, pp. 157–168, 2016.

[5] M. Musielak, I. Piotrowski, and W. M. Suchorska, “Superparamagnetic iron oxide nanoparticles (SPIONs) as a multifunctional tool in various cancer therapies,” Reports Pract. Oncol. Radiother., vol. 24, no. 4, pp. 307–314, 2019.

[6] M. Safi, J. Courtois, M. Seigneuret, H. Conjeaud, and J. Berret, “The effects of aggregation and protein corona on the cellular internalization of iron oxide nanoparticles I – Introduction,” no. July, pp. 1–21, 2011.

[7] M. Galli et al., “A new catechol-functionalized polyamidoamine as an effective SPION stabilizer,” Colloids Surfaces B Biointerfaces, vol. 174, no. October 2018, pp. 260–269, 2019.

[8] G. Kandasamy, S. Soni, K. Sushmita, N. S. Veerapu, S. Bose, and D. Maity, “One-step synthesis of hydrophilic functionalized and cytocompatible superparamagnetic iron oxide nanoparticles (SPIONs) based aqueous ferrofluids for biomedical applications,” J. Mol. Liq., vol. 274, pp. 653–663, 2019.

[9] S. Mohapatra, M. Asfer, M. Anwar, S. Ahmed, F. J. Ahmad, and A. A. Siddiqui, “Carboxymethyl Assam Bora rice starch coated SPIONs: Synthesis, characterization and in vitro localization in a micro capillary for simulating a targeted drug delivery system,” Int. J. Biol. Macromol., vol. 115, pp. 920–932, 2018.

[10] M. Ricci et al., “PPARs are mediators of anti-cancer properties of superparamagnetic iron oxide nanoparticles (SPIONs) functionalized with conjugated linoleic acid,” Chem. Biol. Interact., vol. 292, no. July, pp. 9–14, 2018.

[11] I. Blazkova, H. V. Nguyen, S. Dostalova, P. Kopel, and M. Stanisavljevic, “Apoferitin Modified Magnetic Particles as Doxorubicin Carriers for Anticancer Drug Delivery,” pp. 13391–13402, 2013.

[12] R. Massart, “Preparation of Aqueous Magnetic Liquids in Alkaline and Acidic Media,” IEEE Trans. Magn., vol. 17, no. 2, pp. 1247–1248, 1981.

[13] T. Y. Pratama, “SINTESIS DAN PENCIIRAN CORE/SHELL NANOPARTIKEL SUPERPARAMAGNETIK OKSIDA BESI (Fe 3 O 4 / γ - Fe 2 O 3 )/APOFERITIN (EQUEEN SPLEEN) TEGUH YANUAR PRATAMA,” 2016.

[14] E. G. R. Putra, Bharoto, and B. S. Seong, “Recent Development of a 36 meter Small-Angle Neutron Scattering BATAN Spectrometer (SMARTer) in Serpong Indonesia,” J. Phys. Conf. Ser., vol. 012010, no. 247, pp. 1–7, 2010.

[15] S. Soontaranon and S. Rugmai, “Small Angle X-ray Scattering at Siam Photon Laboratory,” Chinese J. Phys., vol. 50, no. 2, pp. 204–210, 2012.

[16] C. A. Scarff, M. J. G. Fuller, R. F. Thompson, and M. G. Iadaza, “Variations on negative stain electron microscopy methods: Tools for tackling challenging systems,” J. Vis. Exp., vol. 2018, no. 132, pp. 1–8, 2018.

[17] C. Dewhurst, “GRASP User Manual,” Tech. Rep. No. ILL03DE01IT, 2003.

[18] S. Rugmai and S. Soontaranon, “SAXSIT,” 2013. [Online]. Available: https://www.slri.or.th/en/index.php?option=com_content&view=article&id=3&itemid=85.

[19] S. R. Kline, “Reduction and analysis of SANS and USANS data using IGOR Pro,” J. Appl. Crystallogr., vol. 39, no. 6, pp. 895–900, 2006.

[20] J. Teixeira, “Small-Angle Scattering by Fractal Systems,” J. Appl. Cryst., vol. 21, pp. 781–785, 1988.

[21] K. L. Sarachan, J. E. Curtis, and S. Krueger, “Small-angle scattering contrast calculator for protein and nucleic acid complexes in solution,” J. Appl. Crystallogr., vol. 46, no. 6, pp. 1889–1893, 2013.
