Study of doxorubicin release on apoferritin-magnetic-doxorubicin nanoparticle

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Abstract. Conjugation of nanoparticle with some substance can be a solution to a theragnostic of cancer cells. The release of doxorubicin from conjugated apoferritin-magnetic-doxorubicin (APO-NPM-DOX) was studied in different pH conditions and incubation time. The preparation of magnetic nanoparticle (Fe₃O₄) was done through the co-precipitation method using FeCl₃ and FeCl₂ (mol ratio 1:1) as the precursor. The encapsulation process was started by conjugation of magnetic nanoparticle with doxorubicin, then followed by incubation of the mixture in apoferritin solution for 2 hours in pH 3. The mixture was then set to pH 8 using NaOH and dialyzed in Tris-HCl. The doxorubicin release from APO-NPM-DOX was studied by incubation at 36.5 °C in different time variations in pH 5 and different pH conditions (4, 5, 6, 7) for a week. The doxorubicin release trend becomes steady after three days of incubation in pH 5 with 4.6% of the doxorubicin had been released. The highest percentage of doxorubicin release was found in pH 4, which is more than 1.2 times higher than in pH 5.

1. Introduction
Cancer has been a serious problem until now. Based on World Health Organization data, 7.6 million people were dying because of cancer every year, and this number is estimated to increase to 13.1 million in 2030 [1, 2]. Many cancer therapy methods have been widely developed, and one of them is through chemotherapy.

However, cancer chemotherapy still has a problem in the off-targeting distribution of conventional drugs due to their side effects, where cytotoxic drugs are internalized by healthy cells beside the primary target, such as cancer cells. As an effect, healthy cells have also been attacked by the drugs [3, 4]. Moreover, the drugs’ translocation, activation, and excretion process are hard to be tracked in vitro and in vivo, due to most anticancer drug compounds are intrinsically non-fluorescent or weakly fluorescent [4].

Tackling this issue, controlled targeted and stimuli-responsive drug delivery system for cancer therapy has been widely developed, such as conjugating doxorubicin to magnetic nanoparticle-PLGA (Fe₃O₄-PLGA) [3], Fe₃O₄-chitosan [5], Fe₃O₄-liposome [6], Fe₃O₄-dextran [7], Fe₃O₄-polydopamine [8], or maghemite-apoferritin [9]. Combining Fe₃O₄ with doxorubicin can offer several advantages for cancer therapy. The Fe₃O₄ nanoparticle can be used as a probe for hyperthermia therapy and MRI agent [6, 9], while the doxorubicin can be used for chemotherapy.

Apoferritin, an empty ferritin protein, is a spherical protein shell consisting of 24 subunits covering an aqueous cavity of approximately 8 nm in diameter. This hollow protein can be loaded with many kinds of metallic and non-metallic species [9, 10]. Apoferritin can be used as a potential theragnostic
agent because of its small size, biocompatibility, and non-immune tolerance [11, 12]. Thus it can increase the biocompatibility of Fe$_3$O$_4$ nanoparticle and reducing doxorubicin cardiotoxicity effects [13]. Chen [14] compared the use of free doxorubicin and doxorubicin encapsulated in apoferritin in mice. The results showed that the encapsulated doxorubicin has a lower weight loss percentage and lower liver damage, which indicates apoferritin can reduce the off-target doxorubicin and lower the toxicity.

Only a few research reports the combination of Fe$_3$O$_4$ nanoparticle, apoferritin, and doxorubicin for cancer therapy [15], mostly research studied the combination of apoferritin and doxorubicin [14, 16, 17]. Moreover, the release of doxorubicin from the nanoparticle is an important issue to study since it will prevent healthy cells damage and maintain a high concentration of cancer drugs in the cancer cells for a long time [9]. Thus, in this research, we synthesize Fe$_3$O$_4$-apoferritin nanoparticle loaded with doxorubicin (APO-NPM-DOX). Then the doxorubicin release from APO-NPM-DOX was investigated in different pH conditions and time.

2. Materials and Methods

2.1. Chemicals
The materials used in this research were HCl 37%, NaOH, NH$_4$OH, FeCl$_3$·6H$_2$O, FeCl$_2$·4H$_2$O, NaNO$_3$, HNO$_3$, all obtained from Merck with pro-analysis grade. Doxorubicin 2 mg/mL purchased from Dankos and 0.2 µm filtered apoferritin obtained from Sigma-Aldrich.

2.2. Synthesis of apoferritin-nanoparticle magnetic-doxorubicin (APO-NPM-DOX)
Magnetite nanoparticle was synthesized using the co-precipitation method of Fe (II) salt with Fe (III) salt with the mol ratio of 1:1. In this process, 3.58 g of FeCl$_3$·6H$_2$O and 1.32 g of FeCl$_2$·4H$_2$O were dissolved in 20 mL HCl 0.3 M. Then, 16.75 mL of NaNO$_3$ 6 M was added into the mixture and followed by ultrasonication using an ultrasonic probe for 2 minutes. The mixture of Fe salts-NaNO$_3$ was added into 185 mL NH$_4$OH 0.6 M and ultrasonicated for 10 minutes. The mixture was dried for 2 hours in an atmospheric condition. The precipitate was separated from the supernatant using an external magnet followed by dispersion in 20 mL of HNO$_3$ 1 M and ultrasonication for 2 minutes. Then the mixture was separated using centrifuge 9000 rpm for 10 minutes. The obtained precipitate was dispersed in 30 mL DI-water and ultrasonicated for 5 minutes.

Encapsulation of magnetic nanoparticle and doxorubicin by apoferritin was started by dissolving 30 µL of doxorubicin (2 mg/mL) in the magnetic-HNO$_3$ solution (1 mg/mL) and was shaken using a rotary shaker for 1 minute. Then 27.8 µL of apoferritin (25.6 mg/mL) was added into the mixture and shook gently using a rotary shaker. HCl 1 M was added into the mixture of apoferritin-nanoparticle magnetic-doxorubicin (APO-NPM-DOX) until pH 3 followed by incubation for 2 hours. Next, NaOH 1 M was added into the mixture until pH 8. The mixture then was dialyzed in Tris-HCl media pH 7 using a dialysis cassette (molecular weight cut-off 20 kDa) inside a refrigerator and stirred at 200 rpm for 24 hours.

2.3. Release doxorubicin from apoferritin-nanoparticle magnetic-doxorubicin (APO-NPM-DOX)
The release study was conducted at two different conditions, pH variation and time variation. The time variation was conducted at 1, 8, 24, 72, 122, 145, and 169 hours, at pH 5 (using acetate buffer 0.1 M media). In brief, 2 mL of APO-NPM-DOX was incubated in 20.0 mL acetate buffer pH 5 in a Slide-A-Lyzer® Mini Dialysis Devices (molecular weight cut-off 10 kDa). For the time variation, 1 mL was withdrawn from the tube and replenish an equal volume of pH 5 acetate buffer. The concentration of doxorubicin released was measured using a microplate reader (BioTek™ Eon™ Microplate Spectrophotometers) at a wavelength of 480 nm.

The pH variation was conducted at pH 4, 5, 6, and 7 (using acetate buffer 0.1 M media for pH 4 and 5, while PBS buffer 0.1 M media used for pH 6 and 7). The APO-NPM-DOX samples (500 µL each) were incubated in the media (1.5 mL) for a week at 36.5 °C using a Slide-A-Lyzer® Mini
Dialysis Units (molecular weight cut-off 10 kDa). Since, after one week, the samples have dried, then the samples need to be re-dispersed again using 400 µL from each buffer. The concentration of doxorubicin released was measured using a microplate reader (BioTek™ Eon™ Microplate Spectrophotometers) at a wavelength of 480 nm.

3. Result and discussion

APO-NPM-DOX nanoparticles have been synthesized by co-encapsulation of doxorubicin with APO-NPM through the pH driven assembly-disassembly of apoferitin. The assembly-disassembly ability of apoferitin is useful, and it was used in this research to encapsulate doxorubicin and magnetic nanoparticle. Hopefully, the apoferitin will increase the biocompatibility through the nature of apoferitin as a biomolecule and by preventing the agglomeration of magnetic nanoparticle and uncontrollable doxorubicin [18, 19].

In acidic conditions (in this research pH 3), apoferitin will disassemble into their 24 subunits. In this condition, doxorubicin was added into the mixture of APO-NPM. Increasing the pH condition until more than 8 will led the APO re-assembly again into the spherical form with NPM and doxorubicin inside the spherical APO [9, 10, 14, 15, 17]. The dialysis process after the co-encapsulation process was necessary for purifying the APO-NPM-DOX from the free doxorubicin and to uniform the size of the APO-NPM-DOX [20]. The illustration of the co-encapsulation process is shown in Fig. 1.

![Figure 1. Co-encapsulation process of doxorubicin in apoferitin-magnetic nanoparticle (APO-NPM) [15].](image)

The release of doxorubicin in different time variation was conducted in pH 5 due to the disassembly of apoferitin in low pH that led to the increase of doxorubicin release. pH lower than 5 increases the doxorubicin release. However, it can make the doxorubicin release uncontrolled, which can also risk the healthy cells into damage. Moreover, lowering the pH of more than 5 will make the release condition far from mimicking the pH of cancer cells, especially the extracellular, which represents the cancer cell's environment. Cancer cells are believed to have the pH range from 6.0 to 7.5, with the intracellular pH (pH_i) 7.12-7.65 is higher than the extracellular pH (pH_e) 6.2-6.9. This pH range is lower than the normal cell pH 7.2-7.5 [21, 22]. Thus, instead of only conducting the time dependence release in pH 5, we also conducted the pH variation release, from pH 4 to 7, to confirm the trend that the doxorubicin release increases as the pH decreases.

Based on our data, the doxorubicin concentration in our APO-NPM-DOX sample is 320 µg/mL or 32 µg/100 µL. Fig 2. illustrates the release of doxorubicin in a range of time. The graph shows that the release of doxorubicin reaches its steadiness after 72 hours of incubation APO-NPM-DOX in acetate pH 5 buffer media. Around 4% of the doxorubicin has been released after 72 hours of incubation or equal to 12.8 µg/mL. The steadiness of the graph indicates that an equilibrium point has been reached. It is interesting to notice that a high accumulated percentage of doxorubicin released in the first 24 hours (more than 3% doxorubicin released). This phenomenon was probably because of doxorubicin's interactions with the hydrophobic and hydrophilic channels of the protein molecule, which impeded the diffusion release of doxorubicin [9].
Figure 2. (a) Accumulated released doxorubicin trend from APO-NPM-DOX in pH 5, PBS buffer media, (b) photographs of the samples, the samples show an increase of red colour after several hours (indication of doxorubicin release).

Fig 3. shows that the highest concentration of doxorubicin released from the APO-NPM-DOX was in pH 4, with the doxorubicin has been released is 1.26 times higher than in pH 5 or 1.1% has been which is equal to 3.59 µg/mL. While the lowest release of doxorubicin occurred in pH 7, with only 0.16% of doxorubicin has been released or equal to 0.512 µg/mL. The data were normalized to pH 5 because it can be misinterpreted with the time dependence release data since the time dependence also the percentage unit. The different release percentage data (in pH 5) between the time dependence and the pH variation can be caused by the different doxorubicin release methods and the re-disperse procedure in the pH variation release. However, the overall trend of the doxorubicin released decreases while the pH increase. The release trend is reasonable due to the stability of apoferritin spherical form in higher pH will inhibit the release of doxorubicin, while in lower pH, the spherical form of apoferritin disassembly into their subunits, making the release of doxorubicin more easily [9, 23]. Besides that, the potential of the inner surface protein is more positive when the pH decrease, thereby reducing the electrostatic adsorption force with positively charged doxorubicin molecules [9, 24].

Figure 3. Percentage of doxorubicin released in different pH condition after a week of incubation.

Based on the results, especially from the time-dependent doxorubicin released data, the APO-NPM-DOX can be a potential theragnostic agent. It is believed that an ideal theragnostic agent should gradually dissociate from its delivery carrier to retain a high concentration in the cancer cells for a long time [9]. However, a low percentage of doxorubicin release in higher pH could be a problem, since cancer cells have a pH around 7. This issue should be explored further, but since the APO-NPM-DOX offers multi-modal cancer treatment, the low release of doxorubicin in high pH could not be a problem.

The magnetic nanoparticle (NPM) core could offer another alternative of cancer therapy through hyperthermia. In theory, applying an external magnetic field would flip the magnetic polarity of the NPM rapidly. This flipping phenomenon will cause a hysteric loss, which manifests as heat [25]. The heat would probably heat up the cancer cell, which led to hyperthermia or damage such as apoptosis or cell death caused by a small temperature rise around 40-45 °C [25, 26]. The rise of temperature will probably increase the amount of doxorubicin released from the APO-NPM-DOX. In
the previous study, the amount of DOX released from magnetic nanocomposites-doxorubicin increased with the increase in temperature [27]. However, the apoferritin thermal stability up to 80-100 °C should also be considered [28]. Lastly, until now, there is no research about the drug release from apoferritin in higher temperatures (more than 40 °C).

4. Conclusion
The time-dependent doxorubicin release experiment shows that a high release percentage occurs in the first 24 hours of incubation. More than 3% of doxorubicin has been released, and then the release remained to increase but not significantly, indicating an equilibrium point has been reached. As predicted before, for the pH variation, the highest percentage of doxorubicin release from the APO-NPM-DOX occurs in low pH (pH 4), with approximately 1.2 times higher than in pH 5. Overall, the synthesized APO-NPM-DOX shows a promising result as a potential theragnostic agent for cancer therapy. It shows a slow release of doxorubicin that can retain high concentration doxorubicin near the cancer cell for a long period. However, further experiments should be done for increasing the doxorubicin release in cancer cells pH (6.0 – 7.5), so the APO-NPM-DOX can be more efficient in use.

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