Synthesis and characterization of targeted superparamagnetic MRI contrast agent  MnFe$_2$O$_4$-chitosan-folate

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Abstract. Superparamagnetic materials MnFe$_2$O$_4$ in nanosized form, is useful for some applications such as magnetic storage, electronic devices, biosensing and MRI contrast agent. Efficiency of the contrast agent (CA) delivery to cellular targets generally depends on the rate of vascular extravasation and pharmacokinetics in the plasma. Contrast agents with longer circulation time could show better kinetics at the target site. In this research, strategies to prolong the plasma life is conjugating MnFe$_2$O$_4$ NPs with chitosan-folate. MnFe$_2$O$_4$ NPs were synthesized from MnCl$_2$ with FeCl$_3$ using a reducing agent NaOH. In other side, chitosan was conjugated with folic acid to produce chitosan-folate compounds. Then chitosan-folate was reacted with MnFe$_2$O$_4$ nanoparticles to produce MnFe$_2$O$_4$-chitosan-folate. MnFe$_2$O$_4$ nanoparticles was characterized using UV Vis, FTIR, PSA and SEM EDX. The characterization of MnFe$_2$O$_4$-chitosan-folate is also carried out using radioactive agent by labeling with $^{99m}$Tc radionuclide which will produce the MnFe$_2$O$_4$-($^{99m}$Tc) Chitosan-folate complex. The characterization tests using $^{99m}$Tc radionuclides showed yields and radiochemical purity of more than 95%. It indicates that the compound of MnFe$_2$O$_4$-chitosan-folate has been successfully synthesized. MnFe$_2$O$_4$-chitosan-folate nanoparticles as finished product will be used for targeted MRI Contrast agent.

Keywords : superparamagnetic, $^{99m}$Tc, MnFe$_2$O$_4$ nanoparticles, contrast agent, MRI

1. Introduction

MRI is one of the imaging modalities that is widely used in many hospitals in Indonesia. Increasing the utilization of this modality to the level of "molecular imaging" to detect cancer more accurately needs to be developed and applied by utilizing the potential that is already available in Indonesia [1]. The quality of MRI (Magnetic Resonance Imaging) for diagnose can be improved by using contrast agents that are still imported. Contrast agents for MRI have been widely used since the last decade to improve the image of organs / tissues that are difficult to distinguish through ordinary MRI imaging techniques, especially in the soft tissue of the central nervous system, liver, digestive system, lymphatic system, breast, cardiovascular system and pulmonary. The two major classes of MRI contrast agents are paramagnetic contrast agents, usually based on chelates of Gd generating T1 positive signal enhancement, and super-paramagnetic contrast agents that use mono- or polycrystalline iron oxide to generate strong T2 negative contrast in MRI images. Gadolinium (Gd) is one of the very strong paramagnetic elements and meets important requirements as a contrast agents, because these complex have a high stability, safe, also provides some mild side effects such as slight headaches,
nausea, whereas allergic reactions rarely occur. The pathological analyses have revealed that residual gadolinium is not only deposited in these brain regions, but also found in extracranial tissues such as liver, skin and bone [2-3]. The average detected residual Gd concentration in the brain was approximately 15-fold higher for linear than for macrocyclic Gadolinium Based Contrast Agents. The highest amounts of Gd found in brain corresponded to less than 0.0002% of the injected dose per gram of tissue [4-5].

The development of targeted MRI contrast agents using certain specific molecules will expand their application to be specific to these molecular targets. The nanostructures of a magnetic particle, will expand its use in the field of biomedical drug delivery, cellular signaling and hyperthermia. The results of imaging with MRI provide good anatomy details with high spatial resolution without using radioisotope or X-ray, but MRI sensitivity is still inadequate for detecting a little changes in tissue, which is very important for early detection of the disease [6-7].

Material in the form of nanoparticles has an important role in the field of medicine and biotechnology. Spinel ferrite which has the formula as MnFe₂O₄ (M = Mn, Fe, Co, Ni, etc.) is a very promising candidate for understanding and controlling the magnetic properties of nanoparticles at the atomic level. In the magnetic nanoparticle group, the ferrite spinel MnFe₂O₄ is widely used in several applications such as high-density magnetic recording, magnetic resonance imaging, treatment of hyperthermia and also as a drug carrier. The interesting fact of ferrite spinel MnFe₂O₄ are the magnetic and electrical properties of the MnFe₂O₄ NPs can be tuned by changing the identity of the divalent M2+ cation or by partial substitution. Manganese metal (Mn) belongs to the same paramagnetic group as gadolinium and copper which is able to increase the intensity of the T1 signal in the Magnetic Resonance (MR) images [8-13].

Cancer cells, unlike normal cells can express folate receptors with a high affinity for folic acid. These receptors can be used for uptake of folic acid in cell. There are two major forms of folate receptors: folate receptor alpha (FR-α) and folate receptor beta (FR-β). FR-α primarily exist on cancer cells and FR-β exists on monocytes and macrophages in a cluster on chromosome [14].

![Figure 1. Diagnosed cancer patients over express folate receptors in Us, Europe and japan.](image)

Chitosan is a cationic poly-saccharide and pH sensitive polymer. Nanoparticles which contain chitosan can be used to investigate their cell binding on normal and cancer cells and to explore their potential for tissue targeting [16-18]. Also radiolabeled drugs or formulations can be used for in vitro cell binding studies by measuring the radioactivity in different types of cells after administration of labeled formulations to the cells. After preparation, MnFe₂O₄-chitosan-folate was labeled with a commonly used radioisotope ⁹⁹mTc to form MnFe₂O₄-(⁹⁹mTc)chitosan-folate where, chitosan forms complex through its dextran moiety (Beta(1,4)-D-glucosamine) with reduced ⁹⁹mTc ions and the amine groups of polymer is also expected to bind to ⁹⁹mTc ions.
The present study was aimed to synthesis, optimize and characterize MnFe$_2$O$_4$-chitosan-folate as a promising targeted MRI contrast agents for cancer diagnosis that can be expressed by folate receptors. Characterization of the MnFe$_2$O$_4$-chitosan-folate compound was carried out by chemical methods and also by using radioisotope $^{99m}$Tc. In the labeling of MnFe$_2$O$_4$-chitosan-folate compound with $^{99m}$Tc, optimization parameters on the labeling has been done through labeling time and pH variations [19].

2. Materials and methods

2.1. Materials.

All materials used in this research are MnCl$_2$.4H$_2$O, FeCl$_3$.6H$_2$O, NaOH and PEG 400 supplied from E. Merck. Chitosan medium molecular weight supplied from Sigma Aldrich. All chemical were purchased without purification.

2.2. Synthesis of MnFe$_2$O$_4$ nanoparticles

MnFe$_2$O$_4$ nanoparticles were synthesized by coprecipitation method using MnCl$_2$, FeCl$_3$ and precipitated with NaOH solution at pH 11. After the precipitate has formed, the reaction was continued for 1 hour, then 1 ml of PEG 400 was added. The whole mixture continued to reaction for 2 hours at the temperature 70 °C with continuous stirring. The product was separated by using a magnet. The precipitated washed 2 times with water and dried at 80 °C for 5 hours. Characterization of MnFe$_2$O$_4$ nanoparticles compound was carried out using a UV / Vis spectrophotometer, SEM EDX, FTIR and TEM.

2.3. Synthesis of Chitosan-folate

Chitosan-folate complex was synthesis by reacting ester of folic acid with chitosan, the reaction in the dark at room temperature 16 h and Chitosan-folate was precipitated using acetone, centrifugation 13000 rpm for 30 min, and washed by centrifugation three times with ultrapure water, freeze-dried to obtain Chitosan-folate.

2.4. Synthesis of MnFe$_2$O$_4$-Chitosan-folate nanoparticles

MnFe$_2$O$_4$-chitosan-folate nanoparticles were synthesized by reacting MnFe$_2$O$_4$ with chitosan-folate at room temperature for 16 hours, protected from light. The product a MnFe$_2$O$_4$ -Chitosan-folate compound was separated using a magnet. The compound was centrifuged and washed several times with water, then dried at 50°C C for 8 hours. Characterization of MnFe$_2$O$_4$-chitosan-folate nanoparticles compounds was carried out using a UV / Vis spectrophotometer, FTIR and labeling the complex with radionuclide Technetium-$^{99m}$Tc.

2.5. Labeling of MnFe$_2$O$_4$-Chitosan-folate nanoparticles with $^{99m}$Tc radionuclide

Labeling of the MnFe$_2$O$_4$-chitosan-folate complex with radionuclide $^{99m}$Tc was carried out by reducing TcO$_4^-$ with SnCl$_2$ solution. Labeling of the MnFe$_2$O$_4$-chitosan-folate complex with $^{99m}$Tc radionuclides was carried out at pH 2.5, 4, 5, 6, 7 and 9. The reaction time were varied from 10 minutes, 20 minutes, 30 minutes and 45 minutes. Yield and purity of radiochemistry of labeling results was calculated.
3. Results and discussion

The superparamagnetic MRI-targeted contrast agents MnFe$_2$O$_4$-chitosan-folate in this study was synthesized based on 3 reaction steps, namely synthesize of MnFe$_2$O$_4$ nanoparticles, synthesize of Chitosan-folate and reacting chitosan-folate with MnFe$_2$O$_4$ nanoparticles to produce MnFe$_2$O$_4$-chitosan-folate. In this research, spinel ferrite MnFe$_2$O$_4$ nanoparticles was synthesized by coprecipitation method from MnCl$_2$ and FeCl$_3$ using NaOH solution as reductor.

![Figure 2. FTIR spectra of MnFe$_2$O$_4$ nanoparticles](image)

The characterization of MnFe$_2$O$_4$ nanoparticles using FTIR are shown in Figure 2. The IR spectra indicated that two main metal–oxygen bands due to vibrations of the Fe–O and Mn–O at wave numbers that appear at 885 and 584 cm$^{-1}$ respectively.

| No. | Element       | % Mass      | % atom      |
|-----|---------------|-------------|-------------|
| 1   | (O)           | 27.87±2.78  | 43.09±8.4   |
| 2   | Manganese (Mn)| 12.56±5.17  | 5.77±2.86   |
| 3   | Iron (Fe)     | 25.04±9.88  | 11.31±5.38  |
| 4   | C             | 8.82±4.68   | 17.43±7.74  |
| 5   | Na            | 14.14±5.67  | 14.74±4.84  |
| 6   | Cl            | 11.44±7.25  | 7.61±4.18   |
| 7   | Zn            | 0.13±0.23   | 0.05±0.08   |

![Figure 3. SEM-EDX micrograph of MnFe$_2$O$_4$ nanoparticles](image)

The SEM-EDX micrographs analysis confirm the presence of the elements Mn, Fe and O. The relative ratio is given in the table was shown in the Figure 3. From Figure 3, the elemental value from the composition obtained from nanoparticle synthesis was showed that the compound formed was MnFe$_2$O$_4$. 
Figure 4. Particle Size Analyzer (PSA) spectra of MnFe$_2$O$_4$

The particle size distribution using PSA as shown on Figure 4 of the MnFe$_2$O$_4$ compound showed that the majority of the size dominated was 100-150 nm. The particle size certainty of the MnFe$_2$O$_4$ compound using the TEM can be seen as shown in Figure 5.

Figure 5. TEM images of MnFe$_2$O$_4$ nanoparticles

As seen in Figure 5, the results of TEM images was seems less clear which might be due to the poor dispersion of the sample but from this figure it still can be concluded that the formed particle size was smaller than 50 nm.

Figure 6. Chromatogram UV/Vis from Chitosan-folate, MnFe$_2$O$_4$ and MnFe$_2$O$_4$-chitosan-Folate
UV-visible spectroscopy is a simple technique in optical characterization to investigate the optical properties of manganese ferrite-based nanoparticles. Using mineral-free water as a solvent, a colloidal solution of MnFe$_2$O$_4$-chitosan-folate nanoparticles was prepared and the absorption spectrum observed at wavelengths between 200-800 nm. It can be observed that the sample was absorbed in the wavelength of 219 nm, which is 1 nm shift from the initial wavelength of MnFe$_2$O$_4$ which is 218 nm, which indicates the occurrence of a batochromic effect. As seen at Figure 6, UV/Vis chromatogram of Chitosan-folate, MnFe$_2$O$_4$ and MnFe$_2$O$_4$-chitosan-folate were very different.

Figure 7. UV/Vis Spectra characteristic of MnFe$_2$O$_4$-Chitosan-Folate at pH 2.5, 5 and 9

At Figure 7, the chromatograms of spectrofotometer UV/Vis indicated that at pH 5 the MnFe$_2$O$_4$ was trapping inside chitosan-folate complex. The results of this chemical examination are consistent with the radiochemical purity testing of the MnFe$_2$O$_4$-Chitosan-folate complex which has been labeled with radionuclide $^{99m}$Tc (Figure 10 and Figure 11).

Figure 8. FTIR spectra of MnFe$_2$O$_4$-chitosan-folate

At Figure 8, wave numbers that appear at 885 and 584 cm$^{-1}$ respectively identified that there are Mn-O and Fe-O vibrations from the MnFe$_2$O$_4$ component. The adsorption broad band at the range of 3200–3450 cm$^{-1}$ represents a stretching mode of –OH groups and H$_2$O molecules. The adsorption band at 1,419 (O–H bending vibration), and 1,630 cm$^{-1}$ (N–H bending vibration).
Figure 9. Effect of reaction time to yield of $\text{MnFe}_2\text{O}_4(99m\text{Tc})\text{chitosan-folate}$

Labeling a complex or compound by using radioactive materials can help in the case of characterization of these compounds to be more quickly and accurately. The use of $99m\text{Tc}$ radionuclides for labeling of $\text{MnFe}_2\text{O}_4$-chitosan-folate nanoparticles have been done by reduction of $99m\text{Tc}$ using tin(II) ion, then reduced $99m\text{Tc}$ will react with $\text{MnFe}_2\text{O}_4$-chitosan-folate to form $\text{MnFe}_2\text{O}_4(99m\text{Tc})\text{chitosan-folate}$ complex. To obtain the optimal labeling results, optimization has been done through labeling time variations and pH, the results can be seen in Figure 9 and Figure 10. The parameters used to see the labeling efficiency are yield and radiochemical purity. The optimization results showed that the highest yield was obtained from labeling time of 30 minutes.

Figure 10. Effect of pH to yield of $\text{MnFe}_2\text{O}_4(99m\text{Tc})\text{Chitosan-Folate}$

Variations of pH in the synthesis of the complex of $\text{MnFe}_2\text{O}_4(99m\text{Tc})\text{Chitosan-folate}$ was showed different results in the yields as shown in Figure 10 where in pH 5 to 6 complex compound produced more than 90% yield whereas at pH < 5 and pH > 7 the yields were decreased less than 90%. The results of paper chromatography with whatman 1 paper as the stationary phase and acetone as the mobile phase showed that the percentage of the compound $\text{MnFe}_2\text{O}_4(99m\text{Tc})\text{chitosan-folate}$ was obtained more than 95% at the reaction pH of 5 and 6 (Figure 11). Radiochemical purity (RCP) is defined as the percent of the total radioactivity present in the desired chemical form in a radioactive compound.
4. Conclusion

Labeling a contrast agent MnFe$_2$O$_4$-chitosan-folate by using $^{99m}$Tc radionuclides can help in the case of characterization of these compounds to be more quickly and accurate. The results of all chemical tests including test using $^{99m}$Tc radionuclide on MnFe$_2$O$_4$-chitosan-folate compound showed conformity. Based on the observations results on synthesize and characterization of MRI targeted contrast agent MnFe$_2$O$_4$-chitosan-folate that have been carried out, it can be concluded that the compound of MnFe$_2$O$_4$-chitosan-folate has been successfully synthesized.

5. References

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