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Research Paper

A Study on the Synthesis of [Gadolinium-(1,4,7,10 -Tetraazacyclododecane 1,4,7,10 Tetraacetate Acid)-Folate] as a Targeted Contrast Agent for Cancer Diagnosis Using Magnetic Resonance Imaging

Erianti Siska Purnamasari[1*], Husein Hernadi Bahti[2]

[1*]International Women University, Indonesia
[2]Universitas Padjadjaran, Indonesia

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Abstract

Gd-DOTA is a contrast agent approved by the Food and Drug Administration and the European Medicines Agency, but it is less specific when used to diagnose cancer. The purpose of this study was to synthesize a Gd-DOTA-Folate compound as a targeted contrast agent for the diagnosis of cancer using MRI. Synthesis is carried out through three stages of the reaction, namely: 1). The reaction for the formation of EDA-Folate (ethylenediamine-folate), 2). The reaction for the formation of DOTA-Folate, and 3). Gd-DOTA-Folate formation reaction. The product of the Gd-DOTA-Folate complex compound produced was purified, and then characterized using ultraviolet spectrophotometry, infrared spectrophotometry, and mass spectrometry methods. This study also aimed to examine the interaction and stability of Gd-DOTA-Folate on folate receptors through docking simulations and molecular dynamics. The resulting spectroscopic data show that the Gd-DOTA-Folate compound has a maximum UV wavelength of 253 nm, a specific infrared spectral wave number of 460.22 cm-1, and a peak in the mass spectrum with m/z 1025.6561, which corresponds to the calculated molecular mass m/z 1024.1070. Furthermore, data generated from docking simulation experiments and molecular dynamics showed that the addition of Gd-DOTA to the folate structure increased the affinity of folate for folate receptors. The interaction of Gd-DOTA-Folate with α-folate receptors, has produced an energy of -7.91 kcal/mol and -37.8196 kcal/mol, indicating that the Gd-DOTA-Folate product was stably bound to the folate receptors.

Keywords: *Gd-DOTA-Folate, targeted contrast agent, MRI, docking simulation, molecular dynamics*

INTRODUCTION

Cancer is a major global health burden. The World Health Organization (WHO) mentions cancer as one of the primary causes of death worldwide. Data from the Global Burden of Cancer (GLOBOCAN) released by the World Health Organization (WHO) indicated that the number of cases and deaths from cancer up to 2018 amounted to 18.1 million cases and 9.6 million deaths in 2018. Deaths from cancer will continue to increase to more than 13.1 million in 2030 (Pangribowo, 2019). Cancer cases are found at an early stage and receiving prompt and appropriate treatment will provide healing and longer life expectancy. Therefore, it is important to prevent and detect cancer early (Purwoko, 2018).

Magnetic Resonance Imaging (MRI) is a medical imaging process that utilizes magnetic fields and radio frequency energy to produce images of anatomical structures, the presence of diseases, and various biological functions in the human body (Sprawls, 2018). MRI produces very different images from those produced by other imaging modalities. The main difference is that MRI can selectively image several different tissue characteristics (Sprawls, 2018). MRI can detect cancer

Corresponding author's email: eriantisiska@iwu.ac.id

quicker than other diagnostic techniques.

To improve the quality of the images produced, diagnostic techniques with MRI are assisted with a contrast agent (Mutalib et al., 2015). The contrast agent that has been recommended by the US FDA since 1988 is Gd-DTPA with the trademark Magnevist (Mutalib et al., 2015). Another contrast agent that was approved by the FDA and EMA in 2013 is Gd-DOTA with the trademark Dotarem (Grogna et al., 2011). Gd-DOTA is known as a contrast agent that has better chemical stability than Gd-DTPA because the DOTA ligand has five times higher complex stability than the DTPA ligand (Grogna et al., 2011). The two contrast agents can detect abnormalities in the human body, including cancer, but cannot be used to detect cancer specifically. For this purpose, a new directional contrast agent was developed, namely a directional contrast agent (Mutalib et al., 2015). Directional contrast agents in their molecular structure contain ligands that have a high affinity for receptors on cancer cells. This ligand serves to direct all the molecules of the contrast agent to go to the target, namely cancer cells (Mutalib et al., 2015).

Folate receptors can be used as cancer markers because they are overexpressed, especially in 80% of epithelial ovarian cancers (EOCs), breast, endometrial, kidney, lung, and brain cancers. Folate receptors are a glycophosphosphatidylinositol (GPI) protein with a high affinity for the vitamin folic acid (Fauzia et al., 2015). Cancer cells that divide rapidly can express folate receptors because of their relatively high affinity of folate receptors for drugs or contrast agents. Those that use folic acid as a carrier will enter cancer cells through a folate-mediated endocytosis mechanism (Adang et al., 2012).

Kalber et al., (2011) have successfully synthesized a low-molecular-weight contrast agent Gd-DOTA-Folate through seven reaction steps. In this study, the linker used to conjugate folic acid with Gd-DOTA, namely ethylenediamine (EDA), was different from the linker used by Kalber et al., (2011) and its synthesis approach, which was carried out in the solution phase, whereas Kalber et al., (2011) involved one of the stages of its synthesis in the solid phase. Other studies were also conducted by (Fauzia et al., 2015), namely the synthesis and characterization of the directed compound Gd-DTPA-Folate. The drawback of this compound is that the ligand used is DTPA in the linear form. The European Medicine Agency (EMA) has recommended limiting linear chelating-based contrast agents to prevent the dangerous release of gadolinium ion (Gd^{3+}). Therefore, there is a need for a chelating agent that can provide stable and safe macrocyclic chelators (Jeong & Na, 2018).

(Yusuf et al., 2021) conducted a docking and molecular dynamic (MD) simulation study on the structure of the Gd-DTPA-Folate directed contrast agent with an EDA linker. The simulation results showed that Gd-DTPA-Folate with an EDA linker has a binding energy of -42 .78 kcal/mol, which is stable during the simulation. EDA is also widely used as a linker to conjugate folic acid with good compounds of protein, cholesterol, and polymers (Li et al., 2015; Trindade et al., 2014).

The synthesis process is carried out through the initial stage of forming the EDA-Folate precursor by first activating folic acid using N,N. dicyclohexylcarbodiimide (DCC) and N-hydroxysuccinimide (NHS) (Razjouyan et al., 2015; Li et al., 2015) producing NHS-Folate which is then conjugated with EDA to obtain the EDA-Folate precursor (Fauzia, et al., 2017; Trindade et al., 2014; Li et al., 2015). The next stage of DOTA-Folate precursor synthesis from DOTA-NHS and EDA-Folate produced from the first and last stages, namely, conjugation of gadolinium ions (Gd^{3+}) with DOTA-Folate precursors to become Gd-DOTA-Folate. The resulting Gd-DOTA-Folate complex compound product will then be purified and characterized using ultraviolet, infrared, and mass spectrometry methods.

Based on the background presented, the main problems studied are as follows: what Gd-DOTA-Folate is synthesized through the reaction of the formation of the main precursor EDA-Folate and DOTA-Folate with gadolinium ions and what is the effect of the addition of Gd-DOTA on the folate

structure of the folate receptor through docking and molecular dynamics simulation. The purpose of this research was to synthesize Gd-DOTA-Folate compounds through the reaction of the formation of the main precursors EDA-Folate and DOTA-Folate with gadolinium ions and to determine the effect of adding Gd-DOTA on the affinity of folate with folate receptors through docking and molecular dynamics simulations.

LITERATURE REVIEW

Magnetic resonance imaging (MRI) is one of the most well-established modalities in the field of noninvasive medical imaging. Since its inception, molecular imaging has been commonly used to further the understanding of disease progression and monitor treatment efficacy. This has naturally led to the advancement of targeted imaging (Koudrina & DeRosa, 2020).

Contrast agents are chemicals used in Magnetic Resonance Imaging (MRI) scans that are injected into the body to improve the quality of MRI. Contrast agents consist of ions and chelating agents that are chemically bonded (Ferris & Georgen, 2017). One of the metal atoms that is often used in contrast agents is gadolinium metal (Gd^{3+}). Contrast agents, which are the most crucial medical image enhancement methods, are required to achieve targeted image enhancement of the target area. Traditional contrast agents are mostly small molecules with several disadvantages, such as rapid metabolism and poor targeting in human circulation and tumour, limiting their clinical efficacy (Lai et al., 2024).

Folate receptor α is overexpressed in 80% of epithelial ovarian cancers (EOCs), and its expression correlates with histological grade. This overexpression is a marker of cancer aggressiveness. Overexpressing folate receptors reduces the level of response to chemotherapy agents. However, several studies have shown that folate receptors expressed on the tumor surface do not differ between samples before and after chemotherapy. This indicates that chemotherapy agents do not affect the antigen expression of the disease, highlighting the potential for folate receptors to be used as targets for therapeutic and disease diagnosis (Cheung et al., 2016).

Eukaryotic cells cannot synthesize folate, so folate is delivered into cells either via reduced folate carriers, which are present in all cell types, or FRs, which are expressed in limited amounts in normal cells. Although folate is transported into cells via either system, folate conjugates are designed for diagnostic and therapeutic purposes. By exploiting the high affinity of folate receptors in cells, compounds that bind to folate enter diseased cells via a mechanism called “folate-mediated endocytosis”..

1,4,7,10-Tetraazacyclododecane-1,4,7,10-Tetraacetic acid, or better known as DOTA, is a dual-function chelating agent or Bifunctional Chelating Agent (BFCA), which is a compound that has two active groups, where the first group functions to complex metal ions stably while the second group forms bonds with biomolecules such as peptides, antibodies, and other biomolecules. Like DTPA, DOTA has a chelating ligand ion that can strongly form 8 coordination bonds with gadolinium. DOTA is an excellent ligand for Gd(III) complexation due to its high thermodynamic stability. Gd-DOTA has been known as a contrast agent that has a lower toxicity potential, so its chemical stability is better compared to Gd-DTPA. Gd-DOTA (DOTAREM) has been extensively evaluated in patients with kidney disease and has not shown any side effects to date. The higher relaxivity and stability of Gd-DOTA makes it possible to use it as an alternative to Gd-DTPA for MRI.

A low-molecular-weight contrast agent Gd-DOTA-Folate, was successfully designed and synthesized through seven steps and was able to bind to tumors expressing FR in vivo, with increased imaging levels (Kalber et al., 2011). The synthesized Gd-DTPA-Folate was formed from the reaction of Gd3+ with DTPA-Folate, which was freshly synthesized via the reaction between DTPA-dianhydride and EDA-Folate, which was previously prepared by adopting and modifying seven route i.e folic acid was consecutively converted into pyrofolic acid, pteroyl hydrazide, pteroyl

azide, methyl ester of folic acid, and finally into EDA-Folate, DTPA-Folate was synthesized through a substitution reaction of DTPA-dianhydride with EDA-Folate, and Gd-DTPA-Folate was synthesized via a complexation reaction of Gd^{3+} ion with DTPA-F (Fauzia et al., 2015).

Fauzia et al. (2017) conducted a comparative study of the EDA-Folate precursor synthesis method routes through direct and indirect synthesis methods. In the direct synthesis method, it is carried out through two main reaction stages (1). activation of folic acid using DCC and NHS to produce NHS-folate, (2). conjugation of NHS-folate with ethylenediamine to produce EDA-Folate. The indirect synthesis is carried out through five reaction stages i.e., formations of: (1). pyrophoric acid, (2) petrol hydrazide, (3) petrol acid, (4) methyl ester of folic acid and (5) EDA-Folate as the final product.

In this study, the direct method was chosen because it is simpler than the indirect method because it consists of only two reaction steps to produce EDA-Folate. The formation of DOTA-Folate was carried out through the reaction between EDA-Folate and DOTA-NHS. The final reaction is the conjugation reaction of DOTA-Folate with gadolinium ion (Gd^{3+}) to become Gd-DOTA-Folate.

RESEARCH METHOD

In this study, equipment for synthesis and study in silico was used. The synthesis equipment used in this research were a GENESYS 50 UV thermoscientific spectrophotometer, Perkin Elmer Spectrum FTIR Infrared Spectrophotometer, Waters LC-MS ESI-TOF Mass Spectrometer, Waters High Performance Liquid Chromatography, magnetic stirrer, evaporator, vacuum desiccator, and tools - other glassware in the laboratory. The study in silico equipment consisted of a set of PCs (Personal Computers) and software as follows: Biovia Discovery Studio 2016, Autodock 4.2, Amber14, Amber Tools 15, and Visual Molecular Dynamics (VMD).

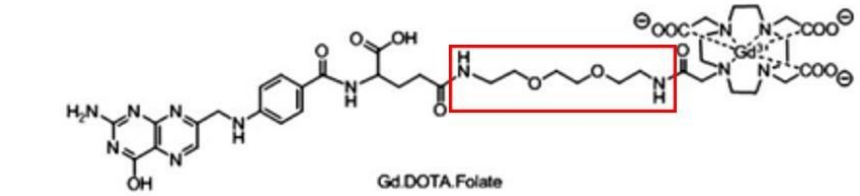
The synthetic materials used in this study were from Sigma Aldrich and Merck, such as aquabides, folic acid, nitric acid, 1,4,7,10-tetraazacyclododecane-1,4,7,10-tetraacetic acid (DOTA), trifluoroacetic acid., hydrochloric acid, acetone, acetonitrile, diethylether, dicyclohexylcarbodiimide (DCC), anhydrous dichloromethane (DCM), N,N- diisopropylethylamine (DIPEA), dry dimethylsulfoxide (DMSO), ethyl acetate, ethylenediamine (EDA), gadolinium oxide (Gd_2O_3), chloroform, methanol, sodium hydroxide, N-hydroxysuccinimide (NHS), and pyridine. The in silico materials used in this study were the structure of the α folate receptor with the 4LRH code downloaded from https://www.rcsb.org/structure/4LRH and Gd-DOTA-Folate structure in pdb format.

The reaction for the formation of EDA-Folate as the main precursor is carried out by activating folic acid using DDC. The results are then reacted with NHS (N-hydroxysuccinimide) to form NHS-Folate. Furthermore, NHS folate was conjugated with ethylenediamine to produce EDA folate. The main precursor reaction (Stage 2), namely the reaction for the formation of DOTA-Folate was carried out through the reaction between EDA-Folate from Stage 1 and DOTA-NHS. The final reaction (Stage 3) is the conjugation reaction of DOTA-Folate with gadolinium ion (Gd^{3+}) to become Gd-DOTA-Folate. The product of the Gd-DOTA-Folate complex compound produced was purified using reversed-phase High-Performance Liquid Chromatography (HPLC) and then characterized using ultraviolet spectrophotometry, infrared spectrophotometry, and mass spectrometry.

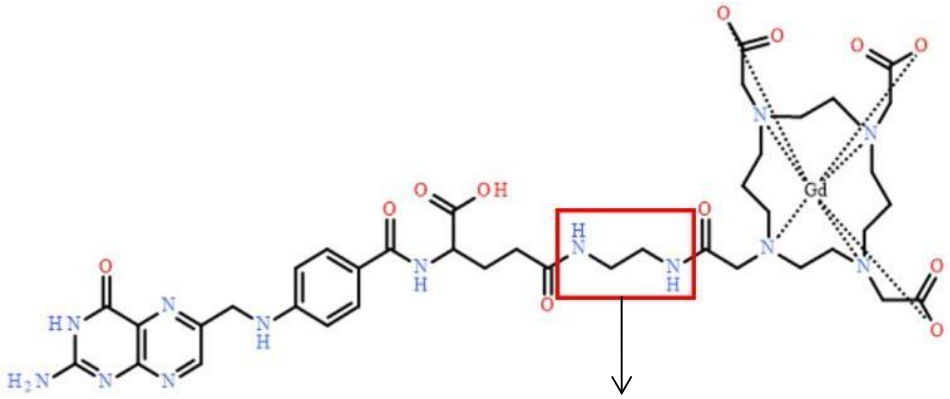
FINDINGS AND DISCUSSION

The novelty of this research is that the combination of the Gd-DOTA-Folate structure using ethylenediamine as a linker to conjugate folic acid with the Gd-DOTA complex has never been done, as well as the use of docking simulation methods and molecular dynamics in studying the interaction of Gd-DOTA-Folate with folate receptors.

The synthesis of the Gd-DOTA-Folate contrast agent in this study differed from the Gd-DOTA-Folate synthesis that had been carried out by Kalber et al., 2011. In this study, the linker used was ethylenediamine (EDA), and the synthesis process involved three stages of reaction: synthesis of EDA-Folate precursors, synthesis of DOTA-Folate precursors, and conjugation of gadolinium metal with DOTA-Folate precursors.



(a)



(b)

Figure 1. Structure of the Gd-DOTA-Folate directed contrast agent (Kalber et al., 2011) (a) and the structure of the Gd-DOTA-Folate contrast agent with an ethylenediamine linker (b)

Ethylenediamine is used as a linker because it is widely used as a linker to conjugate folic acid with protein, cholesterol, and polymer compounds (Li et al., 2015; Trindade et al., 2014). Yusuf et al., (2021) conducted a docking and molecular dynamic (MD) simulation study on the structure of the Gd-DTPA-Folate directed contrast agent with an EDA linker. The simulation results showed that Gd-DTPA-Folate with an EDA linker has a binding energy of -42 .78 kcal/mol, which is stable during the simulation. The novelty of this research is that a structural combination using ethylenediamine as a linker to conjugate folic acid with a Gd-DOTA complex has not been previously reported.

Synthesis of an EDA-Folate Precursor

The synthesis of the EDA-Folate precursor began with folic acid activation using dicyclohexylcarbodiimide (DCC) and N-hydroxysuccinimide (NHS) to produce activated folic acid (NHS-Folate) and dicyclohexylurea as side products, as shown in Figure 2.

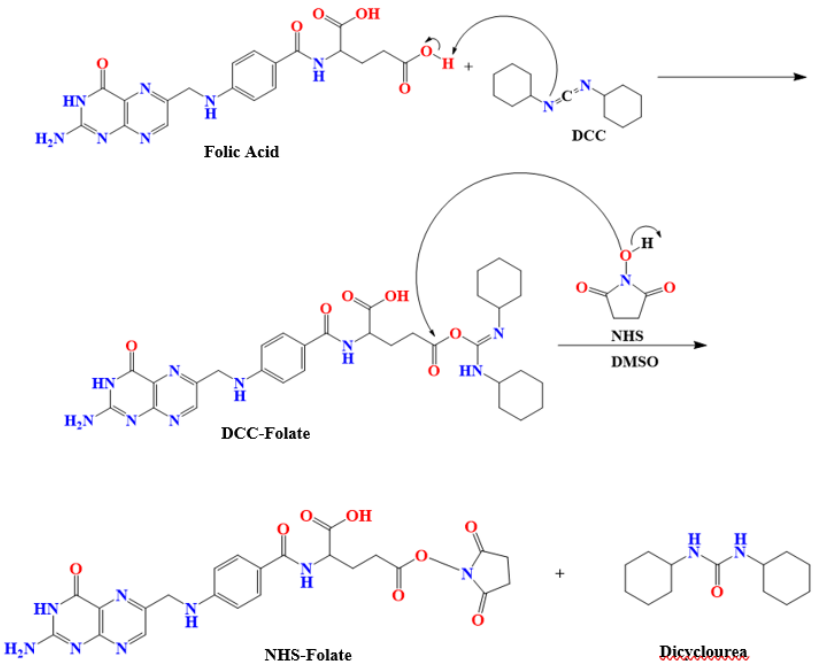


Figure 2. Folic acid activation reaction with DCC and NHS

In this study, folic acid activation was performed via a direct method. The NHS conjugation of folic acid is a direct method that is often used to conjugate the primary amine in ethylenediamine with the carboxylic group in folic acid to form an amide bond in EDA-folate. NHS-Folate synthesis was carried out in a dark-colored beaker because folic acid is sensitive to light. If folic acid is exposed to light, it will degrade and decompose, and it will even become inactive to form 2-amino-4-hydroxypteridine-6-aldehyde. The NHS-Folate filtrate was directly conjugated with EDA with the help of pyridine, which served as a catalyst, as shown in Figure 3.

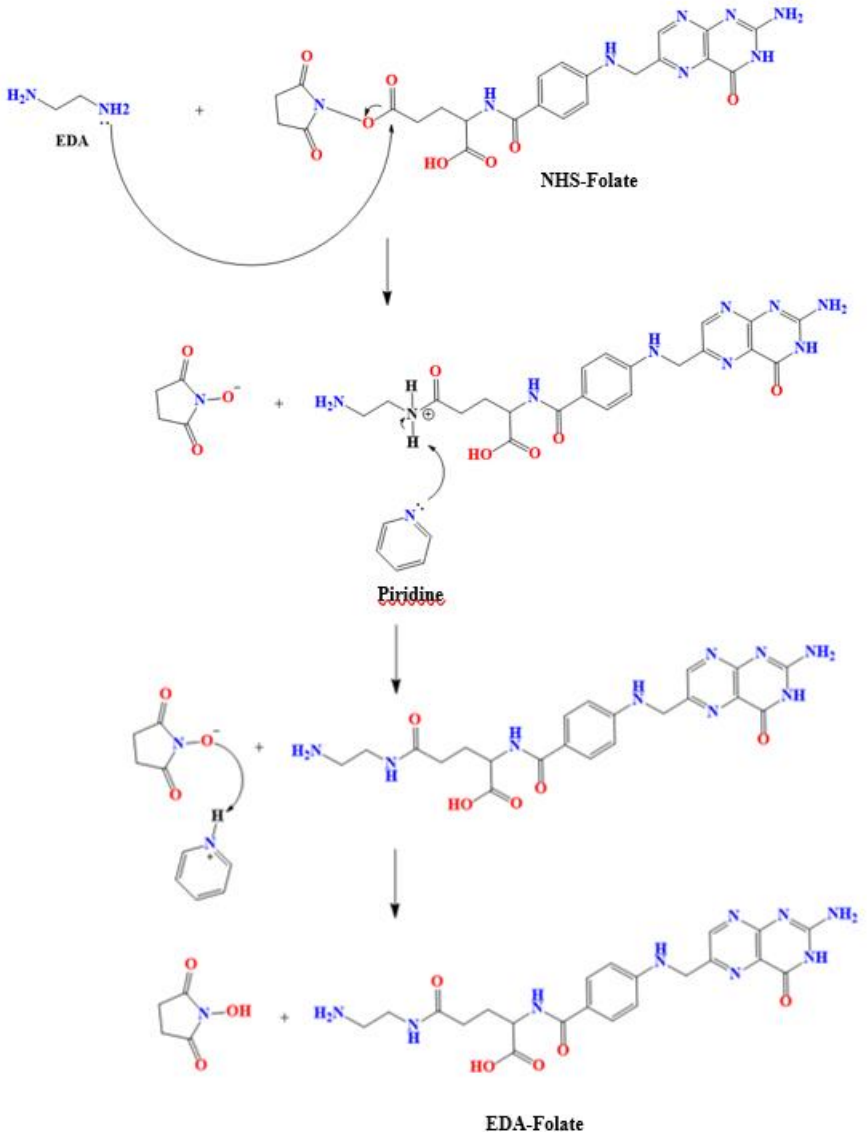
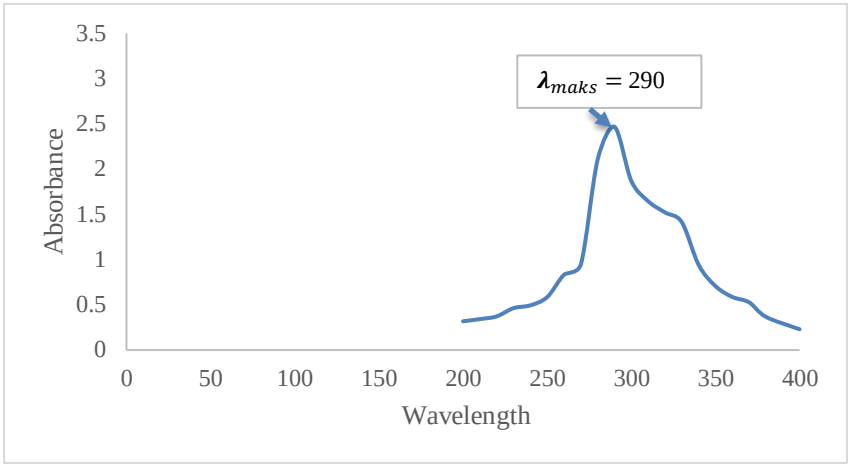


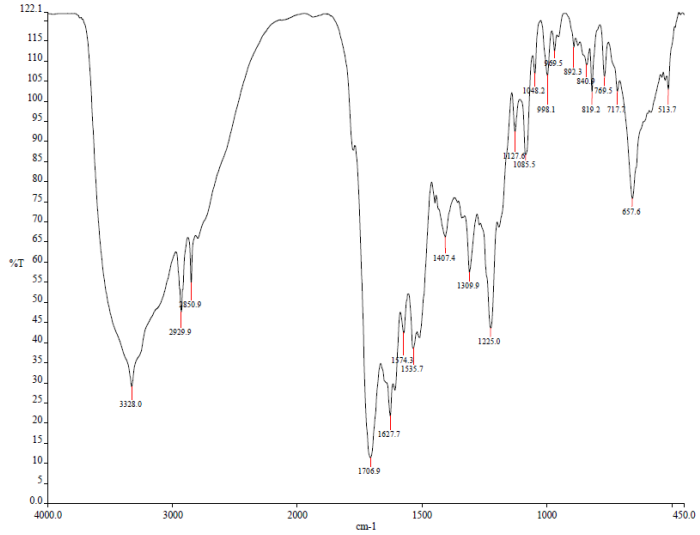
Figure 3. Ethylenediamine conjugation reaction with NHS-Folate

The results obtained are in the form of a yellow solution in which 20% acetone in diethyl ether is added and then stirred overnight. The yellow light precipitate was then centrifuged and washed with acetone 4 times followed by 2 times diethyl ether. The result is 74%. The obtained results were then purified using the reverse phase High Performance Liquid Chromatography (HPLC) method with Column C18. The mobile phase used was gradient elution, namely a mixture of water (0.3% TFA): acetonitrile (0.3% TFA) (8:2).

Furthermore, EDA-Folate was characterized by ultraviolet spectrophotometer, infrared spectrophotometer, and mass spectrophotometer. In Figure 4a. shows the maximum wavelength of EDA-Folate, namely at a wavelength of 290 nm, which is thought to originate from the transition of electrons from the n to π* orbitals caused by the presence of a double bond in C=O.



(a)



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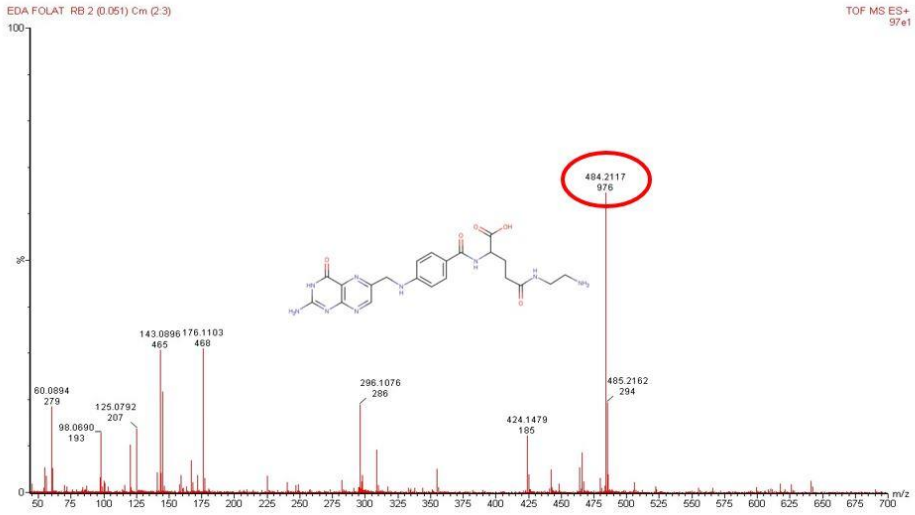


(c)

Figure 4. Ultraviolet spectrum (a), Infra-red spectrum (b), Mass spectrum (c) of EDA-Folate precursors

In Figure 4b. shows the existence of several distinctive groups, namely, at wave number 3328.0 cm^{-1} is a group typical of OH and NH strains. Wave number 2929.9 cm^{-1} is a typical group for the CH stretch, while wave numbers 1706.9 and 1627.7 cm^{-1} are a typical C=O groups for amides and carboxylates. At 1407.4 cm-1 is a group that is typical for CH bending, and at 1085.5 cm^{-1} is a group of CO and CN. Meanwhile, the wave number at 819.2 cm^{-1} is a typical wave number for CH benzene. The EDA-Folate mass spectrum is shown in Figure 4C. EDA-Folate ($C_{21}H_{25}N_9O_5$) theoretically has a molecular mass m/z of 483.4760. In terms of molecular mass, the results of measurements of the EDA-Folate compound produced an HR-TOFMS ES^+ m/z spectrum of 484.2117.

Synthesis of the DOTA-Folate Precursor

The synthesis of the DOTA-Folate precursor begins with the synthesis of the DOTA compound to become DOTA-NHS. The DOTA compound has four active groups that can react with other compounds. Therefore, one of the carboxylic groups in the DOTA compound needs to be activated first using DCC and NHS to form an active ester, which makes it easier to conjugate with EDA-Folate, as shown in Figure 5.

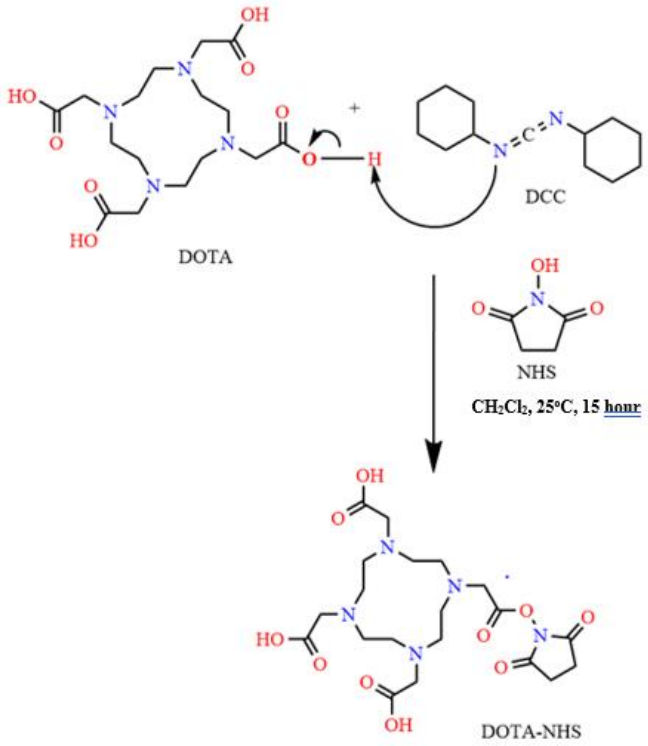
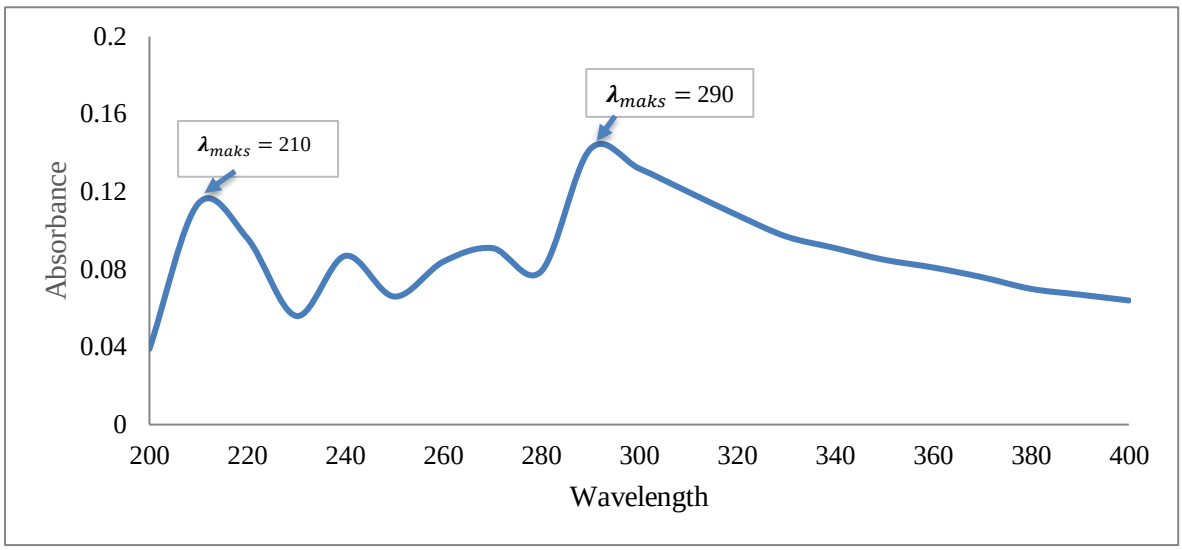


Figure 5. The reaction for the formation of DOTA-NHS from DOTA precursors activated using DCC and NHS at 25°C for 15 hours

The reaction for the formation of DOTA-NHS was carried out at 25°C for 15 hours with the addition of dichloromethane (DCM). The reaction product is then evaporated to remove the remaining solvent, and a white precipitate is obtained with a yield of 73%, then purified using the reverse phase High Performance Liquid Chromatography (HPLC) method with Column C18. The mobile phase is water (0.1% TFA): acetonitrile (0.1 % TFA) (1:7) with a flow rate of 1 mL/min, UV detectors λ 216 nm and 254 nm. Furthermore, the purified DOTA-NHS was characterized by an ultraviolet spectrophotometer, an infrared spectrophotometer and a mass spectrophotometer.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0.16
0.12
0.08
0.04
0
Absorbance
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λ_{maks} = 290
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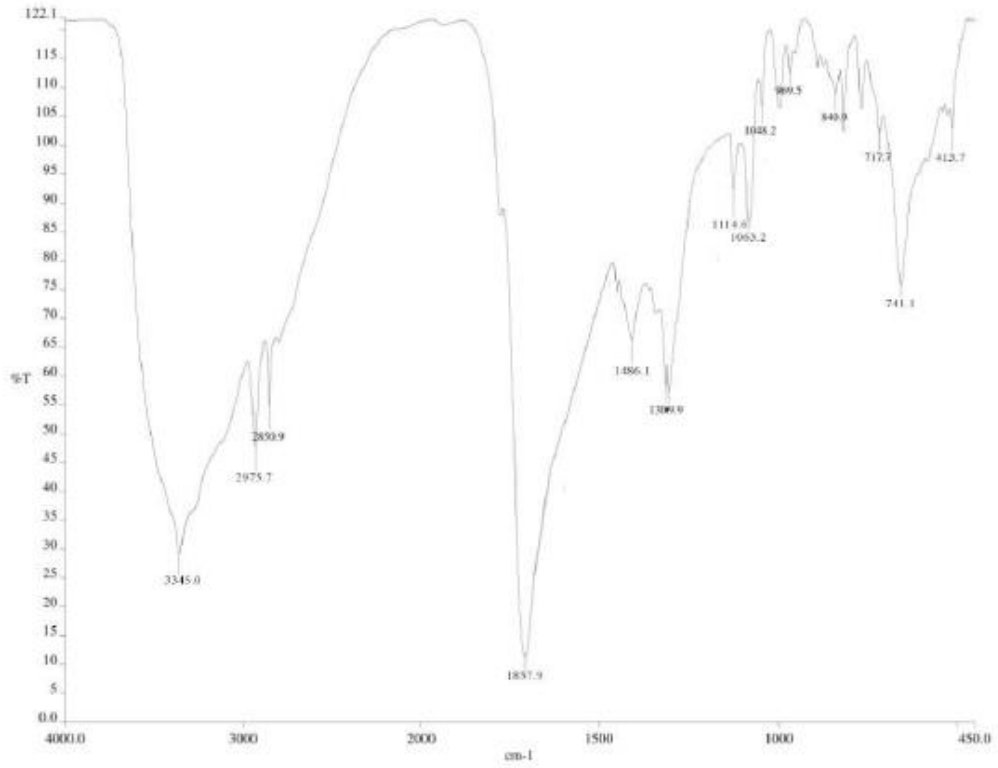
280
300
320
340
360
380
400
Wavelength

(a)


%T
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2975.7
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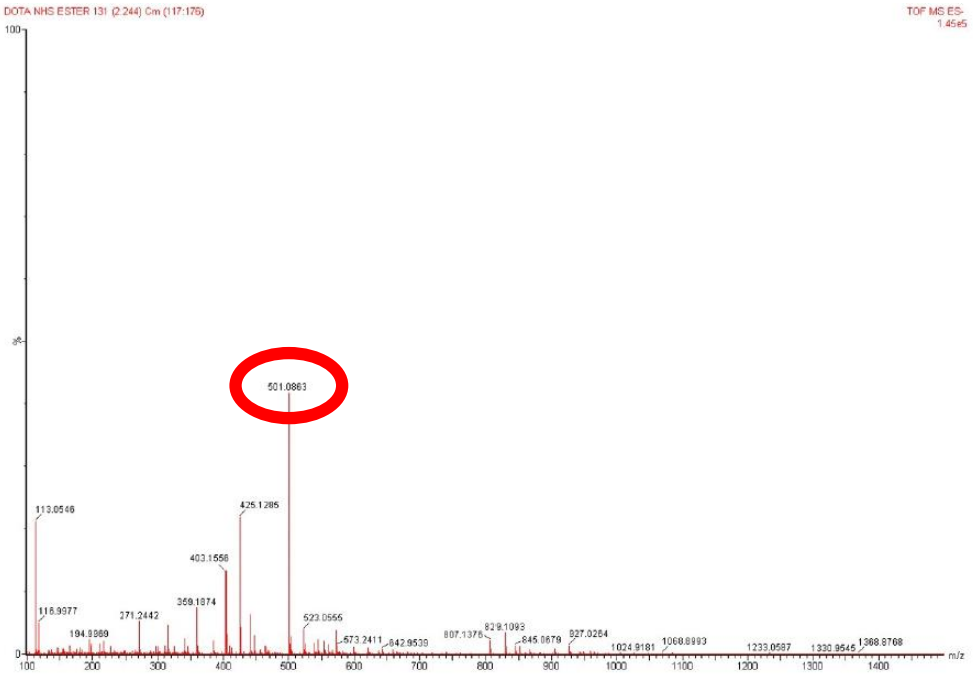
(b)



(c)

Figure 6. Ultraviolet spectrum (a), infrared spectrum (b), Mass Spectrum (c) of DOTA-NHS precursors

In Figure 6a. shows the maximum wavelength of DOTA-NHS, namely, at a wavelength of 290 nm, this wavelength is thought to originate from the transition of electrons from the π to π* orbitals caused by the presence of a double bond in C=O. Next in Figure 6b. shows the infra-red spectrum of the DOTA-NHS precursor in Figure 6b shows the presence of several distinctive groups, namely at wave number 3345.0 cm^{-1} is a group typical of OH and NH strains. Wave numbers 2975.7 and 2850.9 cm^{-1} are typical groups for CH stretching, while wave numbers 1857.9 are typical C=O groups for amides and carboxylates. At 1486.1 cm^{-1} is a typical NH group, and the wavenumbers 1114.6 and 1063.2 cm-1 are CO and CN groups. Figure 6c. shows the mass spectrum of DOTA-NHS, DOTA-NHS ($C_{20}H_{31}N_5O_{10}$) theoretically has a molecular mass m/z of 501.4880. On the basis of the molecular mass measurement results of the DOTA-NHS compound, the HR-TOFMS ES^- m/z spectrum is 501.0863.

Furthermore, the synthesis of the DOTA-Folate precursor is under the reaction shown in Figure 7. The amino group in EDA-Folate attacks the carbonyl group, releasing the NHS group, which is a good leaving group. In this synthesis, a DCM solvent is used, and the addition of a base of N, N-diisopropylethylamine (DIPEA) is used to improve the efficiency of the amine acylation process.

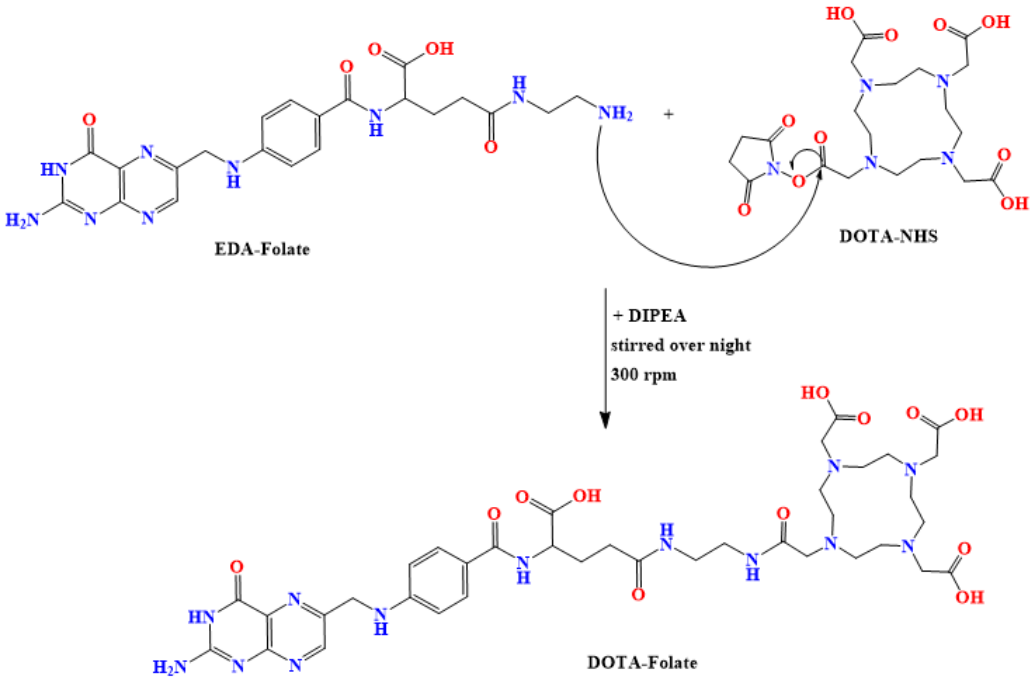
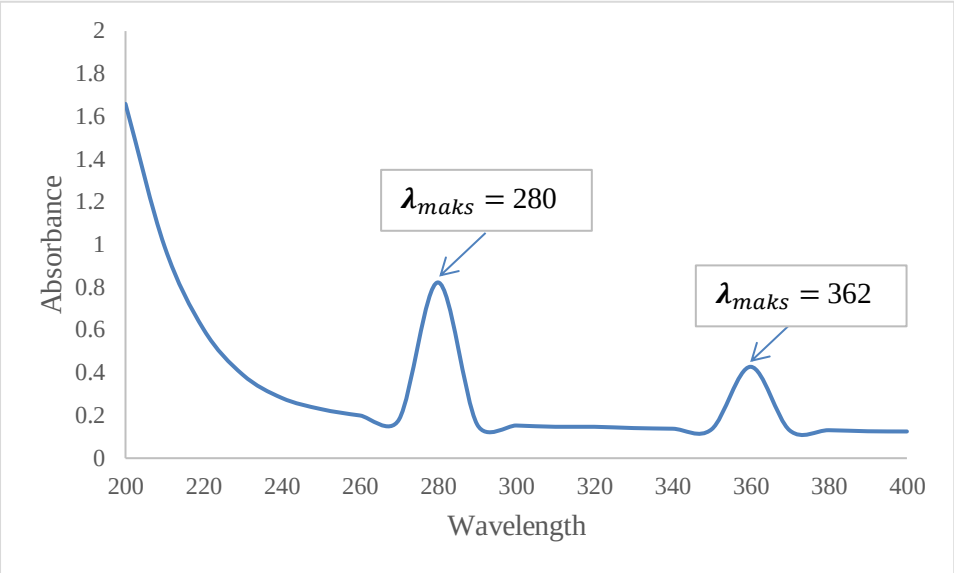


Figure 7. Reaction for the formation of DOTA-Folate precursors from DOTA-NHS and EDA-Folate precursors at 25°C; stirred overnight; at a speed of 300 rpm

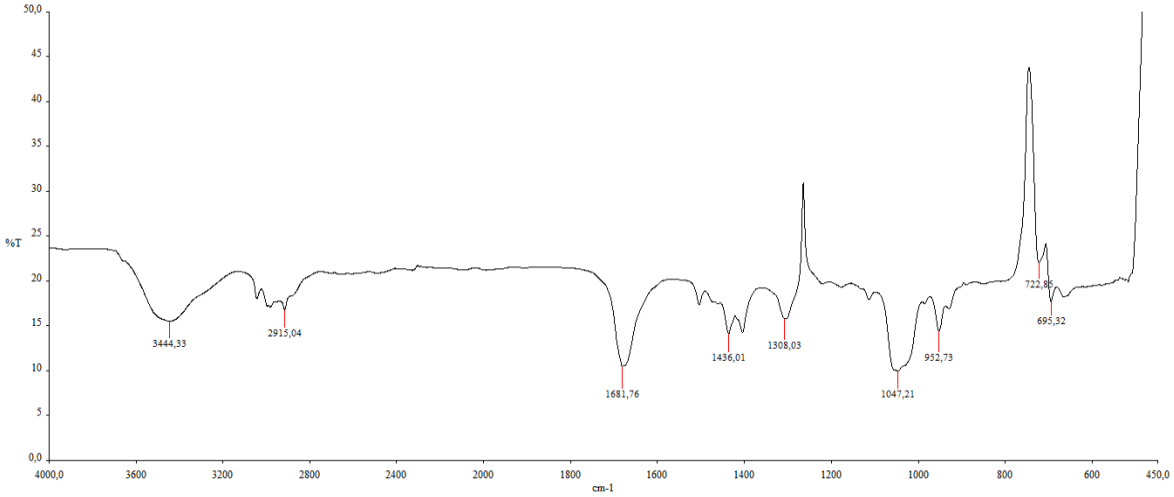
The reaction was carried out at 25°C, stirring overnight at a stirring speed of 300 rpm. The reaction product is then evaporated to remove the remaining solvent, and a white precipitate is obtained with a yield of 72%, then purified using the reverse phase High Performance Liquid Chromatography (HPLC) method with Column C18. The mobile phase is water (0.1% TFA): acetonitrile (0.1 % TFA) (1:7) with a flow rate of 1 mL/min, UV detectors λ 216 nm and 254 nm.

DOTA-Folate was characterized by its ultraviolet spectrum, as shown in Figure 8a. In the ultraviolet spectrum of DOTA-Folate, the maximum wavelength at 280 nm is a π to π* transition due to the presence of the C=C group of benzene, and the maximum wavelength of 362 nm is the n to π* transition due to the presence of a conjugated C=O group in the carboxylic group of DOTA - Folate. Then, we characterized DOTA-Folate using the infrared spectrum, as shown in Figure 8b. In the infrared spectrum of the DOTA-Folate precursor, there are several distinctive groups, namely at wave number 3444.33 cm-1 which is a group that is typical for both OH and NH strains. Wave number 2915.04 cm-1 is a typical group for the CH stretch, while wave number 1681.76 cm-1 is a typical C=O group for amides and carboxylates. At 1436.01 cm-1 is a typical NH group, 1308.03 cm-1 is a typical C=C group for benzene, 1047.21 cm-1 is a CO group and for wave number 722.85 cm-1 is a group typical for CH benzene in the para position.

In Figure 8c. shows the mass spectrum of DOTA-Folate as measured using the HR-TOFMS ES+ mass spectrometer. On the molecular mass measurement results of the DOTA-Folate compound, the HR-TOFMS ES+ m/z spectrum is 870.7224. The peak of 870.7224 shows that DOTA-Folate has experienced the addition of a proton [M-H+], for m/z 295.1296 is [M+5H+] triple charged ions.


Absorbance
Wavelength
$\lambda_{maks} = 280$
$\lambda_{maks} = 362$
2
1.8
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
200
220
240
260
280
300
320
340
360
380
400

(a)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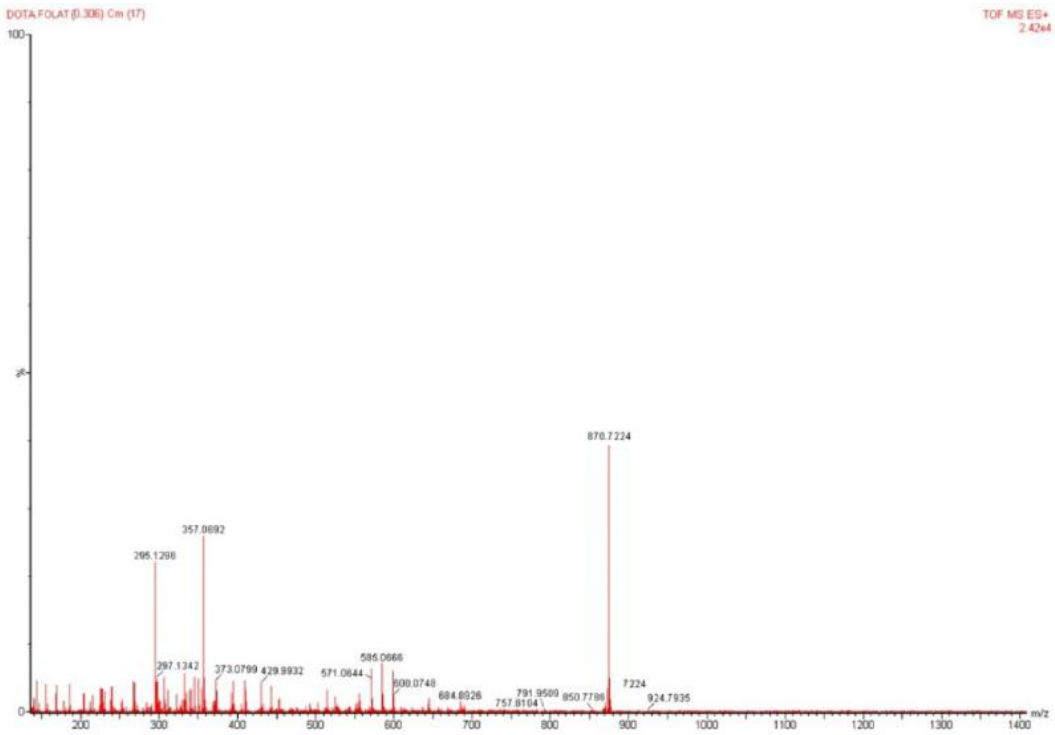
(b)



(c)

Figure 8. Ultraviolet spectrum (a), infrared spectrum (b), and mass spectrum (c) DOTA-Folate

Synthesis of a Targeted Contrast Gd-DOTA-Folate

The Gd-DOTA-Folate directed contrast agent was formed from a complex formation reaction between Gd^{3+} and the DOTA-Folate precursor, as shown in Figure 9. In the Gd-DOTA-Folate synthesis process, the DOTA-Folate precursor is made in excess to produce the optimum yield gain and maximize DOTA-Folate which reacts perfectly with Gd^{3+}.

Figure 9. shows that Gd^{3+} binds to the lone pair electrons from O and N, which DOTA-Folate owns. When viewed from the structure of Gd-DOTA and Gd-DOTA-Folate, there is a difference, namely the bond between Gd^{3+} and the lone electrons from O and N. Gd-DOTA has 8 lone pair electrons, 4 lone pair electrons from N and 4 lone pair electrons from O, whereas Gd DOTA-Folate.

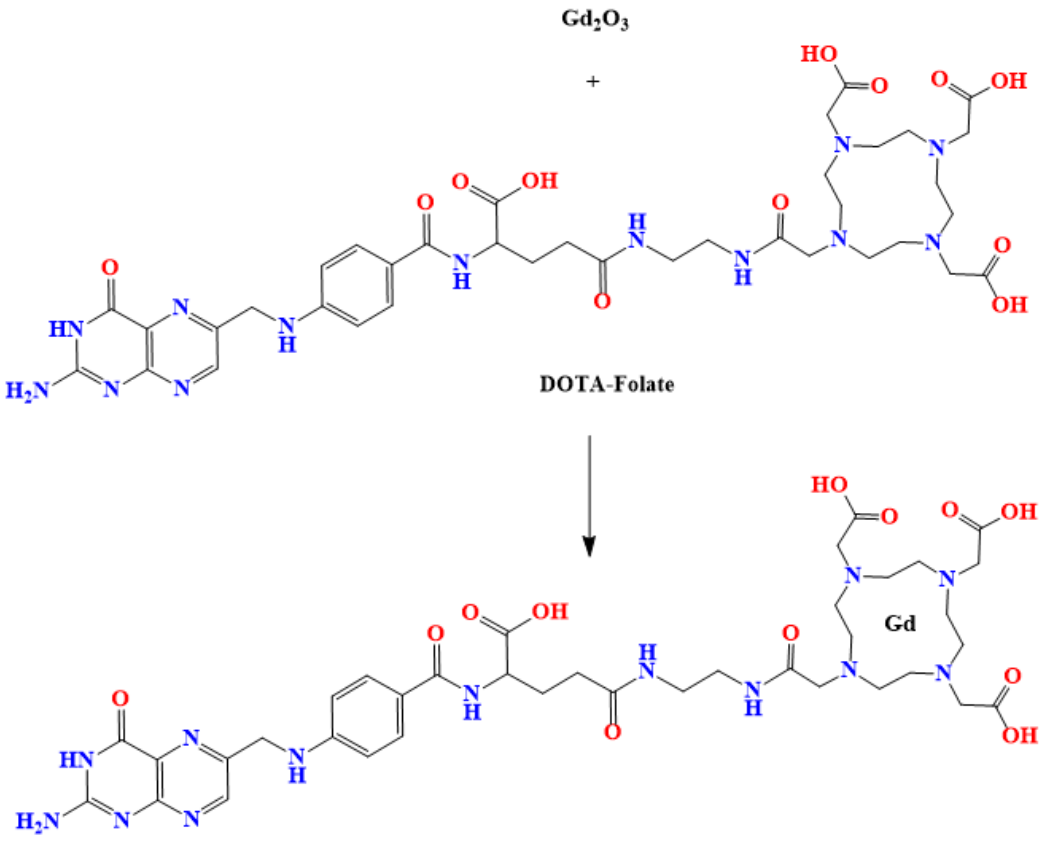


Figure 9. Reaction for the formation of a Gd-DOTA-Folate directed contrast agent with an ethylenediamine linker

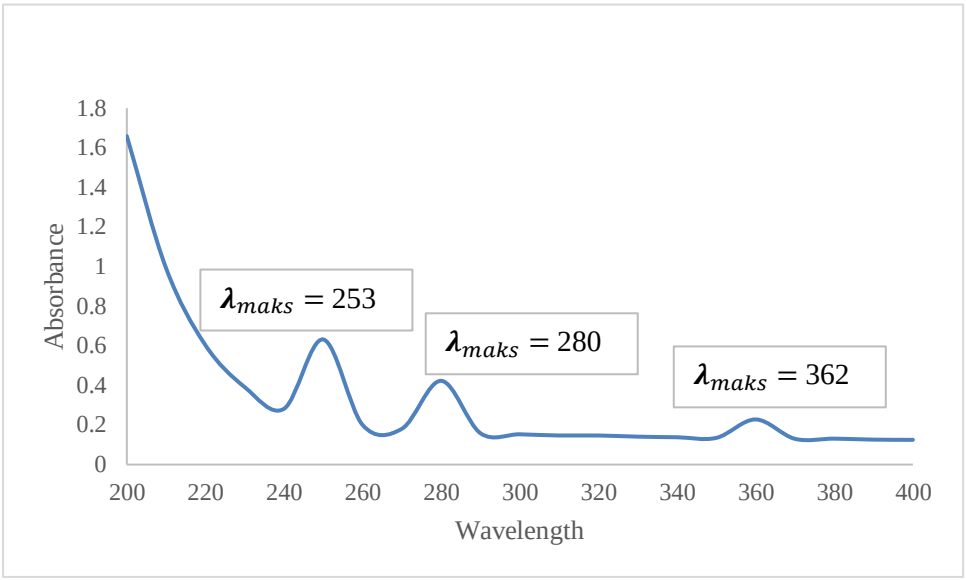
Gd-DOTA-Folate synthesis results were then purified using a 1 kDa MWCO dialysis membrane to remove impurities and residual precursors. The yellow solution in the freezer produced a yield of 76%. Furthermore, Gd-DOTA-Folate was purified using the reverse phase High-Performance Liquid Chromatography (HPLC) method with Column C18. The mobile phases were water (0.1% TFA): acetonitrile (0.1% TFA) (1:7) with a flow rate of 1 mL/min, UV detectors λ 216 nm and 254 nm.

The purified Gd-DOTA-Folate compound was characterized using ultraviolet spectrophotometry, infrared spectroscopy, and mass spectrometry. Figure 10a. shows the ultraviolet spectrum of Gd-DOTA-Folate. The maximum wavelength at 250 nm occurs a π to π* transition due to the presence of the C=C group from benzene, and the maximum wavelength of 360 nm occurs n to π* transition due to the presence of a conjugated C=O group in the carboxylates of DOTA-Folate. The spectrum of Gd-DOTA-Folate is almost the same as DOTA-Folate, however, the absorbance at this wavelength decreases slightly, which is due to the hypochromic effect, which causes a decrease in absorption intensity caused by diluting the solute. This is due to the presence of groups from DOTA-Folate that have reacted with Gd^{3+}.

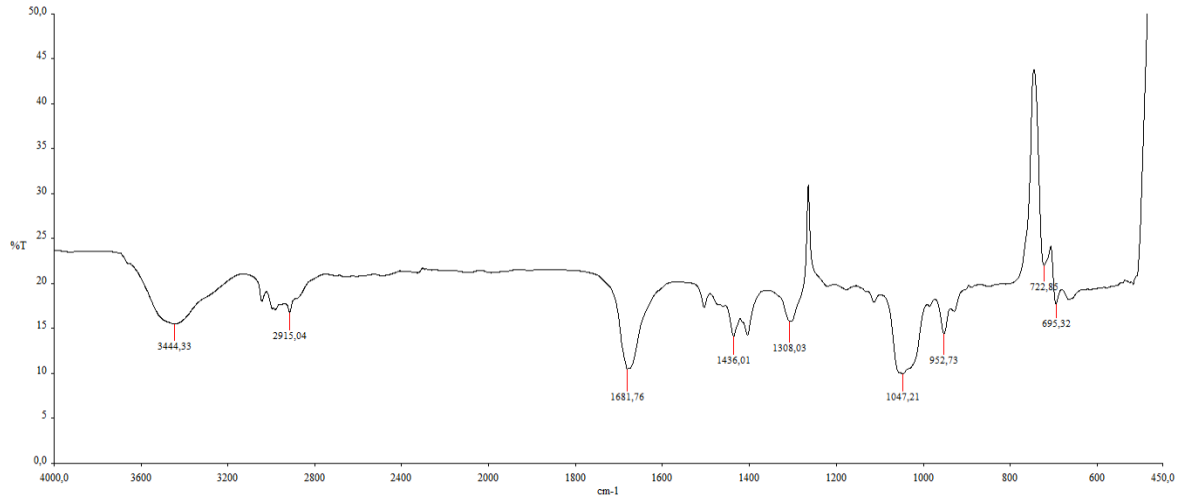
In Figure 10b. shows the infra-red spectrum of the Gd-DOTA-Folate precursor. wave number

3399.26 cm^{-1} is a typical group for both the OH and NH strains. Wave number 2918.42 is a typical group for the CH sp3 stretch. The wave number 1644.64 is a typical C=O group in amides and carboxylates. The wave number 1445.21 is a typical NH group, the wave number 1310.44 cm^{-1} is a typical C=C group for benzene, the wave number 1082.61 cm^{-1} is a CO group, the wave number 722.85 cm^{-1} is a typical group for CH benzene in the para position, and the wave number 460.22 cm^{-1} is the Gd-O bond of Gd-DOTA-Folate.

In Figure 10c. shows the Gd-DOTA-Folate mass spectrum measured using the HR-TOFMS ES+ mass spectrometer. The molecular mass measurement results of the Gd-DOTA-Folate compound yield an HR-TOFMS ES+ m/z spectrum of 1025.6561. The peak of 1025.6561 shows that Gd-DOTA-Folate has added a proton. [M-H+], for m/z 512.8699 is [M-H+]/2 double charged ions, and m/z 102.5734 is [M-H+]/10 from Gd-DOTA-Folate.



(a)



(b)

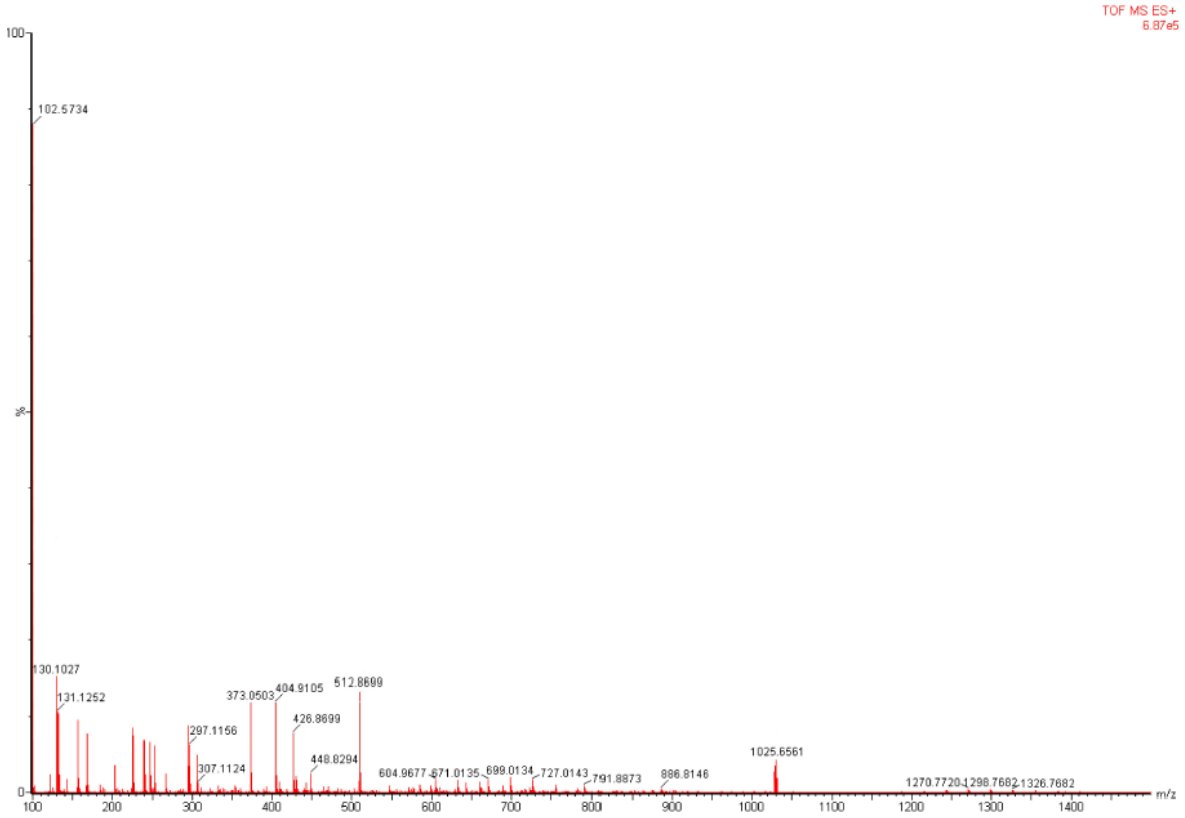


Figure 10. Ultraviolet spectrum (a), infrared spectrum (b), mass spectrum of the Gd-DOTA-Folate directional contrast agent

Simulation of Folic Acid Docking with Folate Receptors

The docking simulation was performed using Autodock 4.2 on the Linux operating system. The center point of the grid map is set to the ligand (folic acid), because the ligand used comes from the same crystal structure as the receptor. Set grid parameters with Number of Specified Grid Points (46, 84, 46) and Coordinates of Central Grid Point of Maps (3.784, 9.750, 12.870).

Conformation number 52 (run = 52) exhibited the best conformation with an estimated total binding energy of -11.70 kcal/mol based on the positive control docking simulation results. The total binding energy is the sum of the intermolecular energy (van der Waals, hydrogen bonds, desolvation energy, electrostatic energy), internal energy, torsion energy, and system energy, as shown in Table 1. In addition, RMSD values of 0.777 Å were also obtained which indicated the average deviation of the docked structure from the initial structure before docking (crystal structure). The RMSD value generated from the docked structure≤2Å, this indicates that the docking method used is valid, and the parameter settings used meet the validation criteria. This indicates that the structure of the docking results is not significantly different from the crystal structure.

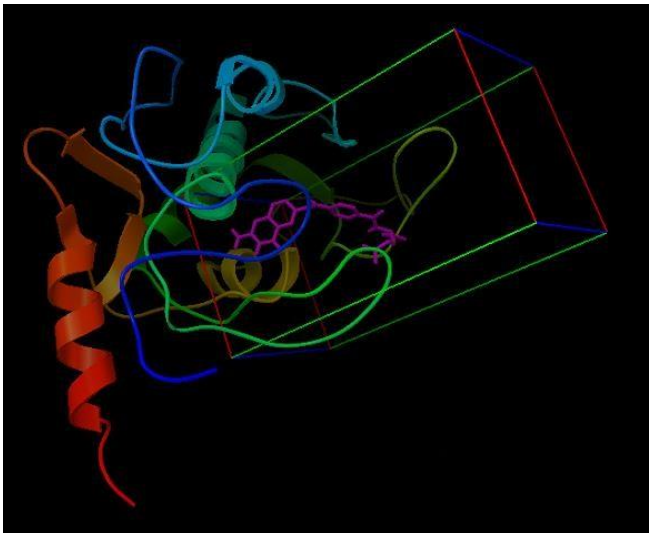


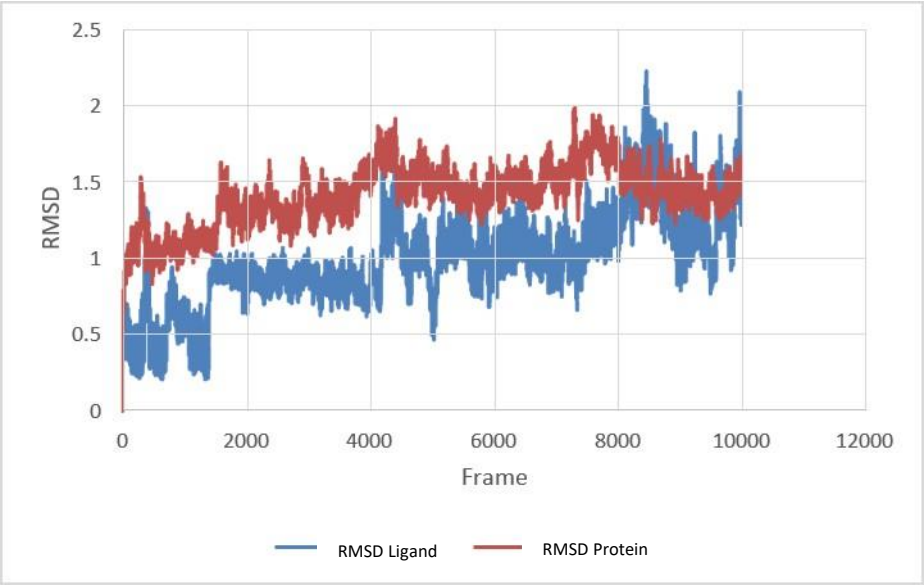
Figure 11. The structure of folic acid ligands in the grid map

Table 1. Energy Generated from Docking Conformation

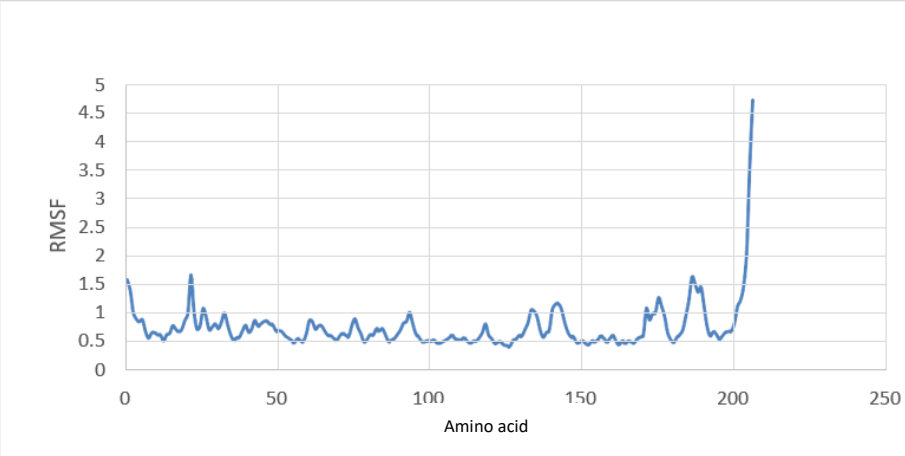
```
Run = 52
DPF = dock.dpf

Estimated Free Energy of Binding    =  -11.70 kcal/mol  [=(1)+(2)+(3)-(4)]
Estimated Inhibition Constant, Ki   =    2.65 nM (nanomolar)  [Temperature = 298.15 K]

(1) Final Intermolecular Energy     =  -14.68 kcal/mol
    vdW + Hbond + desolv Energy     =  -12.62 kcal/mol
    Electrostatic Energy            =   -2.06 kcal/mol
(2) Final Total Internal Energy     =   -1.40 kcal/mol
(3) Torsional Free Energy           =   +2.98 kcal/mol
(4) Unbound System's Energy  [=(2)] =   -1.40 kcal/mol
```

Molecular Dynamics Simulation on Positive Control

MD simulations were conducted using the Amber14 program on the Linux operating system. The amino acids in the protein structure are first changed by the appropriate type of atom under their native conditions. Furthermore, the parameterization of the ligands was carried out using semi-empirical quantum calculations with the AM1_BCC code in the antechamber program. The parameterization is intended to allow the Amber 14 program to recognize the type and charge of constituent atoms. Parameterization was also performed by adding a charged atom to neutralize the charge on the receptor and ligand. Based on the calculation results, the charge on the ligan-receptor complex is +1, so one Cl^- atom is added to neutralize the charge to 0. Then, into the complex system, a TIP3P-type water molecule was added in the form of MAP with a distance of 10 Å from the outer surface of the complex 8980 water molecules. Then, the system was minimized, heated to 37˚C, and equilibrated for 600 ps before entering the production stage.



(a)



(b)

Figure 12. RMSD graph (a) and RMSF graph (b) positive contra

Based on the RMSF data or the average distance of atomic fluctuations from the initial state (Figure 12b., Folate receptors only experience very high fluctuations in the 208 amino acid residues. This is because this residue acts as a terminal so that its position moves freely and experiences very high fluctuations, but these fluctuations do not affect the stability of the protein structure (Yusuf et al., 2021).

Energy calculations during the simulation were carried out using the Molecular Mechanics-Generalized Born Surface Area (MM-GBSA) protocol in the Amber14 program. The MMGBSA uses the Generalized Born approach, which is a faster approximation and treatment of the Poisson-Boltzmann equation. With this program, the system calculates the energy magnitude of the receptor-ligand complex, receptor energy, and ligand energy. The difference between the three energy quantities produced is the amount of binding energy between the receptor and the ligand, which is shown in units of kcal/mol. The amount of binding energy resulting from the results of the positive control MD (folic acid-folate receptors) was -31.1456 kcal/mol.

Table 2. Calculation of the binding energy between the receptor and folate ligand

Differences (Complex - Receptor - Ligand):			
Energy Component	Average	Std. Dev.	Std. Err. of Mean
BOND	0.0000	0.0001	0.0000
ANGLE	-0.0000	0.0001	0.0000
DIHED	0.0000	0.0000	0.0000
VDWAALS	-43.2369	2.5959	0.8209
EEL	-317.2483	30.8536	9.7568
1-4 VDW	-0.0000	0.0000	0.0000
1-4 EEL	0.0000	0.0000	0.0000
EGB	334.4453	25.8534	8.1756
ESURF	-5.1057	0.3164	0.1000
DELTA G gas	-360.4852	29.4023	9.2978
DELTA G solv	329.3395	25.6263	8.1038
DELTA TOTAL	-31.1456	4.0785	1.2897

Gd-DOTA-Folate Docking Simulation with Folate Receptors

The prepared Gd-DOTA-folate structure was then modified on the Gd atom to become a Fe atom. This is because the Autodock 4.2 program does not yet have specific parameters for lanthanide elements such as gadolinium, whereas the element Fe was chosen because Fe in its free state has the same charge as Gd, namely +3. Torque adjustment was then carried out on the structure, with the number of active torques equal to the positive control, from 32 total active torques to 9 units by deactivation of the torques on the ethylenediamine and DOTA structures. Torsional deactivation of the DOTA structure is also intended so that the DOTA structure can support Fe atoms by forming coordinate covalent bonds.

Furthermore, the grid map setting uses parameters from the results of positive control docking because these parameters already contain coordinates suitable for the location of the active site of the folate receptor.

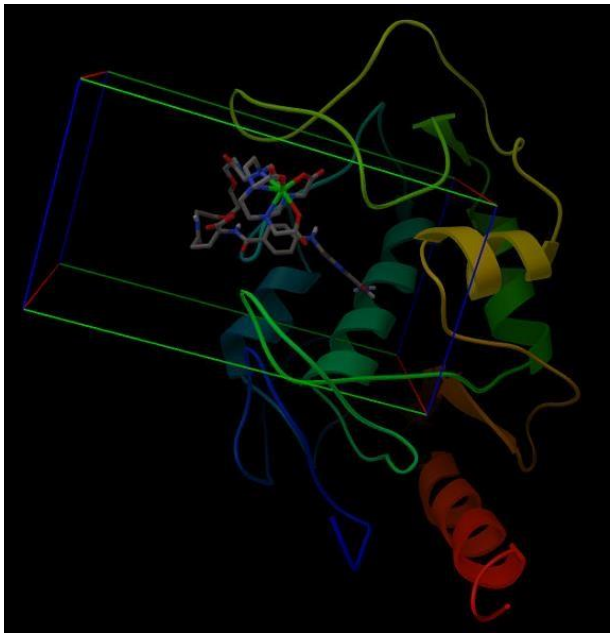


Figure 13. The structure of the Gd-DOTA-Folate ligands in the grid map

Conformation number 14 (run = 14) exhibited the best conformation with an estimated total binding energy of -7.91 kcal/mol from the positive control docking simulation results. The total binding energy is the sum of the intermolecular energy (van der Waals, hydrogen bonding,

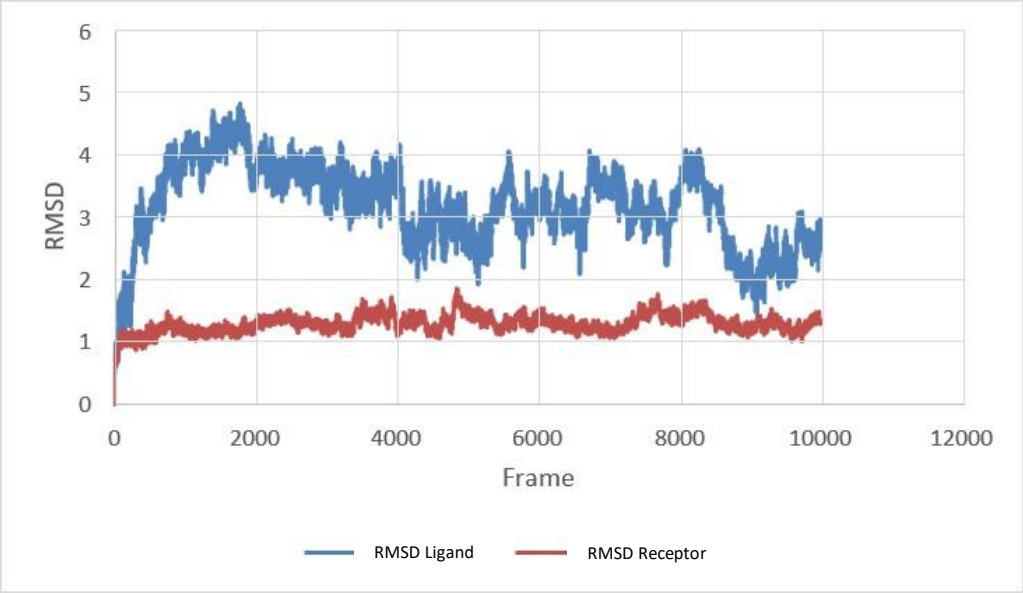
desolvation energy, electrostatic energy), internal energy, torsional energy, and system energy. In addition, an RMSD value of 3.555 Å was also obtained. The docking simulation results show that Fe-DOTA-Folate produces greater energy than the positive control, which has an energy of -11.70 kcal/mol.

Table 3. Energy Generated from Gd-DOTA-Folate Docking Conformation

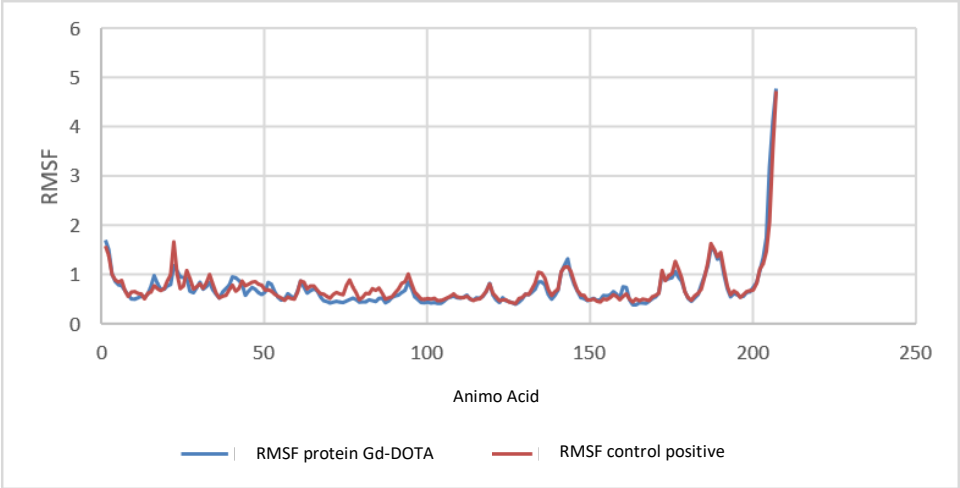
```
Run = 14
DPF = dock.dpf

Estimated Free Energy of Binding    =   -7.91 kcal/mol  [=(1)+(2)+(3)-(4)]
Estimated Inhibition Constant, Ki   =    1.59 uM (micromolar)  [Temperature = 298.15 K]

(1) Final Intermolecular Energy     =  -14.17 kcal/mol
    vdW + Hbond + desolv Energy     =  -13.60 kcal/mol
    Electrostatic Energy            =   -0.57 kcal/mol
(2) Final Total Internal Energy     =   -5.12 kcal/mol
(3) Torsional Free Energy           =   +6.26 kcal/mol
(4) Unbound System's Energy  [=(2)] =   -5.12 kcal/mol
```

Molecular Dynamics Simulation on Gd-DOTA-Folate

The simulation results show that the Gd-DOTA-Folate bound to the receptor is stable during the simulation, although the RMSD calculation results shown in Figure 14a indicate that the ligand undergoes a significant conformational change from the initial conditions.



(a)



(b)

Figure 14. (a) RMSD graph of Gd-DOTA-Folate ligand and folate receptor and (b) RMSF graph of Gd-DOTA-folate receptor with positive control

Based on the RMSF data shown in Figure 14b, Folate receptors only experience very high fluctuations in the 208 amino acid residues, the same as the positive control results; thus, the structure of the folate receptor was assumed to be stable during the simulation. Comparisons with the MD results of the positive controls were performed to observe the stability of the Gd-DOTA-folate MD results. Comparison of the RMSD values of the receptors in the positive control with the receptors in the Gd-DOTA-folate simulation showed that the Gd-DOTA binding receptors were stable during the 10 ns running simulation.

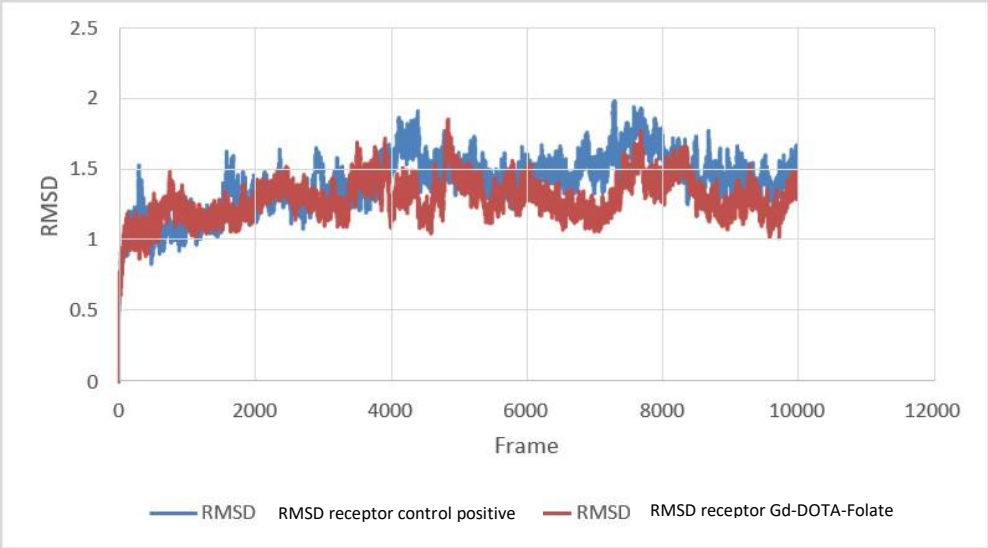
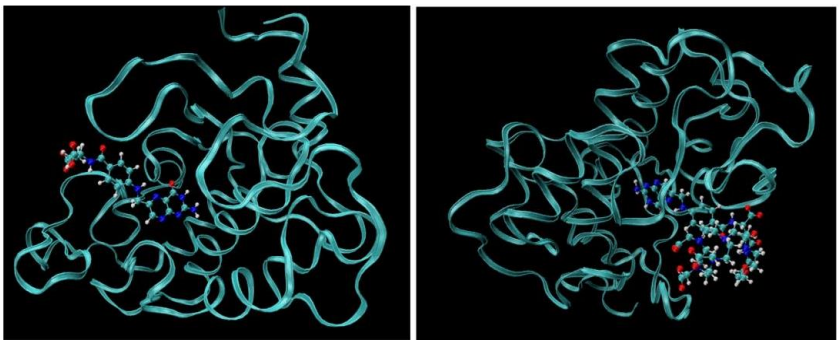


Figure 15. Graph of RMSD differences between Gd-DOTA-Folate and positive control ligands

Based on the results of the RMSD comparison shown in Figure 15. of the ligand in the positive control with the Gd-DOTA-folate ligand, it was shown that Gd-DOTA-folate was very unstable. Instability is indicated by the formation of several peaks on the RMSD graph, indicating that the Gd-DOTA-folate structure often undergoes conformational changes. Based on the results of visual analysis using the VMD program, frequent conformational changes during the simulation were caused by the movement of the Gd-DOTA structure, which moves freely outside the folate receptor.

However, the movement of Gd-DOTA does not affect the conformation of folate, which is present on the folate receptor binding site; thus, Gd-DOTA-folate is not released from the folate receptor during the simulation.



(a) (b)

Figure 16. (a) Visual Analysis of Folic Acid—Folate Receptors and (b) Visual Analysis of Gd-DOTA-Folate—Folate Receptors

The energies of the ligand and receptor complexes were calculated using the MM-GBSA protocol. During the simulation, the total energy generated between the interaction of Gd-DOTA-folate with folate receptors is -37.8196 kcal/mol.

Table 4. Calculation of bond energy between receptors and Gd-DOTA-folate

Differences (Complex - Receptor - Ligand):

Energy Component	Average	Std. Dev.	Std. Err. of Mean
BOND	-0.0000	0.0000	0.0000
ANGLE	-0.0000	0.0000	0.0000
DIHED	-0.0000	0.0000	0.0000
VDWAALS	-79.4986	4.1901	1.3250
EEL	-205.2269	12.4769	3.9455
1-4 VDW	-0.0000	0.0000	0.0000
1-4 EEL	-0.0000	0.0000	0.0000
EGB	255.4488	10.6117	3.3557
ESURF	-8.5430	0.3568	0.1128
DELTA G gas	-284.7254	13.3188	4.2118
DELTA G solv	246.9058	10.4967	3.3193
DELTA TOTAL	-37.8196	6.2566	1.9785

These results indicate that the energy produced decreases when compared with the binding energy between folate and folate receptors, namely -31.1456 kcal/mol. This indicates that the addition of the Gd-DOTA group increases the affinity of folate interactions with folate receptors. Thus, Gd-DOTA-Folate is stably bound to folate receptors.

CONCLUSIONS

The Gd-DOTA-Folate directed contrast agent was successfully synthesized through three reaction steps, namely: 1). Reactions for the formation of EDA-Folate as the main precursor 2). The reaction of forming DOTA-Folate from EDA-Folate with DOTA-NHS and 3). Reaction for the formation of Gd-DOTA-Folate from DOTA-Folate with gadolinium ions. UV and IR spectrophotometric characterization yielded a wavelength of 253 nm and a wave number of 460.22 cm-1 which was specific for Gd-DOTA-Folate and by mass spectrometry obtained m/z 1025.6561

for Gd-DOTA-Folate, and the addition of Gd-DOTA to the folate structure increases the affinity of folate for folate receptors, so Gd-DOTA-Folate is stable and bound to folate receptors. The interaction of Gd-DOTA-Folate with folate receptors, based on docking and MD simulation results, produces energy of -7.91 kcal/mol and -37.8196 kcal/mol.

LIMITATION AND FURTHER RESEARCH

The limitations of the study are the equipment for the synthesis of targeted contrast agents Gd-DOTA-Folate, the linker used to conjugate folic acid with Gd-DOTA, namely ethylenediamine (EDA), and study in silico was used docking simulation dan molecular dynamic45. Further research will increase the synthesis scale to obtain pure Gd-DOTA-Folate products in larger quantities and conduct biodistribution and toxicity tests of Gd-DOTA-Folate targeted contrast agents.

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