Analysis and Characterization of Complex Compound Gadolinium-(1,4,7,10-Tetraazacyclododecane,1-4-7-10-Tetraacetic Acid)n-Poliamidoamine Generation 3-Trastuzumab as a Novel Contrast Agent for Magnetic Resonance Imaging

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Abstract

Currently, cancer is one of the leading causes of death worldwide. One type of cancer that women fear is breast cancer. One tool that can be used for early cancer detection is Magnetic Resonance Imaging (MRI). MRI can diagnose cancer quickly compared to other diagnostic techniques. One of the contrasting compounds used to produce a good image in MRI is Gd-DOTA. Gd-DOTA is a complex compound that can be used as a contrasting compound recommended by the Food and Drug Administration and the European Medicines Agency.

Gd-DOTA is known to be a contrasting compound with low toxicity potential, so its chemical stability is better than other contrast compounds, but it is still less specific when used to diagnose cancer, especially HER-2 positive breast cancer, therefore research into improving its contrasting capability needs to be done.

The purpose of this research was to synthesize complex compounds (Gd-DOTA)n-PAMAM G3-trastuzumab. DOTA-NHS ligand was conjugated with the dendrimer PAMAM G3, then conjugated with a protein trastuzumab. The resulting DOTA-PAMAM G3-trastuzumab was finally conjugated with gadolinium. Characterization using the UV-Vis spectrophotometric method obtained specific wavelength for (Gd-DOTA)n-PAMAM G3-trastuzumab at 254 nm.

The bounding functional groups were characterized using FT-IR spectrophotometry, obtaining the specific wavelengths for Gd-O at 500.26 cm^{-1} and Gd-N at 415.79 cm^{-1}. The number of Gd-DOTA attached to PAMAM G3-trastuzumab was determined by HPLC to be 2 pieces. The determination of free Gd^{3+} was done using a UV-Vis spectrophotometer with xylenol orange indicator and the result is 0.4594 ppm in (Gd-DOTA)n-PAMAM G3-trastuzumab.

Keywords: Cancer, DOTA, Magnetic Resonance Imaging, PAMAM G3, Trastuzumab.

Introduction

Cancer is currently the leading cause of death worldwide and its incidence continues to rise. Every year, about 12.7 million people are diagnosed with cancer and 7.6 million die as a result of it, making up 13% of the world's cause of death[1]. In 2016, 246,660 women and 2,600 men were diagnosed with breast cancer. This number increased by 61,000 new cases in women. Breast cancer is a cancer that is disproportionately diagnosed in women[2].

One of the cancer diagnoses that have been developed in recent years is Magnetic Resonance Imaging (MRI). MRI can detect cancer quicker compared to other diagnostic techniques. MRI has the additional advantage of being a non-invasive diagnostic tool, thereby possessing a far lower risk of harm compared to other diagnostic techniques like CT-Scan, PET and SPECT[3].

MRI can diagnose cancer quickly with the help of a contrasting compound. MRI is very useful in detecting cancer in women compared to mammography or CT scan[4].

The Gd-DTPA contrast compound (DTPA = diethylethylamenicpentaacetic acid) and Gd-DOTA (DOTA=1,4,7,10 tetraazacyclododecane - 1,4,7,10 - tetraacetic acid) have been approved by the Food and Drug Administration (FDA) and the European Medicines Agency (EMA) and have been widely used clinically for cancer diagnosis. Gd-DOTA is known to be a contrast medium with a lower toxicity potential, so its chemical stability is better than Gd-DTPA[5]. The complex stability of the DOTA ligand is five times higher than the DTPA ligand. Gadolinium is attached to the center of the chelation when the Gd-DOTA complex is formed and the bond angle is uniform such that the Gd-DOTA complex formation is more stable than for Gd-DTPA whose large angle varies widely.

Gd-DOTA contrast compounds can split abnormalities in the human body in general including cancer, so when used as a cancer diagnostic, the Gd-DOTA contrasting compound is

not specific or less specific[6]. Gd-DOTA can only look from anatomical, physiological to metabolic processes and is not very effective at the cellular to molecular levels. Therefore, the development of a contrast medium capable of imaging at the cellular to molecular level must be undertaken. The development is a directional contrast container in which the contrasting means are conjugated to a ligand that has a high affinity to the receptors present in the cancer cell, thereby bringing direct contrast to the target cancer cells and performing specific cancer cell imaging[7].

This study used trastuzumab which can inhibit HER-2 function. Trastuzumab is an antibody designed to target and inhibit the function of HER-2, a protein found in large quantity on the surface of some breast cancer cells. Trastuzumab also stimulates the immune system to destroy cancer cells. Treatment with trastuzumab has resulted in improved responses, higher life expectancy and better quality of life among women with late-stage breast cancer. Clinical studies have evaluated women receiving trastuzumab in combination with chemotherapy and as a single drug for those who have been resistant to treatment[8].

Rahmania et al[9] have performed the synthesis of (Gd-DOTA)n-PAMAM G3-trastuzumab using radiogadolinium (III) for the purpose of biodistribution test and an unknown number of Gd-DOTA successfully attached to compound (Gd-DOTA)n-PAMAM G3-trastuzumab. In this research, a directional contrast compound (Gd-DOTA)n-PAMAM G3-trastuzumab is synthesized and characterized with particular focus on the number of Gd-DOTA bound to the complex.

Material and Methods

The methods and procedures in this study generally refer to Rahmania et al[9]. The product synthesized based on the procedure described by Rahmania et al is then analyzed and characterized. The outline of the synthesis is as follows: Trastuzumab Purification, conjugation of DOTA-NHS with Dendrimer PAMAM G3, activation of DOTA-PAMAM G3, activation of trastuzumab, conjugation of DOTA-PAMAM G3 with trastuzumab, purification of DOTA-PAMAM G3-trastuzumab and conjugation of DOTA-PAMAM G3-Trastuzumab with Gadolinium.

The product of each stage was characterized using high performance liquid chromatography (HPLC) to determine the purity of the complex compounds and FTIR spectrophotometer to determine the functional groups in the complex compounds. The determination of positive fraction at the activation stage was performed by colorimetric test whereas the determination of free Gd^{3+} was performed by UV-Vis spectrophotometer.

Results and Discussion

DOTA-PAMAM G3 synthesis was done by dissolving DOTA-NHS ester in phosphate buffer, then added with a dendrimer. The type of dendrimer used in this study is the 3rd generation of PAMAM dendrimer (PAMAM G3). PAMAM can be applied to deliver drugs in the body[10].

Dendrimer PAMAM G3 can bind a maximum of 32 pieces of the complex Gd-DOTA. After conjugation, the DOTA-PAMAM G3 complex is purified by using a 2 kD MWCO dialysis cassette because the DOTA-PAMAM G3 complex will be separated from impurities having a molecular weight of less than 2 kD.

Dendrimer PAMAM G3 and all complex compound were characterized using HPLC with SEC column (BioSuite, 7.5 × 300 mm), UV-Visible detector, isocratic elution system (PBS 0.05 M pH 7.4) and flow rate 1 mL/min. The result for PAMAM G3 is shown in figure 1.

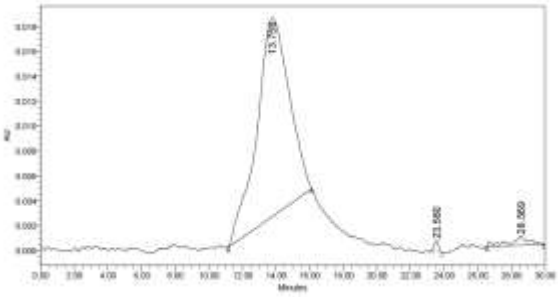

Figure 1: Chromatogram of PAMAM G3 compound and the retention time is 13.758 minute

The DOTA-PAMAM G3 was then characterized using FTIR spectrophotometry. The result is shown in figure 2.

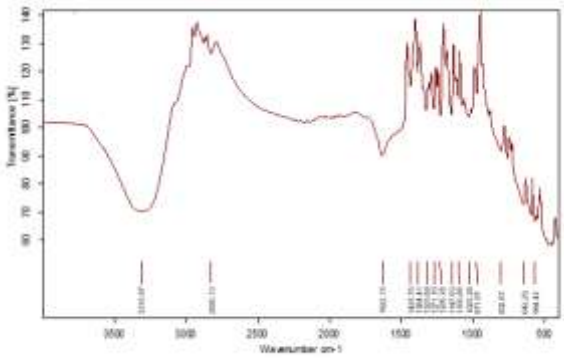

Figure 2: Infrared spectra of DOTA-PAMAM G3 complex compound

The result in figure 2 shows that the -OH groups for carboxylates originated from the DOTA ligands. The N-H group for the 1° amine originated from the unbound PAMAM G3 dendrimer as can be identified in the fingerprint region for N-H bending. This may be due to overlap in the 3600-3500 cm^{-1} absorption area for the N-H and O-H strains and the 3° amine group derived from the PAMAM G3 and more dominant DOTA. Absorption at the wave number for the amide bond indicates that DOTA has been attached to the PAMAM G3 dendrimer such as N-H, C=O and C-N group for amides.

Table 1
Infrared Spectra Interpretation of DOTA-PAMAM G3 complex compound

v (cm^{-1})	Intensity	Prediction
3315. 97	Strong	Strain -OH (carboxylic acid)
2830.13	Weak	Strain C-H sp^3
1632.15	Medium	Strain C=O (amide)
1435.75	Medium	Strain C-N
1384.41 – 1325.69	Medium	Bending -OH (carboxylic acid)
1271.70 – 1025.28	Medium	Strain C-N (amine 1^0, 2^0, 3^0)
802.67 – 643.29	Medium	Bending N-H (amine 1^0, 2^0)

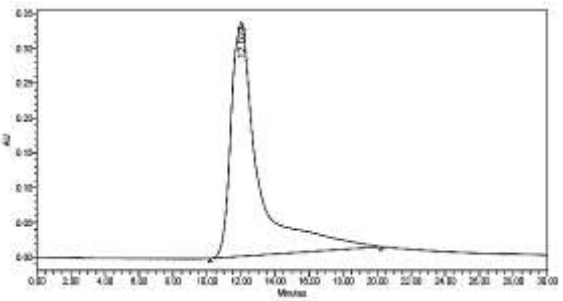

Figure 3: Chromatogram of DOTA-PAMAM complex compound shows a retention time of 12.023 minute

In figure 3, it is seen that DOTA-PAMAM G3 has a retention time of 12.023 minutes and the appearance of a peak on the chromatogram showed that DOTA-PAMAM G3 conjugates have been synthesized and purified after the dialysis process to eliminate excess PAMAM G3 dendrimer. From figure 1 and figure 3, it appears that after PAMAM G3 is conjugated with DOTA-NHS, its retention time decreases from 13.758 minute to 12.023 minute indicating that PAMAM G3 has bound a number of DOTA ligands.

DOTA-PAMAM G3 activation was performed with the aim of adding an -SH (thiol) functional group to the DOTA-PAMAM G3 complex. The -SH (thiol) functional group will be used for DOTA-PAMAM G3 conjugation with trastuzumab which has been activated before-hand. The activation of DOTA-PAMAM G3 is done by adding the Traut's reagent into the pure DOTA-PAMAM G3 conjugate.

Figure 4: Colorimetric test of DOTA-PAMAM G3 fractionation purification result with Coomassie Brilliant Blue G-250 dye protein

Figure 4 shows that B4-C3 (10 fractions) are the positive fractions of DOTA-PAMAM G3 activation. DOTA-PAMAM G3 was activated, and then characterized using FTIR spectrophotometer. The result is shown in figure 5.

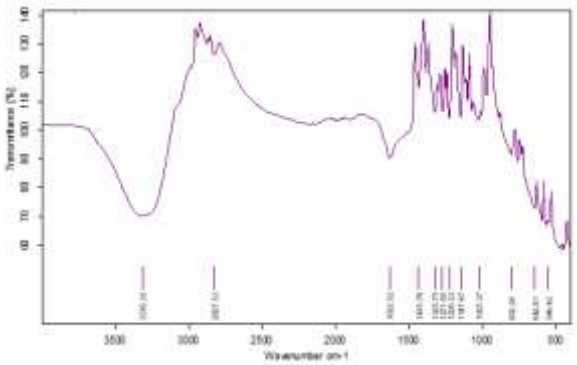

Figure 5: Infrared spectra of DOTA-PAMAM G3 activated with Traut's reagent

Table 2
Infrared Spectra Interpretation of activated DOTA-PAMAM G3

v (cm^{-1})	Intensity	Prediction
3316.35	Strong	Strain –OH (carboxylic acid)
2827.53	Weak	Strain C=N
1632.52	Medium	Strain C=O (carboxylic acid)
1435.76	Medium	Strain C-N
1325.75	Medium	Bending –OH (carboxylic acid)
1271.8 – 1025.37	Medium	Strain C-N (amine 1^0, 2^0, 3^0)
802.26 – 642.81	Weak	Bending N-H (amine 1^0, 2^0, 3^0)
549.42	Weak	Strain C-SH

Trastuzumab activation is carried out by adding a sulfo-SMCC solution dissolved in DMF into the purified trastuzumab.

Figure 6: The colorimetric test of trastuzumab fractionation purification with Coomassie Brilliant Blue G-250 dye protein

Figure 6 showed that B4-C3 (10 fractions) and F7-G10 (14 fractions) are the positive fractions of maleimide trastuzumab.

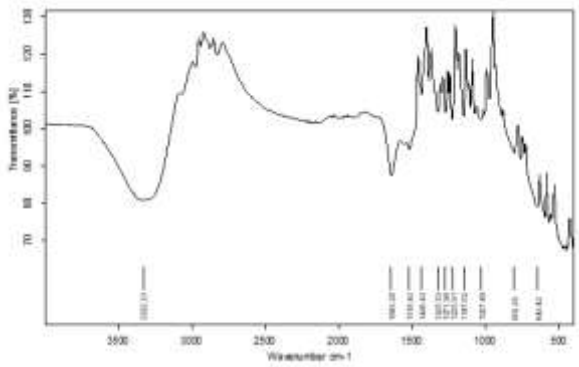

Figure 7: Infrared spectra of trastuzumab activated with sulfo-SMCC

Table 3
Infrared Spectra Interpretation of activated trastuzumab

ν (cm^{-1})	Intensity	Prediction
3332.23	Strong	Strain –OH (carboxylic acid)
2900	Medium	Strain C-H
1643.28	Strong	Strain C=O
1518.43	Weak	Bending N-H
1436.43 – 1325.53	Medium	Bending –OH (carboxylic acid)
1271.98	Medium	Strain C=O (Ar – O)
1225.91	Strong	Strain C-N
1147.02 – 1027.49	Weak	Strain C-N (amine 1^0, 2^0, 3^0)
803.29 – 643.42	Medium	Bending N-H (amine 1^0, 2^0)

The characterization data using FTIR indicates that the addition of the -NH group to the trastuzumab molecule resulted in a shift for the NH-group wavenumber at NH_2. Amino acids usually appear at 1610 - 1580 cm^{-1}, but in the activated trastuzumab, they appear at 1518.43 cm^{-1}.

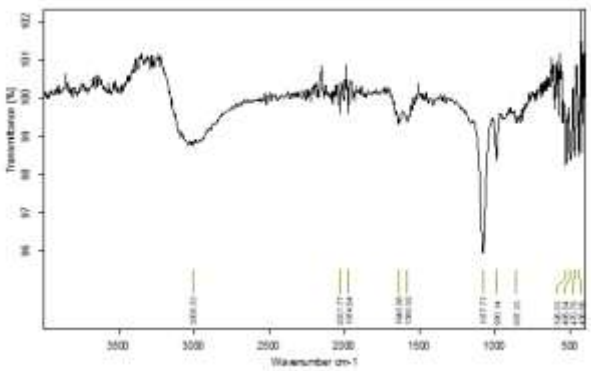

Figure 8: Infrared spectra of DOTA-PAMAM G3-trastuzumab complex compound

Table 4
Infrared Spectra Interpretation of DOTA-PAMAM G3-trastuzumab complex compound

ν (cm^{-1})	Intensity	Prediction
3008.53	Strong	Strain –OH (carboxylic acid)
1974.64	Medium	Strain C-S-C
1640.98	Medium	Strain C=O
1580.92	Weak	Bending N-H (amide)
1077.77	Strong	Strain C-NH_2
990.14 - 857.33	Strong	Strain N-H

In figure 8, the presence of a C-S-C group (sulfur bonded to DOTA-PAMAM G3 and trastuzumab through carbon atoms) is observed. The intensity of 1077.77 cm^{-1} is high enough to indicate PAMAM G3 already bound to one trastuzumab.

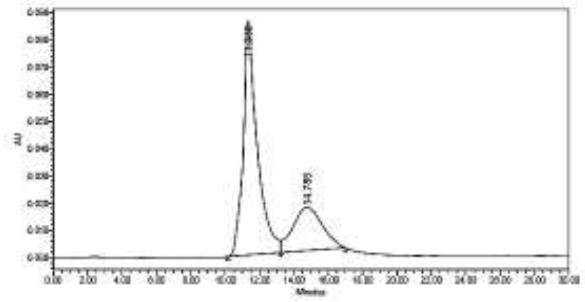

Figure 9: Chromatogram of DOTA-PAMAM G3-Trastuzumab complex compound with a retention time of 11.339 minute

In figure 9 it is shown that the DOTA-PAMAM G3-trastuzumab complex has a lower retention time of 11.339 minute compared to the DOTA-PAMAM G3 complex at 14.786 minute. This is because the molecular weight increases after DOTA-PAMAM G3 activated is conjugated with activated trastuzumab and this change is quite substantial due to the trastuzumab molecular weight of ±145 kD.

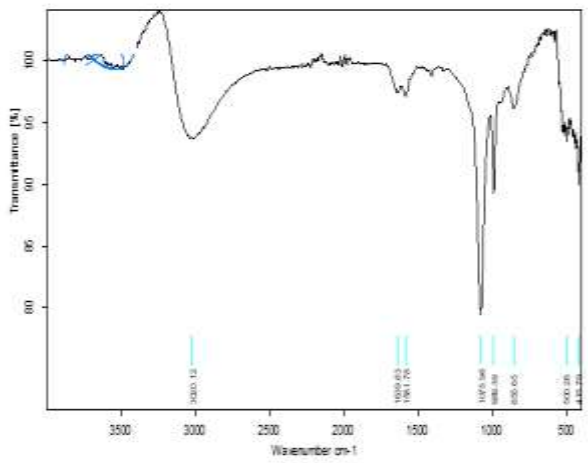

Figure 10: Infrared spectra of (Gd-DOTA)n-PAMAM G3-trastuzumab complex compound

Table 5
Infrared Spectra Interpretation of (Gd-DOTA) n-PAMAM G3-trastuzumab complex compound

v (cm^{-1})	Intensity	Prediction
3020.12	Strong	Strain –OH (carboxylic acid)
1639.83	Medium	Strain C=O
1581.78	Weak	Bending N-H (amida)
1075,98	Strong	Strain C-NH_2
989.39 – 855.65	Strong	Strain N-H
500.26	Weak	Gd-O bonding
415.79	Medium	Gd-N bonding

In the (Gd-DOTA)n-PAMAM G3-trastuzumab complex, it is typical for the Gd-O bonds at the 500.26 cm^{-1} and 415.79 cm^{-1} wave numbers for the Gd-N bonds.

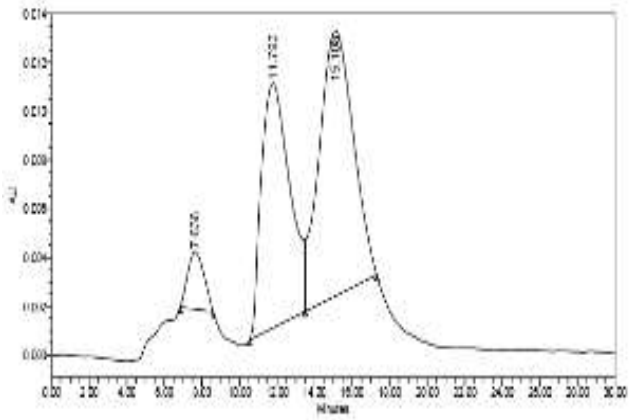

Figure 11: Chromatogram of (Gd-DOTA)n-PAMAM G3-Trastuzumab complex compound where the retention time is 7.636 minute

Figure 11 shows that the compound complex (Gd-DOTA)n-PAMAM G3-trastuzumab appears early at a retention time of 7.636 minute compared to at 11.792 minute for DOTA-PAMAM G3-trastuzumab and 15.108 for DOTA-PAMAM G3 (the first synthesis proccess without trastuzumab and Gadolinium). This suggests a rise in molecular weight in the synthesized compound (Gd-DOTA)n-PAMAM G3-trastuzumab where the presence of a Gd^{3+} ion was conjugated with a DOTA ligand.

In the determination of the number of Gd-DOTA attached to the (Gd-DOTA)n-PAMAM G3-trastuzumab complex compound, characterization was performed using the HPLC approach based on molecular weight (in Log Mr) and retention volume. This approach has been utilized by Brent Irvine in determination of Molecular Size by Section-Exclusion Chromatography (Gel Filtration) for the determination of the molecular weight of proteins by utilizing seven standard proteins with known molecular weights[11].

Figure 12 shows characterization results using HPLC for five standard proteins.

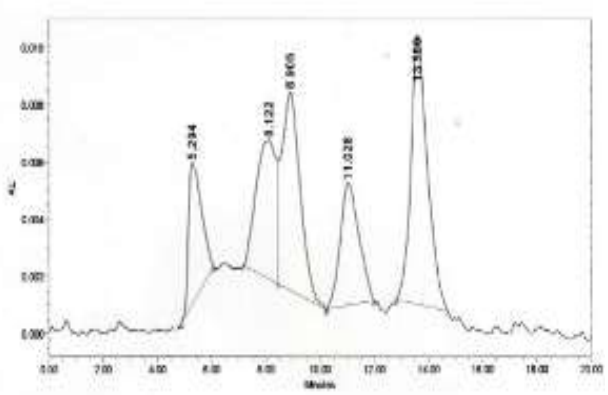

Figure 12. Chromatogram of protein standard

Table 6 shows the protein standard table that is used to determine the molecular weight of (Gd-DOTA)n-PAMAM G3-trastuzumab and their retention volume.

Table 6
Identification of standard protein chromatograms

Peak	Protein	Mr (Da)	Vr	Log Mr
AB	Thyroglobulin	670000	5.294	5.826075
C	γ-globulin	158000	8.122	5.198657
D	Ovalbumin	44000	8.905	4.643453
E	Myoglobin	17000	11.028	4.230449
F	Vitamin B12	1350	13.596	3.130334

From the chromatogram, we get the linear fit as in figure 13.

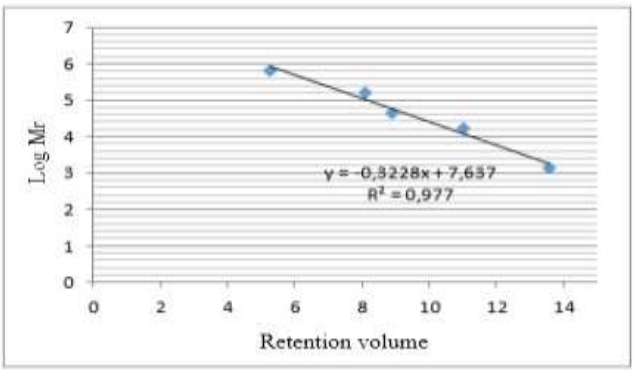

Figure 13: Linear fit to determine the number of Gd-DOTA

The retention volume of (Gd-DOTA)n-PAMAM G3-Trastuzumab is given as 7.636. Thus, the number of Gd-DOTA attached to the complex (Gd-DOTA)n-PAMAM G3-trastuzumab is 2 pieces.

For the determination of harmful free Gd^{3+}, analyze using variation of $GdCl_3$ concentration and was measured by UV-Vis spectrophotometer with Xylenol Orange indicator.

Table 7
Variation of $GdCl_3$ concentration to determine free Gd^{3+}

Concentration (ppm)	Absorbance
2	0.272
4	0.732
6	1.192
8	1.652
10	2.112

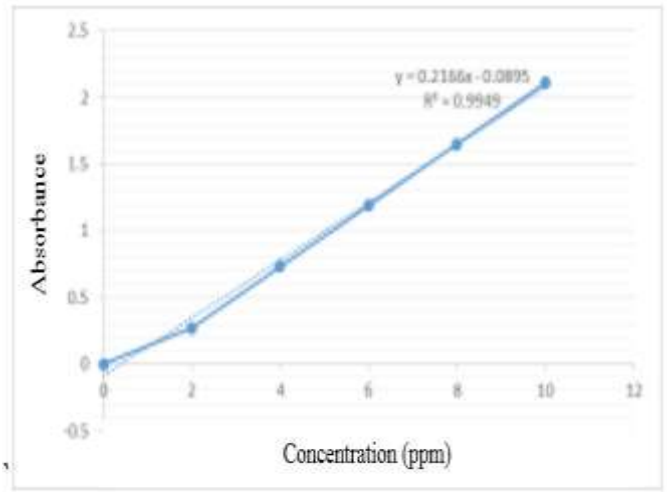

Figure 14. Linear fit to determine free Gd^{3+}

Measurement of sample (Gd-DOTA)n-PAMAM G3-trastuzumab obtained absorbance 0.010. Thus, free Gd^{3+} is present in the (Gd-DOTA)n-PAMAM G3-trastuzumab complex compound as 0.4594 ppm.

Conclusion

From the results of the present study, it can be concluded that:

1. Compound complex (Gd-DOTA)n-PAMAM G3-trastuzumab can be synthesized from DOTA-NHS ester ligand conjugated with PAMAM G3 dendrimer. Then, DOTA-PAMAM G3 is conjugated with trastuzumab and gadolinium by giving a distinctive character.
2. The number of successful Gd-DOTA attached to complex compounds (Gd-DOTA)n-PAMAM G3-trastuzumab is based on the determination of molecular weight. The molecular weight is 150732.881 Da, so the number of bonded Gd-DOTA is 2 pieces expected to improve image quality on MRI.

Acknowledgement

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