Journal of the Korean Magnetic Resonance Society **2003**, *7*, 46 - 54

Synthesis and Characterization of a Receptor-Targeting Contrast Agent

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Received March 13, 2003

Abstract **:** We synthesized a contrast agent for MRI that is capable of binding to the ABP-1 receptor and enhancing the contrast of the targeted cells. We used a lysine dendrimer (G=3)DTPA[Gd] as the contrast agent and synthesized a biotinylated polyclonal antibody for ABP-1 as the first antibody. Lysine dendrimers were prepared using the solid phase peptide synthesis method.[3] Amino-terminated lysine dendrimers were then coupled to DTPA using the anhydride method. Gd was complexed with the DTPA-lysine dendrimer in an acidic solution of 3 eq $GdCl_3$ to one of DTPA. The lysine dendrimer-DTPA[Gd] and avidin were conjugated in MES solution, pH 6.0, using EDC as the coupling reagent. The biotin-avidin system was used to link the polyclonal antibody and contrast agent. K562 cells were used for imaging.

INTRODUCTION

Magnetic resonance imaging (MRI) is a powerful technique that can obtain internal 3D images of biological and non-biological objects non-invasively. MRI is widely used to diagnose disease and detect lesions. Contrast agents are chemicals in which paramagnetic ions, such as gadolinium, are complexed with a chelating agent. The presence of a contrast agent changes the relaxation time in MRI and enhances the image contrast.

Contrast agents that can bind to specific target molecules have a wide range of applications in MRI. The extracellular domain of a membrane protein is one of the best targets for contrast agents, since characteristic proteins are expressed in the membrane of cancer cells or virus-infected cells. The advantage of MRI results from its high spatial resolution.[1] The more specific the binding of the contrast agent to the target, the better the resulting image contrast.[2]

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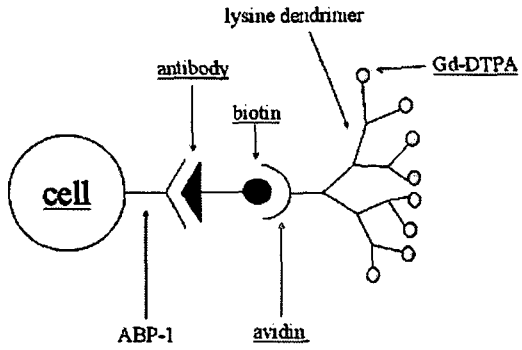


Fig. 1. Schematic representation of the labeling of cells expressing ABP-1 with the targeted lysine dendrimer-DTPA[Gd] using a biotin-streptavidin linker.

This paper presents the synthesis and characterization of a contrast agent designed to target auxin-binding protein-1 (ABP-1), which is anchored in cell membranes. Fig. 1 shows a schematic diagram of the molecular interaction between ABP-1 and the agent. Active targeting utilizes an antigen-antibody interaction and a lysine dendrimer is used to enhance the contrast.[4]

EXPERIMENTAL SECRION

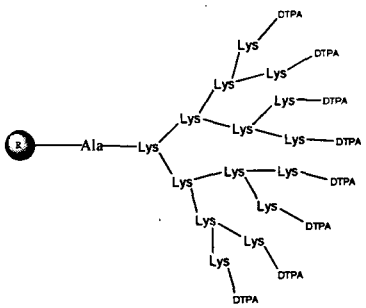
Contrast Agent

Synthesis and characterization of Ala-lysine dendrimer-DTPA [3,4,5]

We prepared DTPA-modified lysine dendrimers with multiple DTPA sites. Wang resin-Ala-lysine dendrimers with Fmoc amino-protective groups were purchased from Advanced ChemTech Co (Louisville, KY, USA). To remove the Fmoc protecting groups, the Wang resin-Ala-lysine dendrimers were placed in a round-bottomed flask to which 30% (v/v) piperidine in DMF (approximately 10 mL/gm resin) was added. The flask was then shaken at room temperature for 30 min. The resin was filtered and washed several times with DMF. The purified Wang resin-Ala-lysine dendrimers with terminal amino groups were placed in a round-bottomed flask and suspended in DCM. Five equivalents of DTPA dianhydride (based on resin substitution) and 5.5 equivalents of HOBt (based on resin substitution) were dissolved in DMF and added to the resin suspension. The mixture was shaken at room temperature under N_2 gas. When the ninhydrin test was negative, the resin mixture was filtered and washed three times with DMF, three times with DIC, and then three times with methanol. To cleave it from the Wang resin, the resin was slurried in 90% TFA in DCM (v/v) and shaken for 1.5 to 2 h. This resin mixture was filtered using a fine sintered

glass funnel and washed three times with small portions of TFA. The combined filtrates were evaporated under reduced pressure to obtain the crude product mixture (Fig. 2).

a)



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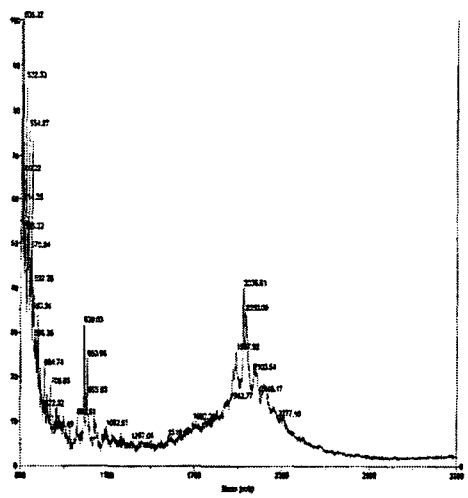

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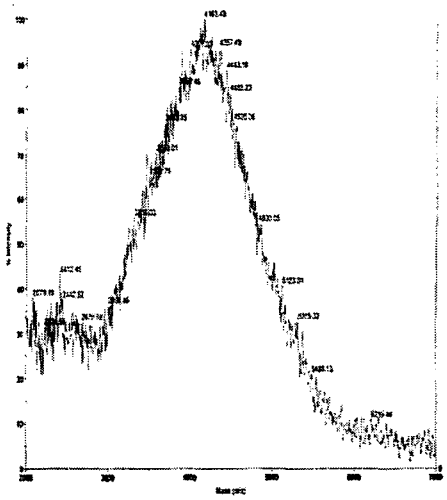

Fig. 2. a) Structure of the Wang resin-ala-lysine dendrimer(G=3)-DTPA. One DTPA was conjugated for each terminal lysine and this dendrimer has eight DTPA molecules. b) Ala-lysine dendrimers with terminal amino groups. MALDI-TOF mass spectrum of the ala-lysine dendrimer. The ala-lysine dendrimer is seen at m/z=2,000. c) MALDI-TOF mass spectrum of ala-lysine dendrimer-DTPA. The DTPA conjugated to the ala-lysine dendrimer is seen near m/z=4100.

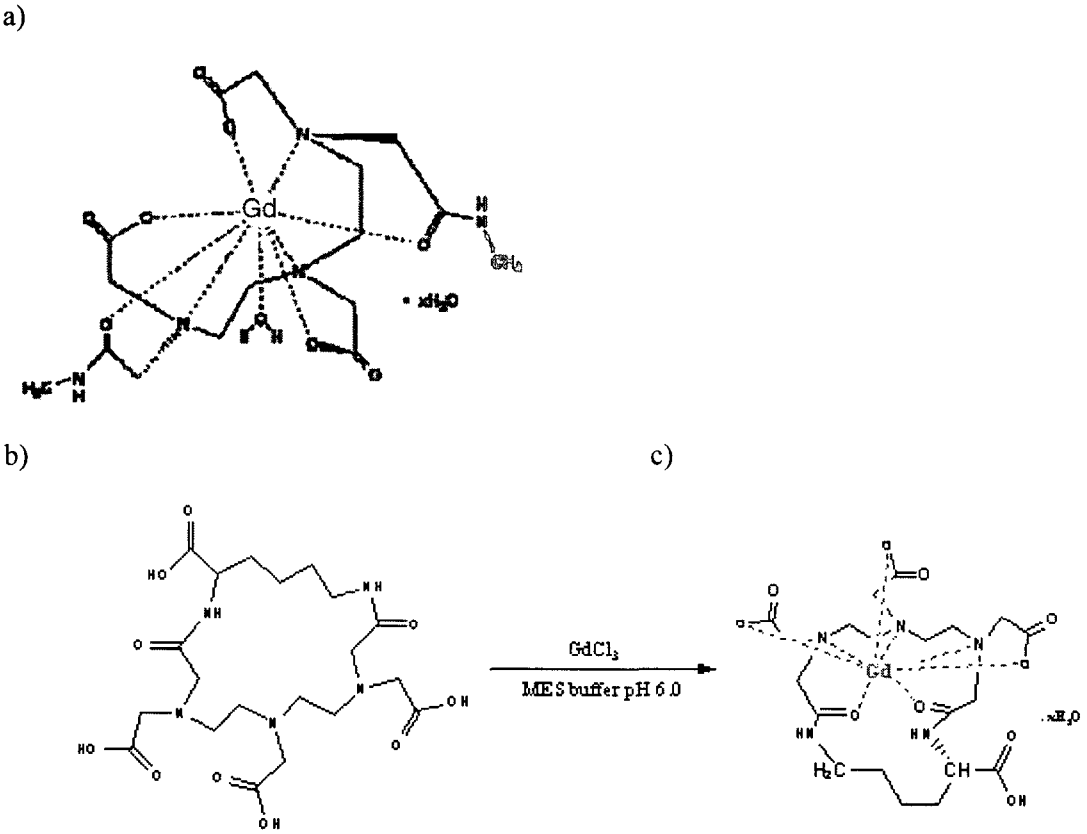

Fig. 3. a) Non-ionic contrast agent: commercially available product (OMNISCAN), b) cyclic structure of lysine-DTPA, c) complexation of Gd in the cyclic structure. This has a structure similar to that of the non-ionic contrast agent in a).

Preparation of streptavidin-ala-lysine dendrimer-DTPA [2, 6, 7, 11, 12]

The ala-lysine dendrimer-DTPA and EDC coupling reagent (10-molar excess relative to the amount present) were added to a cooled solution of streptavidin in 0.1 M MES buffer (pH 6.0).[7] The reaction mixture was then stirred for 12 h at room temperature. After the reaction, the unreacted ala-lysine dendrimer-DTPA was removed by diafiltration using a Centricon (Amicon Co., Millipore, Bedford MA, USA) and dialysis in sodium phosphate buffer pH 7.3.

Preparation of streptavidin-ala-lysine dendrimer- DTPA [Gd][4]

A 3 molar excess of $GdCl_3$ was added to 200-μl aliquots of the streptavidin-ala-lysine dendrimer-DTPA in MES buffer, pH 6.0. This solution was incubated for 1 week, and purified by diafiltration and dialysis (Fig. 6).

Transfection and Immunostaining

K562 human leukemia cells (ATCC) were grown in suspension in RPMI 1640 media (Gibco, Gaithersburg, MD, USA) containing 10% fetal bovine serum. The day before transfection, the cells were counted and plated so that they were 80% confluent on the day of transfection. pDisplay vector containing ABP-1 gene was diluted in serum-free SF transfection medium (RPMI or plain medium). Solutions A (DNA mix) and B (liposome complex) were prepared separately and left at room temperature for 10 min after mixing. To the 200 μl of liposome-DNA complex was added 800 μl of serum-free medium. The cells were washed with fresh media, suspended in 1 ml of liposome-DNA complex, and loaded in a six-well plate containing K562 cells. This plate was incubated for 12 h at 37°C, in 5% CO_2. After transfection, the culture medium was exchanged with RPMI medium containing 10% FBS, G-418 (Geneticin, 500 μg/ml) for selection. After passage number five, the cells were grown in a 25T flask, and then the cells were cultured in bulk.

The transfected cells were washed with PBS buffer solution and fixed with 3.7% formaldehyde in PBS for 1 h. The fixed cells were washed again with PBS buffer solution and treated with blocking solution for 1 h. The cells were then washed twice with PBS buffer. The polyclonal antibody against ABP-1 in blocking solution (1:2000) was added slowly to the K562 cells and placed at room temperature. After 1 h, the cells were washed three times with PBS buffer and gently loaded into a glass capillary tube for MR imaging.

RESULT AND DISCUSSION

Synthesis and characterization of ala-lysine dendrimer-DTPA[Gd] conjugates of streptavidin

Using the lysine dendrimer system, we prepared a receptor-targeting contrast agent to enhance the contrast effect. The generation three dendrimer had eight terminal lysines and DTPAs. One terminal lysine has two amines and can react with one DTPA. Therefore, we synthesized a lysine-DTPA via the carbodiimide reaction and confirmed the cyclization of DTPA to the lysine (Figs. 4 and 5). This streptavidin-ala-lysine dendrimer had eight cyclic forms of DTPA and it was similar to non-ionic contrast agents (Fig. 3). Usually, non-ionic forms are used as contrast agent for brain imaging.

Polyclonal antibody against ABP-1 was purchased from TaKaRa Co (Kyoto, Japan).

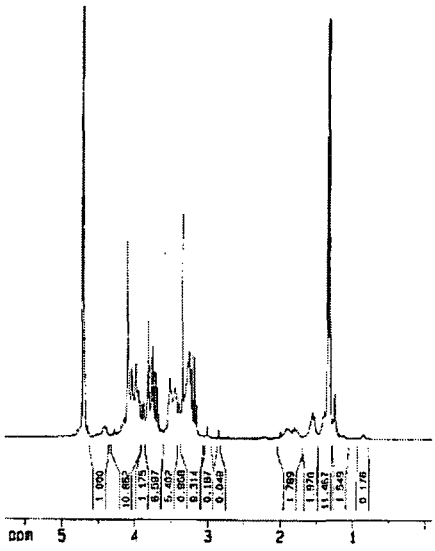

Fig. 4. NMR data for lysine-DTPA. At 1~2 ppm, the lysine backbone is seen, while peaks for the DTPA backbone appear at 3~4 ppm.

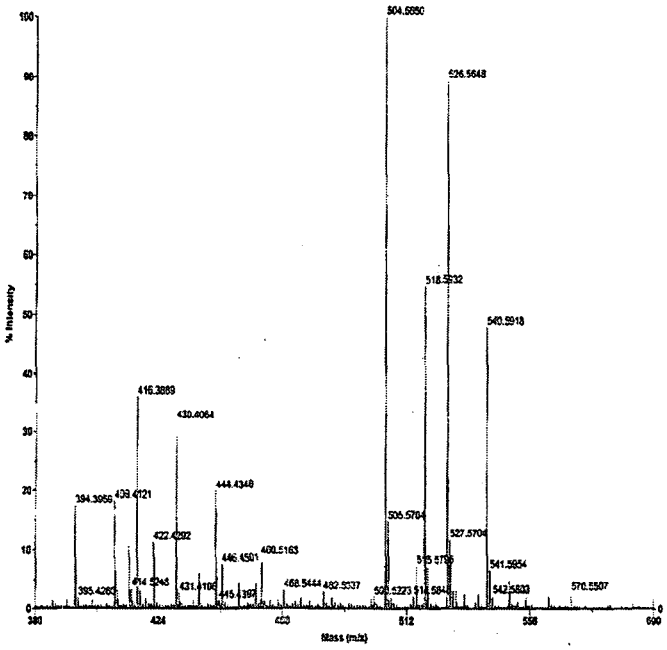

Fig. 5. MALDI-TOF mass spectrum measurement of the cyclic form of lysine-DTPA The compounds at m/z=504.56 and m/z=526.56 are [M+H] and [M+Na], respectively.

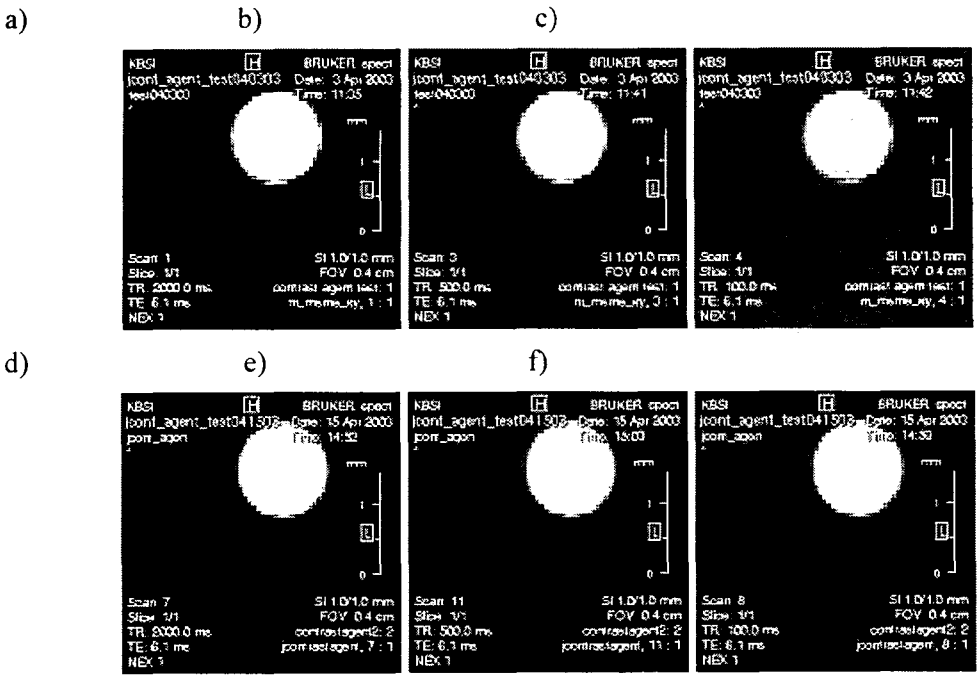

Fig. 6. MRI image of the ala-lysine-dendrimer-DTPA[Gd]: a), b), and c) were obtained using water at 2000, 500, and 100 ms, respectively; d), e), and f) were measured using ala-lysine dendrimer DTPA[Gd] (0.0625 mM) at the same respective times.

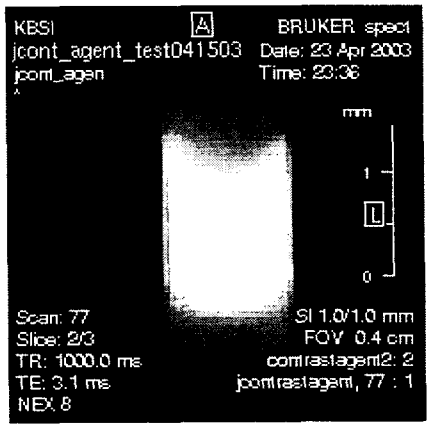

Fig. 7. An image of K562 cells treated with biotinylated polyclonal antibody and streptavidin-ala-lysine-dendrimer-DTPA[Gd].

a) b)

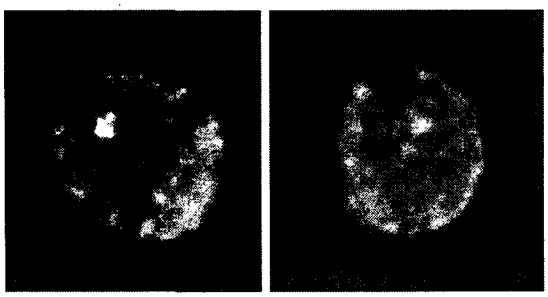

Fig. 8. Confocal image of a K562 cell. K562 cells were transfected with a) pDisplay vector and b) PCAGGS vector. Both were immunostained using polyclonal antibody against ABP-1 as the first antibody and streptavidin-FITC as the second antibody.

Non-specific binding to the ABP-1 receptor might result from the lack of specificity of the polyclonal antibody. For specificity, a monoclonal antibody should be used as the first antibody. Fig. 2(b) shows the distribution Ala-lysine dendrimer and Ala-lysine dendrimer-DTPA at m/z=2,000 and 4,100, respectively. This distribution results from the incomplete reaction. When the dendrimer is synthesized, a generation step is needed to cap each of the free amines so that they do not react.

CONCLUSION

In this study, we synthesized receptor-targeting contrast agent and obtained MR images of the over-expressed receptor on the membrane of K562 cells.

Acknowledgement

This work was supported by Life Phenomena & Function Research Grant and NRL Grant from the Ministry of Science and Technology, the Republic of Korea.

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