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Curcumin Targeted, Polymalic Acid-Based MRI Contrast Agent for the Detection of A β Plaques in Alzheimer's Disease^a

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Abstract

Currently, there is no gadolinium-based contrast agent available for conventional magnetic resonance imaging (MRI) detection of amyloid beta ($A\beta$) plaques in Alzheimer's disease (AD). Its timely finding would be vital for patient survival and quality of life. Curcumin (CUR), a common Indian spice effectively binds to $A\beta$ plaques which is a hallmark of AD. To address this binding, we have designed a novel nanoimaging agent (NIA) based on nature-derived poly(β -L-malic acid) (PMLA) containing covalently attached gadolinium-DOTA (Gd-DOTA) and nature-derived CUR. The all-in-one agent recognizes and selectively binds to $A\beta$ plaques and is detected by MRI. It efficiently detected $A\beta$ plaques in human and mouse samples by an ex vivo staining. The method can be useful in clinic for safe and noninvasive diagnosis of AD.

Graphical abstract

Keywords

Alzheimer disease; curcumin; MRI; nanoimaging agent; polymalic acid

1. Introduction

Alzheimer's disease (AD) is a gradual disease in which memory loss can be accompanied by dementia and even death. Symptoms vary from individual to individual, with increased frequency in older individuals. Currently, there are no effective methods to detect an early onset of AD.^[1–3] Finding a routinely usable MRI-based approach for the detection of AD would be extremely vital for easy management of disease including treatment, quality of life, and patient survival. The standard approach for demonstration of Alzheimer's plaques in human autopsy employs small chemical compounds that attach specifically to the plaques and detected by ex vivo staining or by radioactive scintigram in vivo.^[4] Recent approaches include fluorescent agent-tagged antibodies, and more recently positron emission tomography (PET)^[5,6] for detection of plaques.

However, the problem of all in vivo imaging approaches is that the contrast agents have to cross BBB in order to become effective. Enabling such imaging agents to cross BBB and specifically targeting them to AD plaque using selective plaque-binding compounds like curcumin (CUR), while reducing clearance from circulation could provide the best solution to these problems.

It is well known that neuron deterioration is linked with AD and has lethal effects on the brain. Impaired neurons are often seen with clumps of A β plaques and neurofibrillary tangles (NFT) surrounding damaged dendrites.^[7] The A β plaques are formed from the proteolysis of the amyloid precursor protein (APP) by α - β - γ -secretases and are found in the blood vessels when stained.^[8,9] Accumulation of such plaques due to mutation to the APP or by presenilins (PS1 and PS2) damage neurons and cause neuronal cell apoptosis which eventually leads to the development of AD.^[10,11]

CUR, a common Indian spice is nontoxic and can pass through the blood brain barrier (BBB) when injected into the blood stream. It is now studied extensively and tested as an anti-inflammatory agent and as an inhibitor for A β aggregation.^[12] Besides its therapeutic effects, CUR effectively binds to plaques and has a strong fluorescence signal which can make it a powerful diagnostic tool in the battle against AD.^[12] On the other hand, gadolinium (Gd)-based chelates provide excellent contrast for visualization of variety of tumors and are the most commonly used contrast enhancement agents in clinic. Gd-DOTA chelates have extremely high stability regarding leakage of free Gd which can be highly toxic particularly in the patients with renal problems. PMLA is a natural polymer known to be biodegradable, non-toxic, and non-immunogenic.^[13] PMLA has numerous pendant carboxyl groups which can be easily utilized for the chemical attachment of therapeutic drugs, imaging agents, proteins, RNA-based drugs, and other functional moieties.^[14–21] PMLA provides an excellent drug delivery platform for the variety of drugs against numerous diseases including brain cancer. Here, in this work, we have designed and synthesized a nanoimaging agent (NIA) to detect and image A β plaque in AD using PMLA as a carrier platform. This NIA (Figure 1) contains covalently attached CUR, which recognizes and binds to A β plaque, and a Gd-based contrast enhancement tracer for detection by MRI.

2. Experimental Section

Materials and methods are described in “Supporting Information” section. All experiments with animals were performed in strict accordance with the protocols approved by the Cedars-Sinai Medical Center Institutional Animal Care and Use Committee (IACUC). The human tissues were obtain from post mortem material and qualified for IRB exemption number 4.

2.1. Synthesis of Amino-PEG-Amino-N-Propyl-3-Oxycurcumin (CUR-PEG-Amine)

2.1.1. Attachment of PEG Linker—To a suspension of K₂CO₃ (1.2 mmol) in 2mL acetonitrile was added a solution of BOC-PEG2000-NH₂ (0.2 mmol) dissolved in 2mL of acetonitrile. After stirring at room temperature (RT) for 10 min, 1-bromo-

propyloxycurcumin (0.2 mmol) in 2mL of acetonitrile was added and stirred at RT for 72 h in the dark. The reaction was monitored by reverse phase HPLC.

Upon completion, the mixture was filtered, concentrated, and passed over Sephadex LH-20 in methanol. Products containing fractions were collected. After methanol evaporation, a sticky substance was obtained and used without purification in the next reaction. Reaction yield 73%.

2.1.2. Removal of *tert*-Butyloxycarbonyl (BOC) Group—Methanolic HCl (9 mL, 3M) was added to 200mg of BOC-NH-PEG2000-CUR. After stirring at RT for 16 h, solvent was evaporated to dryness in a rotary evaporator. The thick solid was dissolved in water and freeze dried to obtain a dark yellow solid (yield 96%).

2.2. Synthesis of Gd-DOTA with Amine Side Chain (Gd-DOTA-Amine)

2.2.1. Removal of BOC Group—2-Aminoethyl-mono-amide-DOTA-tris(t-butylester) (2mmol) was dissolved in dichloromethane (10 mL) and the mixture cooled down in an ice bath. Trifluoroacetic acid (TFA, 12mmol) was slowly added under stirring and the reaction mixture was allowed to return to RT. After concentration by rotary evaporator, 3% triisopropylsilane in TFA (4mL) was added and the solution stirred for 40h. Diethyl ether (10mL) was added to form a white precipitate, which was filtered and washed with diethyl ether. Solvent was evaporated under vacuum to obtain the product as a white solid.

2.2.2. Formation of Gd-DOTA Complex—Amino-mono-amide-DOTA (0.5 mmol) was dissolved in water (3 mL), and a slight stoichiometric excess of gadolinium (III) acetate (0.55 mmol) in water (3 mL) was added drop wise. The solution was stirred at RT while maintaining the pH between 6 to 7 by the addition of 1MNaOH, and then stirred for additional 48 h. Free Gd ions were removed with Chelex 100 resin (yield 90%).

2.3. Synthesis of NIA

N-Hydroxysuccinimide (NHS) (0.5 mmol) and DCC (0.5 mmol) dissolved in 1.2mL of DMF were added to the solution of 58mg of PMLA (0.5mmol with regard to malyl units) dissolved in 1mL of anhydrous acetone under vigorous stirring at RT. After 2.5 h to complete the activation of carboxyl groups, the reaction mixture was filtered and acetone was removed by rotary evaporation. Next, 0.05mmol of CUR-PEG-Amine in 1mL DMF (5 mol% with regard to malyl units) was added at RT followed by 0.05mmol of triethylamine (TEA). The reaction was completed within 1 h (assessed by TLC). At this stage, 0.05mmol of Gd-DOTA-amine (10 mol% with regard to malyl units) was dissolved in 2mL of DMSO:DMF (1:4) mixture and was added to the reaction mixture followed by TEA and stirring for an additional 5 h at RT. 5–6mL of 100mMsodiumphosphate buffer containing 150mMNaCl (pH6.8) was added and the reaction mixture was stirred at RT for 45min. After centrifugation at 1 500 g for 10min, the clear supernatant was passed over a PD-10 column pre-equilibrated with deionized (DI) water. Fractions containing the product P/PEG-CUR(5%)/Gd-DOTA(5%) (NIA) were combined and lyophilized to obtain a yellow solid (yield 73%, with regard to PMLA). Content of CUR was determined by UV spectrophotometer with reference to a standard curve of known concentration of CUR.

Amount of Gd was determined by Inductively Coupled Plasma-Mass Spectrometry (ICP-MS) (EXOVA, s cGMP certified laboratory) using gadolinium standards. Percentage (%) of the nanoconjugate loading was calculated by using the formula $\% = 100 \times (\mu\text{mol ligand})/(\mu\text{mol malic acid unit})$.

2.4. MRI Measurement

T1 values of the samples were measured from regions of interest using a single exponential fitting of the intensity for different repetition time scans after saturation. A 9.4 Tesla small animal scanner (BrukerBioSpec 94/20USR, BrukerBiospin MRI GmbH) was utilized to obtain an in-plane resolution of $234 \times 92 \mu\text{m} \cdot \text{pixel}^{-1}$ for a 1 mm thick slice. A 6 cm diameter small animal body coil was used for transmission and detection.

3. Results and Discussion

3.1. Characteristics of the Synthesized NIA

NIA was synthesized (Scheme 1) from two major intermediate products: CUR-PEG-Amine and Gd-DOTA-amine (Scheme 2). Both carried an amino group that could readily form an amide bond with the NHS-activated pendant carboxyl groups of PMLA. NIA resolved as a single elution peak from SEC-HPLC indicating high purity of the synthesis product (Supporting Information). The amount of CUR measured spectrophotometrically was 5% and of Gd by Inductively Coupled Plasma-Mass Spectrometry (ICP-MS) was 5% (with reference to the carboxylic groups in PMLA). NIA carried approximately 35 molecules of Gd-DOTA and CUR each on a single imaging agent. The hydrodynamic diameter of NIA was $8.3 \pm 0.6 \text{ nm}$ and a zeta potential $-19.8 \pm 1.3 \text{ eV}$. Relaxivities were found to be $9 \cdot \text{s}^{-1} \cdot \text{mM}^{-1}$ for free Gd-DOTA, and $12.5 \cdot \text{s}^{-1} \cdot \text{mM}^{-1}$ for Gd-DOTA conjugated NIA. The NIA bound to A β plaque in the subnanomolar concentration range (details are in Supporting Information).

3.2. Detection of A β Plaque by MRI

For proof of concept, we incubated our NIA at 20 μM (in terms of Gd-DOTA concentration) with A β plaques for 3 h in PBS pH 7.4. As a control, a mixture of free CUR and free Gd-DOTA was incubated with A β plaques in a separate tube with equivalent concentrations. After 3 h, the tubes were centrifuged at low speed to settle the plaques. Supernatant was removed and fresh PBS was added. MRI in the presence of NIA and plaques showed a significantly high contrast enhancement (T1 time 1 069ms for the plaques pellet, compared to 2 443 ms for the plaque-free supernatant) (Figure 2A). At the same time, no contrast increase was noticed with plaques incubated with free CUR and free Gd-DOTA. This confirmed the result of the fluorescence binding experiment that NIA was able to bind the plaque via CUR and that this could be detected by MRI.

3.3. Detection of A β Plaques in Human and Mouse Samples

To investigate whether NIA binds to human A β plaques, we incubated human brain sections from AD patients ($n = 2$) and normal or age-matched patient brain ($n = 2$) ex vivo, in solutions containing either NIA (containing covalently bound CUR) or free CUR at 20 μM (CUR concentration). Strong fluorescence intensity was observed in plaque containing tissue

(Figure 2B upper panels) and was absent in control tissue (middle panel). Brain sections stained with hematoxylin and eosin confirmed A β plaques and (NFT) in AD brain (Figure 2C). NIA showed very clean staining pattern without background. In contrast, background was high with free CUR. Similarly, mouse brain sections were incubated with NIA or PBS as negative control. Brain of AD mouse but not the control showed A β plaques with the NIA (Figure 2C bottom panel and D).

4. Conclusion

We have designed and synthesized an all-in-one molecular contrast agent for MRI which recognizes and binds specifically to Alzheimer A β plaques. The binding was followed by the fluorescence of bound CUR, reporter Thioflavin-T and MRI using ex vivo slices of diseased human brain sections or of brain of an AD mouse model, and plaques formed by synthetic peptides. The NIA was able to detect A β plaques in both species (mouse and human). The NIA will have great potential for in vivo application when attached to BBB penetrating agents.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

Schematic presentation of NIA. PMLA was used as a platform to assemble an imaging agent. CUR and Gd-DOTA were attached via amide bonds after forming an activated NHS ester of PMLA's acid groups.

Figure 2.

Detection of A β plaque in brain tissue by MRI and fluorescence microscopy. Panel A, NIA efficiently binds to A β plaques and showed high contrast (red circle) compared with supernatant (blue circle). No contrast was seen for plaques incubated with free Gd-DOTA and CUR (blue circles). Indicated numbers refer to T1 values. Panel B, detection of A β plaques in human and mouse brain sections using fluorescent microscope. Bright staining of A β plaques appeared after staining with NIA, but relatively weak staining was observed with free CUR (upper panel). No signal was detected with either free CUR or NIA in sections from an age-matched healthy control (middle panel). Brain sections of an AD mouse showed similar results (bottom panel). Panel C, hematoxylin and eosin staining of AD human brain. Neurofibrillary tangles and A β plaque are marked by arrows.

A **B** **Scheme 1.**

Synthesis of intermediates. Panel A, introduction of PEG linker to CUR. Heterobifunctional PEG with terminal free amine and BOC-protected amine was attached as a linker to 1-bromo-propyloxycurcumin. BOC group was then removed using methanolic HCl to generate CUR-PEG-amine. Panel B, synthesis of Gd-DOTA with an amido ethylamine side chain. t-Butyl groups on DOTA were eliminated using TFA and triisopropylsilane. Then, gadolinium was chelated using gadolinium acetate in water.

Scheme 2.

Synthesis of NIA. Pendant carboxylates of PMLA were activated by conversion into reactive NHS ester using DCC and NHS. This was followed by sequential addition of PEG-CUR-Amine and then Gd-DOTA-aminoethylmonoamide.