Published in final edited form as:

J Alzheimers Dis. 2017 ; 55(4): 1667–1681. doi:10.3233/JAD-160279.

A Metal-Free Method for Producing MRI Contrast at Amyloid-Beta

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Abstract

Alzheimer's disease (AD) is characterized by depositions of the amyloid- β ($A\beta$) peptide in the brain. The disease process develops over decades, with substantial neurological loss occurring before a clinical diagnosis of dementia can be rendered. It is therefore imperative to develop methods that permit early detection and monitoring of disease progression. In addition, the multifactorial pathogenesis of AD has identified several potential avenues for AD intervention. Thus, evaluation of therapeutic candidates over lengthy trial periods also demands a practical, noninvasive method for measuring $A\beta$ in the brain. Magnetic resonance imaging (MRI) is the obvious choice for such measurements, but contrast enhancement for $A\beta$ has only been achieved using Gd(III)-based agents. There is great interest in gadolinium-free methods to image the brain. In this study, we provide the first demonstration that a nitroxide-based small-molecule produces MRI contrast in brain specimens with elevated levels of $A\beta$. The molecule is comprised of a fluorene (a molecule with high affinity for $A\beta$) and a nitroxide spin label (a paramagnetic MRI contrast species). Labeling of brain specimens with the spin-labeled fluorene produces negative contrast in samples from AD model mice whereas no negative contrast is seen in specimens

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harvested from wild-type mice. Injection of SLF into live mice resulted in good brain penetration, with the compound able to generate contrast 24-hr post injection. These results provide a proof of concept method that can be used for early, noninvasive, gadolinium-free detection of amyloid plaques by magnetic resonance imaging (MRI).

Keywords

Alzheimer's disease; magnetic resonance imaging (MRI); amyloid- β ($A\beta$); amyloid MRI contrast; spin-labeled fluorene (SLF); nitroxide spin label

1. Introduction

Alzheimer's disease (AD) affects over 5 million people in the US and this number is expected to rise dramatically, making it a major public health risk [1]. AD is a progressive, insidious neurodegenerative disease of aging resulting in gradual decline of cognitive function. AD progresses with a complex, multifactorial etiology, however there are two pathological entities present in AD that are targets to both diagnostic and therapeutic endeavors: insoluble extracellular amyloid- β ($A\beta$) plaques, and intracellular neurofibrillary tangles resulting from aggregates of hyperphosphorylated *tau*, a microtubule-associated protein. While the insoluble deposits of $A\beta$ plaques serve as a marker for AD, multiple genetic and biochemical lines of evidence support the hypothesis that the Alzheimer's causative agent is the soluble, oligomeric form of the $A\beta$ peptide [2]. $A\beta$ plaques are generated from the aggregation of soluble $A\beta$ peptides formed when γ and β secretases (proteases) cleave the amyloid precursor protein (APP), a protein constitutively expressed on the plasma membrane of neurons [3]. Because considerable brain damage due to amyloid plaque deposition has already occurred (10–20 yrs) before a definitive diagnosis of dementia can be made [4, 5], it is imperative to develop methods capable of detecting the Alzheimer's disease process at the earliest possible stage. Currently the only definitive diagnostic of AD is post mortem, and clinical diagnosis tools are largely limited to memory testing and PET imaging. Hence, there is a great need for an accessible and noninvasive method capable of the early detection of $A\beta$ levels in the brain. In addition to identifying disease risk, practical measurements of brain $A\beta$ will facilitate the number of clinical trials, disease progression and therapeutic follow-up.

Imaging techniques utilizing radiolabeled positron emission tomography (PET) or single photon emission computed tomography (SPECT) that bind to $A\beta$ peptides in amyloid plaques have the potential to directly assess amyloid burden [6], but have limited availability, are costly, and require use of short-lived radioisotopes. In this regard, a detection method based on Magnetic Resonance Imaging (MRI) for AD risk is highly desirable. MRI provides clear advantages over PET and SPECT such as use of non-radio-active probes, a better resolution for clinical trials and ability to define anatomic details that can be used to quantify amyloid plaque [7].

MRI can detect deposits of amyloid plaques when a sufficient amount of endogenous iron is associated with the plaques [8, 9]. The presence of metals, in particular the iron in the plaques, generates an accelerated T_2^* relaxation rate and negative contrast at regions high in

iron-rich A β [8, 9]. However some regions of the brain contain iron-poor A β depositions [8, 11], making accurate evaluation of total A β load by endogenous iron-induced contrast difficult.

While iron-rich amyloid plaque provides some negative contrast in MRI [9, 12], the lack of signal from iron-poor amyloid limits the significance of the approach for a general assessment of A β levels in the brain. Towards the goal to diagnose AD using MRI, various agents that target A β to generate enhanced contrast in MRI have been created. For example, magnetic nanostructures doped with superparamagnetic iron have been tethered to antibodies directed against the oligomeric form of A β generate contrast in the brains of AD mice [13]. In addition, several Gd(III)-based approaches have explored conjugating a metal chelator to either A β itself [12, 14], or to an Fab directed against A β [15]. Small molecules with affinity for A β assemblies have also been combined with Gd(III) chelators to produce contrast at A β , however addition of the chelated Gd(III) to the targeting molecule diminishes amyloid binding and blood-brain barrier permeability [16]. While metal-containing and/or antibody-directed probes show promise, concerns and limitations drive the need for alternative small molecule agents. For example antibody-directed agents may increase inflammatory cascades [17], and use of immunoglobulin-conjugated Gd(III) agents have low transport in the parenchyma of the brain [12]. In addition, use of chelated Gd(III) is contraindicated in many patients with kidney or liver disease [18]. More importantly, the recent evidence shows that gadolinium is sequestered in the brain many years after contrast-enhanced MRI [19]. These concerns provide an impetus to explore gadolinium-free imaging options for both immunoglobulin-as well as small molecule-based amyloid specific ligands.

To address the need for a Gd(III)-free MRI contrast agent for A β detection in the brain, we have devised a small molecule capable of targeting assemblies of the peptide with high affinity and also producing contrast in MRI. The targeting portion of the compound is based upon the dimehtylamino-fluorene, which carries a demonstrated affinity for A β and good brain penetration [20]. In addition, the molecule contains a nitroxide spin label moiety, a paramagnetic species to enable contrast in MR imaging. We refer to this bi-functional compound as the spin-labeled fluorene (SLF). Its features are identified in Fig. 1, where the nitroxide spin label (black) provides a paramagnetic N-O species that can dock onto assemblies of A β via the fluorene structure (red). Previous, spectroscopic methods have shown that SLF interacts with oligomers of the A β peptide in a similar fashion to the dimethylamino-fluorene molecule [21–23].

Since paramagnetic species increase the microenvironmental magnetic susceptibility, they can be used to enhance contrast in MRI [24]. However, the use of spin labels in contrast enhancement is limited due to the comparatively small magnetic moment of the nitroxide. It has been previously demonstrated that this weakness in nitroxide relaxivity may be overcome if the spins can be arranged into a high local concentration [25–27]. Large amyloid assemblies (as presented in the pathology of AD) can potentially establish such a circumstance, since the SLF is expected to accumulate at high local concentrations in assemblies of both soluble and insoluble A β . Therefore, we examined the ability of the SLF molecule to generate contrast in the brain specimens from transgenic mouse models of AD [28]. Our results support pursuit of the SLF compound as a contrast agent to enable MRI

detection of A β , potentially providing a noninvasive tool for the early determination of AD risk and disease progression.

2. Materials and Methods

2.1 Chemicals and Antibodies

A β (1–40) synthetic peptides (Cat # amp-40-m1) was obtained from EzBiolab, Carmel, IN. The anti Beta-Amyloid, antibody 17–24 (4G8) Monoclonal antibody raised in mouse (Cat # SIG-39220) was purchased from Covance, Emeryville, CA. IRDye[®] 680RD Goat (polyclonal) anti-mouse IgG (H+L) highly cross absorbed, excitation wavelength 676 nm, emission wavelength 694 nm (LI-COR GmbH, Germany). Spin-labeled fluorene HO-4160 (7-Bromo-*N*—[(1-oxyl-2,2,5,5-tetramethyl-2,5-dihydro-1H-pyrrol-3-yl)methyl]-9H-fluoren-2-amine radical) was synthesized as previously described [29]. Hexafluoro-isopropanol (HFIP) was purchased from Sigma-Aldrich (St. Louis, MO). Dimethyl sulfoxide (DMSO) was purchased from Fisher Scientific (Pittsburgh, PA). Thioflavin-S was purchased from Sigma-Aldrich (St. Louis, MO). FSB ((*E,E*)-1-Fluoro-2,5-bis(3-hydroxycarbonyl-4-hydroxy)styrylbenzene) was purchased from Santa Cruz Biotechnology (Dallas, Texas).

2.2 Brain Specimens

Experiments were conducted on female WT mice (14 months of age) and female mice (3-month old) from the Tg6799 5xFAD transgenic AD mice line (5xFAD mice (B6SJL-Tg(APP^{SwFLon},PSEN1*^{M146L}*^{L286V}) 6799Vas/Mmjax (a.k.a. 5xFAD, Tg6799)) were originally obtained from Dr. Robert Vassar at University of Chicago. The 5xFAD mice co-express the human APP-695 protein harboring the Swedish (K670N, M671L), Florida (I716V), and London (V717I) mutations, as well as the human presenilin-1 (PS1) gene harboring two AD-associated mutations (M146L and L286V) thus carrying five mutations responsible for familial AD pathology [30]. This AD transgenic mouse rapidly develops amyloid pathology, with deposition beginning as early as 1.5 months and with plaques spread throughout the hippocampus and cortex by 6 months of age [30]. The mice (n=5 for AD mice and n=5 for WT mice) were sacrificed and their brains were sectioned coronally using a tissue slicer (Siskiyou Inc., OR) into a series of 400 μ m (free floating) and 30 μ m slices (mounted on glass slides), which were then fixed in 4% paraformaldehyde over night at 4–8°C. After fixation, the 400 μ m sections were stored in PBS at 4–8°C while the 30 μ m slices were paraffin embedded and stored at room temperature.. The 400 μ m hemispheric coronal sections containing hippocampus were used for MRI imaging. All animal procedures were approved by the UC Davis Animal Care and Use Committee. Tissues harvested from mice were done in accord with an approved protocol by the Institutional Animal Care and Use Committee.

Confirmatory analysis of MRI contrast was carried out on brain specimens from a 13 months-old PS/APP double transgenic mouse [31]. In the PS/APP strain, A β accumulates in the cerebral cortex and hippocampus starting at approximately 6 months, with later deposition occurring in white matter, cerebrovasculature, and grey matter in the form of diffuse and fibrillar plaques [31].

2.3 In vitro labeling with SLF of amyloid plaques in AD transgenic Mouse coronal sections

Hemispheric coronal brain sections (400 μm thick) in 4% paraformaldehyde from 3-month old 5xFAD AD model mice and from normal control mice (WT mice) were labeled. The free floating 400 μm mouse brain coronal sections were rinsed in 100 mM Tris buffered saline, pH 7.4 (TBS) four times 15 min each and incubated overnight in 80 μM SLF at 4–8 $^{\circ}\text{C}$. After four washes 15 minutes each in TBS, the SLF labeled and control sections were mounted adjacent and in plane in 3% low melting agarose gel that had been degassed and cooled to touch. The agarose slab containing the slices was then mounted within a 35 mm ID volume resonator.

2.4 In vitro MRI imaging

MR images of hemispheric coronal brain slices were obtained at ambient temperature (21 $^{\circ}\text{C}$) using a 7 Tesla (300 MHz) Bruker Biospec system (Billerica, MA) running ParaVision 4.0 software and equipped with a Bruker G060 microgradient set (maximum gradient of 953 mT/m). T_2 -weighted images were performed with RARE sequence, RARE factor 4, and a repetition time/effective echo time (TR/TE) = 1600/12 ms. The Field of view (FOV) was 3.2 $\times$ 3.2 cm^2 and matrix size was 256 $\times$ 256 for an in plane resolution of 125 microns. The slice thickness was 0.2 mm and the number of signal averages was 8. Scan time was 13.8 min. An example of a T_2 -weighted of an entire slab cross-section is given in Fig. S1. T_2^* -weighted images were obtained with a FLASH sequence, flip angle of 30 degrees, repetition time/echo time (TR/TE) = 800/10 ms. The FOV was also 3.2 $\times$ 3.2 cm^2 , and matrix size was 128 $\times$ 128. The slice thickness was either 0.2 mm or 0.3 mm and the signal was averaged over 8 scans.

Quantification of signal suppression in T_2 - and T_2^* -weighted images by SLF in 5xFAD mouse section was calculated using the following equation:

$$\text{SS \%} = [(SI_{\text{ref}} - SI) / SI_{\text{ref}}] * 100 \quad (1)$$

where SI is the average signal intensity of the 5xFAD+SLF section, SI_{ref} is the average signal intensity of 5xFAD+vehicle or WT+SLF control sections.

High resolution T_2^* -weighted imaging was performed with the FLASH sequence, with the flip angle, TR, TE, FOV, and slice thickness as above. The matrix size was increased to 1024 $\times$ 1024 for an in plane resolution of 31 microns. The number of signal averages was doubled to 16 to compensate the decrease of signal to noise ratio due to the large matrix size. Scan time was 3h, 38.5 min.

2.5 Electron paramagnetic resonance spectroscopy (EPR)

Mouse brain sections used for MR imaging were recovered from the agarose slab and washed twice in cold PBS pH 7.4 for 15 min. EPR measurements were performed on a JEOL FA-100 X-band spectrometer using a TE₀₁₁ cavity. Each brain section was loaded into an open-ended flat quartz cell and mounted in the cavity with the flat surface perpendicular to the magnetic field. The EPR spectra were collected at ambient temperature by averaging

three 2-minute scans with a sweep width of 100 G, microwave power of 3 mW and with a modulation width of 0.125 G.

2.6 Antibody staining and histological correlation

Monoclonal antibody 4G8 recognizes residues 17–24 and is reactive against multiple states of both the soluble and insoluble A β peptide. The epitope lies within amino acids 17–22 of beta amyloid (VFFAE). Mouse brain sections used for MR imaging, WT, 5XFAD without SLF and the SLF-labeled 5XFAD brain slices were retrieved from the agarose slabs, and rinsed in 100 mM Tris buffered saline, pH 7.4 (TBS). To unmask the epitope and reverse the protein crosslinking that might have happened during fixation in 4% paraformaldehyde, the brain specimens were incubated in 70% formic acid for 10 min for antigen retrieval, as recommended by the antibody manufacturer. The tissue sections were blocked in TBS containing 0.2% Triton[®]X-100 and 5% goat serum (GS), for 2 hours at room temperature and incubated overnight at 4–8 °C, with affinity purified anti A β antibody (4G8) at 1:1000 dilution in TBS containing 0.1% Triton[®]X-100 and 2.5% GS. After four washes of 15 min. each with TBS containing 0.05% Tween[®] 20 (TBST), the tissue sections were incubated for 2 hours at room temperature with IRDye[®] 680RD Goat (polyclonal) anti-mouse IgG (H+L) highly cross absorbed, excitation wavelength 676 nm, emission wavelength 694 nm at a 1:20000 dilution in TBS containing 0.1% Triton[®]X-100 and 2.5% GS. The tissue sections were washed four times in TBST for 15 min. each and placed in 100 mM Tris (pH 7.4). After a brief rinse in water, the sections were mounted on a glass slide and allowed to air dry for 1 hour at room temperature protected from light. The detection of the fluorescent immunocomplexes was obtained with the LI-COR Odyssey (21 μ m resolution, 2mm offset with highest quality and with sensitivity settings ranged from 0.5 to 3.0, optimized for and applied to each set of sections).

2.7 Staining of 5xFAD specimens with amyloid dyes and co-labeling with 4G8 antibody

Paraffin embedded 30 μ m thick hemi-coronal sections at the bregma region –2.9 prepared as described in 2.3 and mounted on glass slides were deparaffinized and hydrated for thioflavin-S and FSB staining. Briefly, the slides were placed on holders and treated with the clearing agent xylene (paraffin solvent) and a series of graded EtOH as follows: 100% xylene – 5 min, 50%/50% xylene/100% EtOH – 3 min, 100% EtOH – 3 min 95% EtOH – 3 min, 70% EtOH – 3 min, 50% EtOH – 3 min. Sections were washed in water – 2 $\times$ 3 min and stained for 10 min with either a solution of 1% thioflavin-S in 80% ethanol that was filtered through a 0.22 μ m Millipore filter, or in a 5 μ M solution of FSB in 50% ethanol. Stained sections were then washed in 80% ethanol for 1 min and 70% ethanol for another minute and washed in cold tap water 2 $\times$ 3min.

A double staining protocol was used to compare 4G8 (see above) immunoreactivity to Thioflavin-S and FBS staining of plaques. After the Thioflavin-S and FSB staining and to reverse any protein crosslinking that may have occurred during fixation and to unmask the epitope, the brain specimens were incubated in 70% formic acid for 10 min for antigen retrieval [32]. The glass slides containing the sections were then soaked in a solution of blocking buffer, TBS containing 0.2% Triton[®]X-100 and 5% goat serum (GS), for 2 hours at room temperature, to prevent non-specific antibody binding. The samples were incubated

overnight at 4–8 °C, with affinity purified anti β -amyloid antibody (4G8) at 1:1000 dilution in TBS containing 0.1 Triton[®]X-100 and 2.5% GS. After four washes of 15 min. each with TBS containing 0.05 Tween[®] 20 (TBST), the tissue sections were incubated for 1 hour at room temperature with a rabbit anti-mouse secondary antibody labeled with fluorescent Cy3 ($\lambda_{\text{exc}}/\lambda_{\text{em}} = 550/570$ nm) (ThermoFisher Scientific). Finally sections were washed in PBS 4×15 min and mounted to cover slips with Fluoromount (Sigma Aldrich) and sealed with nail polish.

2.8 Characterization of A β deposition in brain specimens by fluorescence confocal imaging

We compared 4G8 immunostaining to thioflavin-S and FSB staining using an Olympus FV1000 spectrum confocal microscope. Each individual field was imaged using an X10 objective. Single plane confocal scans of the hippocampal areas were taken via sequential scanning mode using diode excitation lasers of 405 nm for FSB ($\lambda_{\text{exc}}/\lambda_{\text{em}} = 390/511$ nm) and thioflavin-S ($\lambda_{\text{exc}}/\lambda_{\text{em}} = 430/550$ nm); and 559 nm excitation laser for Cy3 ($\lambda_{\text{exc}}/\lambda_{\text{em}} = 550/570$ nm). As shown in Fig. 2, 4G8 antibody staining of 3-month 5xFAD brain specimens co-localizes with both thioflavin-S and FSB amyloid dyes. Consistent with this phenotype [30], larger deposits are on the order of 20 μm .

2.9 Determination of Blood/Brain Concentrations of SLF

Measurement of SLF brain bioavailability in mice was carried out following the intraperitoneal (IP) injection of three WT mice with 10 mg/kg SLF. Blood was drawn at 20 and 40 min post injection. At 60 minutes post-injection, the animals were sacrificed, and blood and brain harvested. Plasma and brain samples were stored at –80 °C, pending analysis.

Sample separation of plasma and brain tissue was achieved using C18 solid phase extraction cartridges. Eluted fractions were dried under nitrogen and reconstituted in methanol. LC/MS analysis was performed with a Waters Acquity UPLC (Waters, New York, USA) equipped with a Acquity UPLC BEH 1.7 μm C-18 column (Waters, New York, USA) interfaced to a TSQ Quantum Access Max mass spectrometer (MS) (ThermoFisher Scientific, Waltham, MA, USA). An isocratic mobile phase consisting of 20% water and 80% acetonitrile (both containing 0.2% formic acid), with a flow rate of 0.25 mL/min was used for 3 min. Using electrospray ionization MS and selective reaction monitoring (SRM) (capillary temperature 350°C, spray voltage 3000, positive ion mode), SLF was quantified by the product peaks at 258.86, 180.11, 153.07 and 138.20 m/z of its molecular ion peak (412.08, $\text{M}+\text{H}^+$) and its concentration was calculated with a 10-point calibration curve from 5 nmol/L to 10 $\mu\text{mol/L}$ (see Fig. S2).

2.10 Brain uptake of SLF determined by ex-vivo EPR and MRI imaging

Ex vivo analysis of mouse brain following SLF tail vein injection of 100 μM SLF in 50% DMSO (~3.3 mg/kg SLF). After 24-hour, the mice were anesthetized by CO₂ exposure and sacrificed with care to minimize pain. Brains were harvested and sectioned coronally using a tissue slicer (Siskiyou Inc., OR) into a series of 400 μm and 30 μm slices, which were then fixed in 4% paraformaldehyde. The hemispheric coronal sections containing hippocampus

were used for MRI imaging and for immunohistochemical studies. All animal procedures were approved by the UC Davis Animal Care and Use Committee. Mouse brain sections were rinsed in 100 mM Tris buffered saline, pH 7.4 (TBS) four times 15 min each and mounted adjacent and in plane in 3% low melting agarose gel that had been degassed and cooled to touch. The agarose slab containing the slices was then mounted within a 35 mm ID volume resonator. MR images of hemispheric coronal brain slices were obtained at ambient temperature (21 °C) using a 7 Tesla (300 MHz) Bruker Biospec system (Billerica, MA) running ParaVision 4.0 software and equipped with a Bruker G060 microgradient set (maximum gradient of 953 mT/m). T₂-weighted images were performed with RARE sequence, RARE factor 4, and a repetition time/effective echo time (TR/TE) = 1600/12 ms. The field of view (FOV) was 3.2×3.2 cm² and matrix size was 256×256 for an in plane resolution of 125 microns. The slice thickness was 0.2 mm and the number of signal averages was 8. Following MRI measurement, brain slices were removed from the agarose and the presence of SLF measured by EPR spectroscopy as described in section 2.5 above.

3. Results

3.1 SLF labeling facilitates *in vitro* detection of amyloid plaque in brain specimen from an AD transgenic mouse model

Previous biophysical studies of SLF [21, 22], as well as the parent fluorene [23], have demonstrated a direct effect of the molecule on the structure and assembly of soluble A β . These results, in addition to the ability of SLF and the parent fluorene to protect against A β toxicity at sub-micromolar levels, suggests a capacity for SLF to decorate the amyloid- β species in the brain. Although nitroxides provide comparably weak T₁-based relaxivity, macromolecular assemblies of nitroxides have been shown to produce significant contrast in MRI [26, 27]. In a similar vein, we sought to test whether large assemblies of A β can produce a local concentration of paramagnetic nitroxide sufficient to generate contrast in MRI. Thus we obtained MR images of coronal brain slices (400 μ m thick) from an AD mouse model (5xFAD) with and without SLF exposure, where the SLF-negative sample was treated with DMSO vehicle equal to the SLF-exposed sample. 5xFAD transgenic mice exhibit intraneuronal A β at 6 weeks, and develop amyloid deposition, behavioral deficits and impaired memory within 8 weeks [30]. The total amyloid burden increases from ~0.3–0.7 ng/mg protein at 6 weeks to 30-fold at 8 weeks and up to 200-fold at 52 weeks [30]. Following the labeling period with either SLF or the DMSO vehicle control, the brain slices were imbedded into agarose and imaged by MRI. Measurements were made in three separate experiments, using slices from unique AD and WT mice in each experiment (Fig. 3). SLF does not provide observable contrast in the T₁-weighted images. However, both T₂ and T₂*-weighted images of the SLF-treated specimens from AD mice display negative contrast (Fig. 3). 5xFAD brain slices treated with SLF decreases in intensity to a mean value of $1.6 \pm 0.1 \times 10^5$, compared to the T₂-weighted images of the untreated samples, with a mean value of $2.6 \pm 0.1 \times 10^5$. Evaluation of the two sets by the unpaired *t*-test supports a significant difference among these groups (*p* < .01). With respect to T₂*, there also a significant (*p* < .02) intensity difference between the two groups of 5xFAD specimens, with a mean intensity of $1.4 \pm 0.2 \times 10^5$ for the SLF-treated samples, and a mean intensity of $2.3 \pm 0.1 \times 10^5$. In addition, T₂ and T₂*-weighted imaging of corresponding slices from WT mice show no difference

between the SLF-treated and SLF-negative (not shown) specimens. Thus the SLF-induced negative contrast is specific to the AD model mouse specimens that contain a high A β load.

A similar qualitative result is obtained when using brain specimens from the PS/APP mouse model, where SLF-treatment of the sample results in a substantial negative contrast in the T₂*-weighted MRI image (see Fig. S3).

3.2 Quantification of negative contrast from SLF labeled amyloid plaques detected by MRI

To quantify the level of negative contrast due to SLF binding at amyloid-beta, we calculated both T₂ and T₂* signal suppression in each of the three experiments. The average suppression values (calculated according to eqn 1) are plotted in Figure 4, where both the gray and white bars represent the suppression of MRI intensity found in the 5xFAD specimen following SLF treatment. The gray bars show signal suppression relative to the 5xFAD brain in the absence of SLF, while the white bars compare the suppression relative to the SLF-treated specimens from WT mice. Relative to both controls, SLF generates ~40% signal suppression for samples carrying a high amyloid-beta load. In the absence of SLF, the average intensity of the 5xFAD brain sections have a slightly lower T₂/T₂* MRI intensity than specimens from WT mice. This can be attributed to iron bound to deposits of A β , which has been previously observed in the MRI analyses of AD model mice samples [8, 9, 12]. Thus, the SLF treated 5xFAD mice had significantly higher relative signal suppression compared to either vehicle treated 5xFAD, or WT + SLF. Relative suppression is significantly higher when comparing to these controls; but there is little difference between the two controls. This finding clearly demonstrates the ability of SLF to specifically label amyloid plaques and generate negative contrast on MR images.

Because water diffusion and exchange influences contrast enhancement, the observed contrast arises not only from the individual plaques, but also include surrounding tissues and thereby obscuring resolution. To explore the benefit of increased resolution in T₂*-weighted imaging, images of the WT+SLF, untreated 5xFAD, and 5xFAD+SLF specimens were acquired with a matrix size of 1024×1024 for an in plane resolution of 31 microns (Fig. 5, right panel), compared to the 128×128 matrix used to obtain images in the Left panel of Fig. 5 and previous images (Fig. 3). Although the anatomic/structural resolution at the 1024×1024 matrix size is significantly improved, T₂* contrast is lost since T₂* dephasing occurs over larger voxels. Thus, reducing the voxel size (increasing spatial resolution) also reduces the dephasing, and our gain in spatial resolution is offset by poorer T₂* contrast. This effect is quantified in Table 1, when scanning with a matrix of 128×128, the SLF-treated 5xFAD sample suppressed 43% of the signal generated by the untreated 5xFAD specimen. In comparison, the negative contrast achieved at higher resolution was significantly lower (an intensity 74%, or 26% suppression of the untreated 5xFAD slice; Table 1).

3.3. High SLF binding with specificity to the presence of amyloid is supported by EPR analyses

In order to verify SLF binding to the 5xFAD brain specimen, as well as confirm the absence of non-specific binding to WT brain, samples were removed from the agarose, loaded into a

quartz cell, and examined by EPR spectroscopy. As shown in Figure 6, we obtain an intense EPR signal from the SLF-treated 5xFAD brain tissue (Fig. 6, red trace), however no signal is obtained from similarly treated WT brain tissue (Fig. 6, blue trace). Thus, in line with the MRI measurements, the lack of EPR signal from the WT+SLF brain section excludes non-specific binding of SLF in these tissue samples. As expected, the 5xFAD sample lacking SLF treatment shows no EPR signal (Fig. 6, black trace). The broad EPR spectrum from the +SLF 5xFAD reflects a strongly immobilized spin probe, consistent with high affinity binding to a solidified matrix. This highly immobilized state of SLF is not observed in soluble amyloid- β oligomers (A β O), where the molecule displays a high degree of motional averaging due to the fast rotational diffusion within this species [22].

3.4 Anti A β immunohistochemistry correlates to the distribution of amyloid plaques detected by MRI

As shown in Methods, confocal microscopy reveals A β deposition in the 5XAD brain specimens, with anti-A β antibody 4G8 colocalizing with both FSB and thioflavin amyloid dyes (Fig. 2). Using the 4G8 antibody to evaluate the deposition throughout the entire specimen, an InfraRed dye-labeled secondary antibody was applied and the sample scanned for near Infrared fluorescence. To correlate the distribution of MRI negative contrast MRI with histological determined plaque burden the WT, 5XFAD without SLF and the SLF decorated brain slices were retrieved from the agarose slabs and immunostained with the anti-Beta-Amyloid, 17–24 (4G8) monoclonal antibody. Strong indication of amyloid plaque presence in the antibody fluorescence is shown as a pattern of white spots throughout the slice (Fig. 7). These features (apparent in the magnified regions) are typical for what is observed throughout the slice, with spots on the order of 100 μ m in diameter. However due to diffraction of the signal, the source of the intensities are significantly smaller. The observed pattern is similar to the near IR imaging results reported by Skoch et. al. [33] in a PS1/APP mouse brain slice. In the Skoch et. al. study, a longer, higher-resolution scan was able to resolved larger spots on the order of 50 μ m. In our case, we elected to use confocal microscopy to characterize the dimensions of A β deposits in the AD mouse specimens (Fig. 2). This distribution correlates with the diffuse dark pattern (loss of intensity) seen throughout the 5xFAD+SLF mouse brain slice in the T2*-weighted MRI images (Figures 3 & 5).

3.5 Assessment of SLF blood-brain distribution

Table 2 reports the plasma and brain levels of the compound following intraperitoneal injection of 3 mice with 10 mg/kg. Total SLF plasma concentrations were measured from each animal at 20, 40 and 60 minutes. The mice were euthanized 60 min after injection, and the brain removed and stored at –80 °C. UPLC-MS/MS analysis of the SLF in the samples found brain concentrations at roughly 50% of plasma concentrations corresponding to a brain/plasma ratio of 0.5. Taken together these data demonstrate that SLF is sufficiently brain penetrant to be useful as an imaging probe.

In order to determine whether SLF retains its paramagnetism and accumulates to levels sufficient to generate contrast *in vivo*, SLF was injected (i.v., ~3 mg/kg) via tail vein into one 5xFAD mouse and one WT mouse for *ex vivo* analysis of SLF in brain tissue. After 24 hrs

mice were euthanized, with brains removed and fixed in 4% paraformaldehyde overnight at 4–8°C, and 400 μ m coronal slices were prepared and imbedded in agarose. MR images of hemicoronal slices (at bregma –2.12) of both 5xFAD and WT mice were obtained. SLF did not provide observable contrast in T₁- and T₂- weighted images (not shown). As shown in Fig. 8, the T₂*-weighted images of the 5xFAD brain tissue show a slight negative contrast for the 5xFAD mouse coronal slice at the area corresponding to the ventral hippocampus and thalamus. This negative contrast was absent in the specimens from the WT mouse (Fig. 8). MSME sequences were used to measure the relaxation times of the *ex vivo* brain samples. Compared with the WT brain slice (T₁ = 1467 ms), the AD slice showed an obvious decrease in the longitudinal relaxation time (T₁ = 1116 ms, 76% of WT). As for the transverse relaxation times, the T₂ of the WT brain slice was 31 ms, while the T₂ of the AD fell to 28 ms (93% of WT).

To verify that paramagnetic SLF was preferentially retained in the 5xFAD mouse, the brain specimens collected from SLF-injected mice were removed from the agarose slab and analyzed for their nitroxide EPR signal (Fig. 9). Consistent with our results from *in vitro* SLF labeling of brain slices, the WT mouse brain specimen shows no EPR signal, eliminating the non-specific binding of SLF in the tissue samples examined, while the brain slice isolated from the SLF-injected 5xFAD mouse produced a clear EPR signal. Compared to the signal obtained by *in vitro* SLF labeling (Fig. 6), 5xFAD+SLF sample in Fig. 9 generates a weaker EPR signal. However the sample in Fig. 6 was fully bathed in SLF, whereas the SLF labeling of the tissue in Fig. 9 is dependent on accessibility to cerebrospinal fluid (CSF). Furthermore, future studies are needed to determine optimal SLF injection amounts and *in vivo* incubation times.

4. Discussion

Due to their relatively low relaxivity, the application of nitroxides as contrast agents has been limited. However their compact size and organic composition allow for a versatile approach for delivering contrast in certain instances. One such application is the large array of nitroxide docking sites provided by macromolecular assemblies of A β . The fluorene serves as an excellent docking moiety for A β , as it binds to fibrils of A β with an affinity of ~1 nM [20], and to oligomers of A β with affinity of ~15 μ M [23]. Biophysical studies of oligomeric A β (A β O) find similar affinities for the SLF compound [21, 22]. Because a variety of aromatic compounds are known to bind to A β assemblies with affinities ranging from 10^{–10} to 10^{–4} M [34], the combination of small molecules with paramagnetic spin labels provides a flexible approach where the docking moiety can be varied for improved performance.

In this study, we observe SLF-generated negative contrast in T₂- and T₂*-weighted MRI images of coronal brain tissue sections harvested from AD model mice. Given the lack of this effect in samples from WT mice, and the known affinity of SLF for A β , we conclude SLF creates interfaces of high magnetic susceptibility at A β deposits. The specificity of SLF binding to A β deposits is also supported by EPR, as an intense strongly immobilized signal is observed in the AD model specimen, but not in similarly treated tissue from WT mice.

These results are in line with previous studies demonstrating an affinity of the fluorene structure for amyloid beta. Radioiodinated versions were found to bind with nanomolar affinity to A β aggregates as well as display efficient uptake in the mouse brain [20]. In addition EPR [21, 22], circular dichroism [21] and fluorescence correlation spectroscopy [21] measurements demonstrate the SLF directly affects the structure of soluble oligomeric A β O. Finally, as with the parent fluorene [23], preliminary surface plasmon resonance results indicate SLF binds A β monomers and oligomers with high affinity (unpublished).

The SLF generates a diffuse negative contrast throughout the coronal brain section of the 5xFAD mouse in the *in vitro* samples. This correlates with the distribution of A β as detected by monoclonal antibody. The diffuse immunostaining pattern of A β throughout the 5xFAD brain sections correlates with the known pathology of this model, which displays very early amyloid deposition and gliosis [30]. The pattern of the MRI contrast spatially matches that of the histological sections, supporting our conclusion that the decreased signal in the MRI T₂*-weighted images is a consequence of SLF associated with A β . However, in brain specimens isolated from AD mice injected with SLF, negative contrast appears localized at the level of the hippocampus and hypothalamus. As opposed to the *in vitro* bathing of tissue with SLF, the biodistribution of injected SLF *in vivo* will be affected by the selective accessibility of brain regions to the CSF as well as A β content.

As expected by the weak relaxivity of nitroxides and strong immobilization of the spin labels within the AD mouse brain specimens, SLF does not provide observable contrast in the T₁-weighted image intensities. However, negative contrast is observed in both T₂- and T₂*-weighted MRI. The effect on T₂ suggests sufficient spin-spin interaction (dipolar or exchange) with protons, accelerating the dephasing process of local water. In addition, large assemblies of paramagnetic material (with high magnetic susceptibility) can create field inhomogeneities that significantly contribute to the faster T₂* decay. Compared to T₂-weighted images, the lower resolution of T₂*-weighted images is more recognizably darkened over background, although the measured intensity difference in signal suppression with SLF was not significantly different from T₂-weighted images. However, based on studies dependent on elevated iron in amyloid [9, 12], we expect that T₂*-weighted imaging (employing gradient echo sequences that do not refocus decay caused by susceptibility-induced distortions in field homogeneity) may prove to be more robust than T₂ for detecting A β levels by SLF contrast. In addition to determining the ability of SLF to produce contrast in a live 5xFAD mouse, data from future studies will enable a quantitative assessment for the benefit of T₂*-weighted imaging relative to T₂-weighted measurements.

Although extracellular A β serves as the basis for histological AD diagnosis, intraneuronal A β is also recognized for its importance to the AD disease process [35, 36]. Thus having SLF agents capable of intracellular A β detection may be desirable. Although the paramagnetism of nitroxides is chemically stable in CSF and plasma [37], in cells 5- and 6-membered (tetramethyl-pyrrolidinyloxy and tetramethyl-piperidinyloxy, respectively) nitroxides are reduced to non-paramagnetic hydroxylamines [38]. To consider the possibility of intracellular contrast, it may be advantageous to design spin-labeled agents that are resistant to chemical reduction. In a relevant development, Emoto et al [39] described an ascorbic acid resistant, cell permeable nitroxide with a long half-life in mouse brain

(determined by contrast in T₁-weighted MRI). This nitroxide contains tetraethyl substitutions in place of the typical tetramethyl ring substitutions, which shield the N–O moiety from reacting with intracellular reductants.

In a preliminary assessment of acute toxicity, we monitored a cohort of 4 WT mice for 14 days following tail vein injection of 3.3 mg/kg SLF. These mice showed no sign of sedation, agitation or other indicators of acute toxicity. Similarly, the WT mice injected with 10 mg/kg i.p. for the brain penetrations studies showed no signs of acute toxicity. Although it is unknown whether SLF tolerance and bioavailability parameters are suitable for clinical translation, the results here support the rationale for developing a small molecule, Gd(III)-free approach for achieving contrast enhancement in brain regions enriched with the A β peptide. Thus, we are exploring other amyloidophilic compounds that can be combined with spin labels for establishing field inhomogeneities at assemblies of A β . A structurally diverse array of compounds have been found to bind both fibril and oligomeric forms of A β , however many of the agents share the general feature of aromatic rings (containing H-bond donors/acceptors) separated by a rigid linker [34, 40]. Apart from contrast enhancement, an analogous approach has been developed for ¹⁹F-labeled agents using chemical shift imaging (CSI). In this work, a ¹⁹F-labeled curcumin derivative was found to accumulate in the brains of AD model mice sufficient for CSI mapping [41]. As our knowledge on the distribution, specificity and tolerance of small-molecule A β targeting agents becomes more complete, one can envision a multimodal MR-based approach for measuring amyloid in the brain.

A nitroxide-based MRI contrast moiety provides a small, chemically versatile proton relaxation agent. Although clinical studies of nitroxides are limited, animal trials have not observed organ toxicity adverse effects [42, 43]. The results presented here provide proof of principle that the SLF compound represents a novel MRI contrast agent for imaging A β deposits. Such agents have the potential to deliver a much-needed, noninvasive and gadolinium-free method for the early determination of AD risk and monitoring of disease progression. In addition to adverse effects of chelated Gd(III) in patients with kidney or liver disease [18], the growing concern of Gd(III) retention following administration of Gd(III)-based agents [19] promotes the need to develop alternative vehicles for MRI contrast enhancement. This concern is particularly relevant for chronic condition such as AD, where monitoring of disease progression and new therapy evaluation demands imaging methods that can be used on a repetitive basis. Molecules like SLF, which carry an amyloid-beta docking functionality as well as a paramagnetic nitroxide moiety, provide a promising paradigm for achieving this objective.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

This work was supported by a STAIR Award from the University of California Davis to JCV, and in part with funding from the National Institutes of Health (grant P30 AG010129 to JCV and grant EB000993 to AL). The 7 T NMR was made possible by instrumentation grant OSTI 97-24412 from the National Science Foundation. The authors report no conflicts of interest.

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Fig. 2.
Confocal fluorescence analysis of 5xFAD brain tissue (hippocampal region) stained with amyloid dye and 4G8 antibody.

Exp	5xFAD + vehicle	5xFAD + SLF	wt + SLF
T2 -weighted			
A	2.7 ± 0.2	1.7 ± 0.3	2.6 ± 0.2
B	2.5 ± 0.2	1.3 ± 0.2	2.6 ± 0.2
C	2.6 ± 0.2	1.7 ± 0.3	2.7 ± 0.2
T2* -weighted			
A	2.0 ± 0.2	1.5 ± 0.2	2.0 ± 0.2
B	2.4 ± 0.1	1.0 ± 0.1	1.9 ± 0.2
C	2.4 ± 0.2	1.6 ± 0.2	2.8 ± 0.2

Fig. 3.

SLF generates negative contrast in mouse brain with elevated amyloid beta levels. Shown are MR images of mouse brain specimens consisting of a single hemisphere of a coronal slice. Three independent experiments were carried out (A,B,C) on specimens from 5xFAD and WT mice. The sample source and treatment conditions for each column are indicated in the figure header. Each row represents an independent experiment showing images from same coordinates selected from the position providing the most intense slice image under T₂-(upper panel) or T₂*- (lower panel) weighting. Numbers under each box represent

intensity values ($\times 10^5$) of the brain region (absent air or anatomical artifact). In each experiment, the two 5xFAD images are distinct slices from the same animal.

Fig. 4. Magnitude of signal suppression by SLF in 5xFAD slice compared to control (vehicle-treated 5xFAD or WT+SLF). Error bars represent the standard error among values from three independent experiments.

Fig. 5.

T2*-weighted images obtained at standard (left) and higher (right) resolution. Sample labels are given just above the position of the imbedded brain specimen. The left panel shows T2*-weighted image acquired with standard resolution (8 scans, matrix size of 128×128 for an in plane resolution of 250 microns). The right panel shows a T2*-weighted image acquired with a matrix size of 1024×1024 for an in plane resolution of 31 microns. For the higher resolution image, the number of scans was doubled to 16 to compensate the decrease of signal to noise ratio due to the large matrix size.

Fig. 6.

EPR examination reveals specific binding of SLF to A β O as indicated by the presence of an immobilized nitroxide spectrum from the 5XFAD(+)SLF mouse coronal brain slice (red trace), and lack of signal in the 5XFAD(-)SLF(+)vehicle sample (black trace) and the WT(+)SLF sample (blue trace).

Fig. 7.

Near-infrared fluorescence of antibody directed against the A β peptide in the 5xFAD + SLF (L panel) and WT-control (R panel) mice models shows diffuse distribution of A β (white spots) throughout the surface area in the AD mice model, while such features are absent in the control mouse. Zoomed views in the boxes below provide greater detail of selected areas. Typical white spot intensities are on the order of 0.1 mm, however emanate from smaller species due to light diffraction prior to detection. Shown are coronal section of the hemispheres including the hippocampus and cortex. Intensity within the hippocampal region of the 5xFAD sample is indicated by the yellow oval. The stiffness of the AD mouse tissue results in poor surface adhesion at the edges, generating high background signal (regions identified by red ovals). Red scale bar on left image represents 0.2 mm.

Fig. 8.

T2*-weighted MR image of 400 μ m, ex-vivo hemi-coronal brain sections of a 5 mo-old 5xFAD transgenic mouse (top left) and WT (bottom right) following i.v. injection of SLF (~3 mg/kg). The 5xFAD sample shows a marked loss of intensity in the hippocampus area (magenta arrow) versus the WT specimen. Photos of each slice are shown to the right.

Fig. 9.

EPR examination reveals binding of SLF to within the brain of a 5xFAD mouse injected (i.v.) SLF. Shown are the 100 G X-band EPR spectra of brain slices harvested 24-hr post injection from a 5xFAD mouse (black trace) and a wt mouse (red trace). Both mice were injected with ~3 mg/kg SLF.

Table 1

Intensity values for the normal and high resolution T2*-weighted images in Fig. 5. The intensities of comparable ROIs are given for image resolutions of 250 μm (Fig. 5, Left Panel) and 31 μm (Fig. 5, Right Panel).

Specimen	ROI intensity 250 μm resolution	ROI intensity 31 μm resolution
5xFAD+SLF	8.6×10^4	2.3×10^5
5xFAD+Vehicle	1.5×10^5	3.12×10^5
WT+SLF	1.7×10^5	3.2×10^5

Table 2

Post-injection levels of SLF in mouse brain and plasma. Uncertainty of mean is given as Standard Error.

Tissue (time)	[SLF] (nM)		
	Mouse-1	Mouse-2	Mouse-3
Plasma (20 Min)	396	397	663
Plasma (40 Min)	321	431	424
Plasma (60 Min)	492	530	432
Brain (60 Min)	185	268	229
B:P (60 min)	0.38	0.51	0.53
Mean P:B (60 min)	0.47±0.05		