Towards Non-Invasive Diagnostic Imaging of Early-Stage Alzheimer’s Disease

Kirsten L. Viola1,†, James Sbarboro1,†, Ruchi Sureka1,†, Mrinmoy De2, Maíra A. Bicca1,5, Jane Wang1, Shaleen Vasavada1, Sreyesh Satpathy5, Summer Wu6, Hrushikesh Joshi2, Pauline T. Velasco1, Keith MacRenaris4, E. Alex Waters4, Chang Lu1, Joseph Phan1, Pascale Lacor1, Pottumarthi Prasad3, Vinayak P. Dravid2,#,* and William L. Klein1,#,*

1Northwestern University, Neurobiology, Evanston, IL 60208
2Northwestern University, Materials Science and Engineering, Evanston, IL 60208
3NorthShore University Health Systems, Department of Radiology, Evanston, IL 60201
4Northwestern University, Center for Advanced Molecular Imaging, Evanston, IL 60208
5Universidade Federal de Santa Catarina, Department of Pharmacology, SC, Brazil 88049900
6Illinois Math & Science Academy, Aurora, IL 60506

*Northwestern University, International Institute for Nanotechnology (IIN), Evanston, IL 60208

Abstract

One way to image the molecular pathology in Alzheimer’s disease (AD) is by positron emission tomography using probes that target amyloid fibrils. However, these fibrils are not closely linked to the development of the disease. It is now thought that early stage biomarkers that instigate memory loss comprise of Aβ oligomers (AβOs). Here we report a sensitive molecular magnetic resonance imaging (MRI) contrast probe that is specific for AβOs. We attach oligomer-specific antibodies onto magnetic nanostructures and show the complex is stable and it binds to AβOs on cells and brain tissues to give a MRI signal. When intranasally administered to an AD mouse model, the probe readily reached hippocampal AβOs. In isolated samples of human brain tissue, we observed an MRI signal that distinguished AD from controls. Such nanostructures that target neurotoxic AβOs are potentially useful for evaluating the efficacy of new drugs and ultimately for early-stage AD diagnosis and disease management.

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†Authors contributed equally

Correspondence and requests for materials should be addressed to: WLK or VPD, wklein@northwestern.edu; v-dravid@northwestern.edu.

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Study concept and design: K.L.V., J.S., R.S., M.D., V.P.D. and W.L.K. Acquisition of data: M.D., H.J., J.S. and S.V. (MNS development, production, and characterization); K.L.V., J.S., M.A.B., J.W., S.V., S.S., S.W., C.I., J.P. (antibody conjugation, cell experiments, tissue experiments, and immunoprecipitation experiments); K.L.V. and M.A.B. (animal experiments); K.M. (ICP experiments); E.A.W. and P.F. (MR image acquisition). All authors discussed the results and contributed to the analysis of the data. Critical revision of the article for intellectual content: K.L.V., J.S., R.S., M.D., P.T.V., V.P.D. and W.L.K. Obtained funding and supervised studies: W.L.K. and V.P.D.
Alzheimer’s disease (AD) affects 1 in 9 persons over the age of 65. Despite the great personal and economic toll, progress developing effective treatments remains slow. A significant factor is the lack of powerful diagnostic methods. Cerebrospinal fluid (CSF) assays show promise, but spinal taps are invasive and assays of CSF analytes present challenges with respect to accuracy and reliable disease-state discrimination. A promising alternative diagnostic strategy is the detection of AD pathology using targeted brain imaging. The introduction of positron emission tomography (PET) probes for amyloid plaques has been a great technical advance, establishing precedent that brain molecular imaging could become a significant tool for diagnostics and drug development. It is known, however, that amyloid plaques do not correlate well with AD dementia and are not present in the earliest stages of the disease. Probes for alternative markers, especially for the earliest stage of AD, are needed for effective disease intervention and management.

Studies over the last 15 years suggest that pathogenic amyloid beta oligomers (AβOs) provide a more appropriate biomarker than plaques. AβOs cause the synapse failure regarded as responsible for AD memory loss (reviewed in), and they appear early in the disease. Although AβOs offer appealing targets for molecular magnetic resonance imaging (MRI), a specific high-affinity contrast probe needs to be developed. In this context, magnetic nanostructures (MNS) provide an excellent, biocompatible T₂ contrast platform for targeted MRI. MNS below ~ 20 nm diameter exhibit superparamagnetism, lack permanent magnetization at room temperature, and have excellent colloidal stability for both localized and systemic delivery, including blood stream, spinal fluid and nasal passageways. Superparamagnetic MNS create local magnetic field inhomogeneities, yielding negative contrast compared to background. The colloidal form of superparamagnetic MNS also facilitates effective surface functionalization for targeting moieties such as antibodies and fluorescent tags for multimodal imaging and cross-validation of targeting. Conjugation of MNS to target-specific antibodies has been shown to be effective in providing T₂-weighted magnetic resonance (MR) imaging of cellular surface proteins and localized tumours, including those in the central nervous system (CNS).

The current work takes advantage of AβO-specific antibodies that have been introduced to study disease mechanisms (for review) and are now under development for AD therapeutics. Because a successful MRI probe using MNS requires aqueous stabilization of the nanostructure and efficient antibody conjugation, we have produced mono-dispersed, nitro-dopamine (nDOPA) and polyethylene glycol (PEG) stabilized 12–16 nm MNS. These carboxylate functionalized MNS conjugate efficiently to AβO-specific antibodies. MNS-antibody conjugates detect AD-causing toxic oligomers on nerve cell surfaces in vitro, and they quickly reach oligomers in vivo, in a mouse model, following intranasal delivery. Prototype imaging of the mouse model and isolated human brain tissue substantiates the
clinical potential of MR imaging of synaptotoxic oligomers using targeted nanostructure probes.

**Aβ oligomers and the synthesis of a targeted MRI probe**

AβOs are potent neurotoxins that accumulate in the CNS of humans with AD\textsuperscript{23,24,14} and in transgenic (Tg) rodent AD models\textsuperscript{25–27} and are generally accepted as an early event in AD pathogenesis\textsuperscript{28–31}. The usefulness of targeting AβOs as an early AD biomarker is suggested by human neuropathology studies in which AβOs initially appear bound to discrete neurons, localizing to synapses in dendritic arbours\textsuperscript{14} through putative association with clustered cell surface receptors\textsuperscript{11} as seen in Figure 1a. Here, exogenous, fluorescently-labelled AβOs (FAM-AβOs) were added to well-differentiated rat hippocampal neuron cultures, a synapse-forming model that is further used in characterizing the AβO MRI probe. FAM-AβOs bound at discrete sites on dendrites, showing saturable, concentration-dependent synaptic binding. Density and fluorescence intensity of the dendritic puncta increased as the AβO concentration rose to ~150nM (Aβ monomer-equivalent), above which both measures plateaued. Curve fitting indicates a $K_d$ value of ~75nM. The discrete and saturable cell-surface binding of AβOs suggests their potential as a target for a suitable MRI-antibody probe.

To target the MNS, we selected a monoclonal antibody (NU4) that has been shown to recognize AβOs with high affinity in vitro\textsuperscript{19,32} as well as oligomer pathology in vivo\textsuperscript{22}. To ensure that the NU4 antibody specifically detects AβOs, well differentiated hippocampal cells were incubated with FAM-AβOs (as shown in Figure 1) and probed with fluorescently tagged NU4 (633-NU4). Results show a strong association of 633-NU4 with the FAM-AβOs bound to the hippocampal neurons (Supplemental Figure 1). We then compared the pathology detected with NU4 to that recognized by a common marker for amyloid plaques, Thioflavin S (ThioS). Sagittal brain sections from the 5xFAD Tg AD mouse model, and age-matched wild-type (wt) control mice were labelled with fluorescently tagged NU4 (568-NU4) followed by counterstain with ThioS. The 5xFAD transgenic mouse is a model for AD expressing five AD-mutated genes. This model is widely used because it quickly develops AD characteristics.\textsuperscript{33} In the entorhinal cortex, 568-NU4 detected prominent diffuse pathology, sometimes presenting as a halo surrounding the ThioS-positive plaque cores and frequently appearing as diffuse structures lacking a core (Figure 1b). Differences were even more pronounced in hippocampus. No signal was observed in cerebellum or in wt controls. Oligomer-selective NU4 thus targets a pathology that is distinct from the plaque cores detected by ThioS.

A probe for human use must offer sufficient signal sensitivity, stability and biocompatibility, along with high selectivity and specific affinity for relevant targets of interest. Herein we use 12–16 nm Fe$_3$O$_4$ magnetic nanostructures (MNS) as the MRI contrast platform to which specific AβO antibodies are conjugated. The MNS were synthesized following high temperature thermal decomposition of an iron oleate complex as reported earlier.\textsuperscript{34} The MNS prepared by this method exhibit very high monodispersity and enhanced superparamagnetic properties. However, as the surface is coated with hydrophobic ligands which oppose biological applications, additional modification is required.
Previous methods involved MNS that were either stabilized by surface coating with polymer and inorganic layers or by micelle-like bi-layer formation, but maintaining the shell thickness and consistent functional group on the surface is difficult. Moreover, as MRI efficiency depends on water diffusion in proximity to the magnetite core, the surface coating interferes with imaging. Similar problems also arise in the case of micelle-like bi-layer stabilization. Stability can also be an issue for complex biological environments as it relies upon weak hydrophobic interactions.

By comparison, single-layer ligand stabilization by place exchange/removal of a hydrophobic ligand from the MNS surface can enable fine tuning of stability, functionality and overall proton diffusion closest to where its magnetic influence is strongest to enhance its MR properties. Previous use of dopamine, phosphate and amine to stabilize the surface ligand suffered from stability challenges in complex biological environments. Nitro-DOPA (nDOPA) has better affinity for the iron oxide surface and higher stability. We therefore used nDOPA as the surface anchor group and polyethylene glycol (PEG) as a spacer. PEG improves contrast efficiency by enhancing water diffusion in close proximity to the nanocrystal core, provides appropriate biocompatibility, improves colloidal stability, and retains desired magnetic properties such high $r_2$ relaxivity for $T_2$ weighted MR contrast. It is expected that the nDOPA will have no effect on the dopamine receptors of the brain as it is sequestered in the form of a self-assembled monolayer. The nDOPA is used as an “anchoring group” between the MNS and PEG, leaving no free nDOPA in the solution or associated with the probe. Moreover, nDOPA has low agonist efficacy.

The stabilization method involves monolayer formation of a polyethylene glycol ligand on the MNS surfaces, and the ligand was terminated with carboxylate functionality for conjugation with antibodies (Figure 2a). The nDOPA MNS were stable over a month in physiological buffers (Supplemental Figure 2). No agglomeration was observed in the stable colloidal suspension by either TEM (Figure 2b) or dynamic light scattering (Supplemental Figure 2b). The zeta potential and dynamic light scattering (DLS) measurements of the 16 nm particles showed a surface potential of approximately −40 mV and a nominal hydrodynamic radius of 30 nm, indicating excellent stability and colloidal dispersion in aqueous solutions (Supplemental Figure 2). In fact, the MNS solution is sufficiently colloidal to be run through an agarose gel, similar to gold nanoparticles (Supplemental Figure 2a).

The MR relaxivity of the PEG-conjugated MNS was measured using a Bruker minispec 60 MHz (1.41T) relaxometer. We observed a very high $r_2$ value of 385 s$^{-1}$ mM$^{-1}$, significantly higher than commercially available Ferumoxytol (dextran coated iron oxide, 80 s$^{-1}$ mM$^{-1}$) and Ferumoxide (silica coated iron oxide, 120 s$^{-1}$ mM$^{-1}$) (Figure 3c). We attribute the enhanced $r_2$ to the increase in hydrophilic surfaces with carboxylate and glycol ligand tailoring, which interacts strongly to influence surrounding proton relaxation. Potential toxicity of the MNS was assessed in primary hippocampal cells and four different mammalian cell lines. We observed that cell viability was conserved after 24 hr incubation with a high concentration of MNS (1 mM Fe) (Supplemental Figure 2d).
The buffer-stabilized MNS were targeted to AβOs by attaching them to the NU4 antibody. Coupling was by carboxyl-amine conjugation using 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide (EDC). The resulting probe, MNS conjugated to NU4 (NU4MNS), was magnetically separated from free residual antibodies and stored in phosphate buffered saline (PBS) at pH 7.2. NU4MNS remained homogenous and colloidal in PBS for at least 3 hr at room temperature, ample time to allow administration. Additionally, the NU4MNS could be readily re-dispersed to a homogenous colloidal suspension with brief sonication, even several weeks after their preparation.

**NU4MNS probe detects Aβ oligomers in cells and human tissue**

To verify that the NU4MNS probe maintained the high affinity and specificity of the parent antibody, we compared AβO detection by NU4MNS with NU4 using fluorescence microscopy. Mature cultures of hippocampal cells were incubated with FAM-AβOs and probed with NU4MNS (Figure 3, left) or NU4 (Supplemental Figure 3a) and imaged using a fluorescent secondary antibody. The efficacy of the NU4MNS probe for binding AβOs was determined by measuring the percentage of co-localization between FAM-AβOs and NU4 or NU4MNS. Quantification of fluorescence intensity established that NU4MNS showed >90% co-localization with AβOs bound to hippocampal neurons. Cells incubated with vehicle showed virtually no binding by NU4MNS (Figure 3, left), establishing that the high specificity of NU4 for AβOs was retained following conjugation to MNS. Experiments using immunoprecipitation further confirmed that the NU4MNS probe retained its specificity for AβOs in solution (Supplemental Figure 3b & 3c). Immunoprecipitation of AβOs was not due to non-specific adsorption, as non-immune control IgGMNS showed no isolation of AβOs from a 200 nM solution.

To determine the impact of the conjugation procedure on the affinity of the probe for AβOs, hippocampal cultures were treated with FAM-AβOs, then probed with increasing concentrations of NU4 or NU4MNS (0–20μg/ml, antibody concentration). Both NU4 and NU4MNS showed high affinity for oligomers, demonstrating saturable binding with similar doses needed for half-maximal binding (1–2μg/ml) (Figure 3, right). We next tested the prediction that the probe would retain the specificity of the parent NU4 for AD brain tissue relative to control brain tissue. The first experiment compared binding of NU4MNS to cortical slices from human AD and age-matched non-demented (control) patients by immunofluorescence microscopy (Figure 4). Floating human brain sections were labelled using 633-NU4, 633-NU4MNS, or 633-IgGMNS. Sections were imaged at 10X with an epifluorescent microscope. Immunofluorescent signal was detected in the probable AD slices and minimally present in the aged control patients. 633-NU4 and 633-NU4MNS both detected diffuse plaque-like structures as well as smaller clusters of AβOs in AD brain tissue, consistent with 5xFAD results (Figure 1b) and with previous studies of AβO neuropathology. Diffuse plaques of oligomers in human samples have been previously shown to be distinct from and manifest prior to dense-core amyloid plaques that stain with congophilic dyes and currently-used PET probes. Controls established that these structures were not detected using MNS conjugated to non-specific IgG. The data demonstrate the specificity of NU4MNS for AD neuropathology.
NU4MNS distinguishes AD from control non-AD tissue by MRI

Intranasal delivery of NU4 previously was used to bypass the blood brain barrier and bind AβOs in the brain of 5xFAD mice. To confirm that 568-NU4 could detect AβOs in living animals after intranasal delivery, aged 5xFAD and wt littermate mice were inoculated with 568-NU4 and allowed to recover for 4 hours. Sagittal sections were mounted for imaging of antibody distribution. Images show that the 568-NU4 antibody localized throughout the frontal and temporal cortex (not shown) and the hippocampus (Figure 5a) of the 5xFAD mice, binding to diffuse pathology and discrete puncta on cells. Wt mice showed low levels of fluorescence in the pre-frontal cortex (data not shown), compared with the 5xFAD mice, and no labelling in the temporal or hippocampal regions, confirming that the NU4-based probes can be delivered by intranasal inoculation and specifically bind to the target. For comparison, we examined detection of AβOs exposed to 568-NU4 in vitro. The pattern was similar to that seen with in vivo labelling, presenting largely as dispersed puncta around cell bodies and within the neuropil (Figure 5b).

Having shown that NU4MNS retained specificity for AβOs and that NU4 can be delivered to its targets by intranasal inoculation, we tested whether the NU4NMS probe offered sufficient contrast to identify AβO samples by MRI. In a preliminary test, we treated hippocampal neurons as above with AβOs or vehicle, with fluorescence verifying the presence or absence of oligomers. Cells were then probed with NU4MNS and imaged with a 7T MRI scanner. MRI clearly confirmed an AβO-dependent signal, exhibiting more than 300% signal difference in MRI when AβOs were present (Supplemental Figure 4bB). Having confirmed the predicted MR signal in vitro, we next tested whether an AβO-dependent signal could be obtained in vivo. Aged 5xFAD and wt mice, 8 months old, received 5μg (antibody concentration) NU4MNS by intranasal inoculation. Animals were allowed to recover and the probe to distribute for 4 hours before MR imaging. Images from a Tg mouse (Figure 6a) and a wt littermate show a clear signal in the hippocampus of the mouse AD model that is absent in the wt mouse. Signal was also detectable in the cortex (not shown). Following the initial treatment and scans, animals were allowed to recover and were then re-examined by MRI 96 hours later to look for changes in distribution and clearance of the probe (Figure 6b). Results show a change in the distribution and a modest reduction of signal intensity at the hippocampus of the 5X FAD mouse, but not a complete elimination of the probe. Currently, the data would suggest that the probe remains in the brain tissue for longer than 4 days, but it is not yet certain the exact length of residency. The clear difference between the 5xFAD mouse and the control was substantiated by a second NU4NMS inoculation and subsequent MR imaging (Supplemental Figure 4c). Importantly, animals were monitored for signs of infection or distress at the inoculation sites and in general for more than 2 months after treatment. No adverse effects or signs of pain or infection from the inoculation were detected. Overall, the data show that the NU4MNS probe, like the parent antibody, quickly gains access to the hippocampus and that it can provide an MR signal that distinguishes the 5xFAD mouse AD model from controls.

To assess the quality of signal that eventually might be feasible under optimal conditions, we examined MRI signals using brain sections from human and Tg mice. Brain slices, 50μm thick, from 8-month old transgenic 5xFAD and wild-type littermates were probed with 555-
NU4MNS using the procedures described in the Methods. Some sections were imaged for fluorescence to confirm that the probe properly detected pathology, while the remaining slices were layered in agarose for MR imaging. Because of the thinness of the slices, MRI of the sections in-plane could not be readily obtained. Instead, slices were layered on top of each other in agar, with about one cm of agar separating the Tg from the wt slices. Slices were imaged cross-sectionally rather than in-plane (1) to allow multiple slices to appear in the same field of view for better comparison of signal intensity and (2) to address concerns about partial volume artefacts that can result from imaging samples that are thinner than the scan slice depth of the equipment. While this type of analysis can provide information about probe density bound to the tissue in total, it cannot be used to provide localization information. Images show a robust, disease-specific MRI signal in the 5xFAD brain slices that is not detected in the wt slices (Figure 6c). These data further substantiate the specificity of the probe for Alzheimer’s disease pathology.

We concluded by assessing the potential to obtain a disease-relevant MRI signal from human tissue. Again, because the sections were too thin to be imaged in-plane, the sections were layered in agar and cross-sectional images were collected using a 7T scanner. Untreated sections were used as a control to determine baseline MR signal from endogenous iron levels. MR imaging of the human brain samples (Figure 6d) showed that the NU4MNS probe provided a robust, AD-dependent signal.

**Conclusions**

Results here show that an AβO-targeted molecular MRI probe can reach targets within the CNS and is capable of differentiating AβO-containing samples from controls in cells, mouse models, and isolated human tissue. Successful development of the NU4MNS MR probe may have significant value for early AD diagnostics. An AβO-directed diagnostic using MRI would provide both structural information and molecular-targeted contrast for localization of early AβO accumulation. Such information would be of great value for developing effective AD therapeutics by providing a means to track the efficacy of investigational new drugs that target AβOs. Moreover, previous studies with the NU4 antibody have demonstrated a behavioural improvement following NU4 therapy, suggesting a therapeutic potential for the NU4MNS probe in addition to its diagnostic value. Thus, imaging of AβOs with non-invasive in-vivo modalities such as MRI has the potential to revolutionize AD management at its earliest stage for therapeutic intervention and overall disease management.

While a formal study of the pharmacokinetics of the probe has not yet been completed, the data presented show that the probe can be introduced intranasally, cross the blood-brain barrier to distribute to the intended targets within 4 hours, and shows a significant clearance from the brain within 4 days after introduction. It has been shown by Xiao et al. that HRP-NU4 antibody is distributed throughout the brain following the olfactory, rostral migratory stream and trigeminal routes with a residency time in the brain of 96 hours. While distribution of the NU4MNS probe seems to correlate with the published distribution of HRP-NU4, NU4MNS probe residency in the brain appears to be longer.
In conclusion, a molecular MRI probe designated NU4MNS, capable of targeting Alzheimer’s disease-related AβOs, has been synthesized by conjugating AβO-selective antibodies to superparamagnetic nanoparticles. NU4MNS is both specific and sensitive and can distinguish AD brain tissue from non-demented controls by MRI in vitro. In vivo, the probe reaches the brain following intranasal inoculation of mice and can provide an MRI signal that requires transgene-dependent AD pathology. The development of humanized AβO-specific antibodies\textsuperscript{21} substantiates the potential of this new approach for use in AD diagnostics and for measuring the efficacy of investigational new drugs. The nanotechnology-based probe, which can readily be adapted to other targets, provides a strategic and powerful early detection advantage over current PET probes for amyloid by targeting AβOs, toxins putatively responsible for the neuron damage beginning early in Alzheimer’s disease.

**METHODS**

**Animals**

5xFAD Tg mouse model (B6SJL-Tg(APPswFlLon,PSEN1*M146L*L286V)6799Vas\textsuperscript{33} (Jackson Laboratories) is bred on a non-transgenic background (B6SJLF1). Aged transgenic and wild-type littermates, 6–12 months old, were used.

**Cell Lines**

HeLa is a human cervical cancer cell line; NIH/3T3s is a mouse embryonic fibroblast line; HEPG3 is from hepatocellular carcinoma; RAW264.7 is a mouse leukemic monocyte macrophage cell line.

**Synthesis of Magnetic Nanostructures (MNS)**

16 nm magnetite nanoparticles were synthesized by decomposition of iron-oleate at 320°C as described in an earlier report.\textsuperscript{43}

**Synthesis of Iron-oleate complexes**—10.8 g of iron (III) chloride hexahydrate and 36.5 g sodium oleate were dissolved in a mixture of 60 ml distilled water, 80 ml ethanol and 140 ml hexane and heated at 60°C for 4 hr. The organic layer of the biphasic mixture becomes dark, indicating phase transfer of iron (III) ions and formation of iron oleate complex. The resulting dark solution is separated and washed with water three times.

**Synthesis of 16 nm magnetite nanoparticles**—18 g of iron oleate complex and 2.58 g of oleic acid were dissolved in 100 g of octadecene at room temperature and heated to 320°C at a rate of 3.3°C per minute. The reaction mixture is kept at 320°C for 40 min., then cooled down to room temperature. Resulting nanoparticles are separated from the solution by addition of ethanol and ethyl acetate followed by centrifugation.

**Preparation of Dopamine-TEG-COOH and Phase Transfer**

To make the organic phase synthesized MNS suitable for biological application we stabilized the MNS using carboxylate terminated ligand with nDOPA as an anchor.
Synthesis of carboxylate terminated nDOPA ligand and functionalization of the MNS was carried out according to the following protocol. Tetraethylene diacide, N-hydroxy succinimide (NHS), N,N′-Dicyclohexylcarbodiimide (DCC), nDOPA hydrochloride and anhydrous sodium bicarbonate was dissolved in chloroform under argon atmosphere and stirred for 4 hr. Hexane stabilized MNS were added and stirred for another 24 hr. The precipitate formed was separated by magnet, dispersed in water and purified by dialysis.

Conjugation of antibody to MNS

The conjugation of buffer stabilized MNS with antibody was done using a conventional carboxyl-amine crosslinking method. We first activated the carboxyl terminated MNS by sulfo-N-hydroxy succinimide (SNHS) and 1-Ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC) followed by incubation with corresponding antibody (NU4 or IgG1, with or without fluorescent label) overnight. Conjugated MNS were separated by magnet to remove excess reagent and antibody then re-dispersed in working media. Conjugation efficiency was estimated using UV spectroscopy (absorbance at 280nm) of the magnetically separated supernatant.

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\text{Ab conc.} = (\text{total mg added Ab}) - (\text{mg Ab in supernatant})
\]

Of note, because both fluorophore and MNS conjugation use carboxyl-amine crosslinking, fluorescent NU4 that has more than 2.5 mol fluorophore/mol antibody does not conjugate well to the MNS. Optimal concentration of fluorophore/NU4 is ~1.0–2.5 mol fluorophore/mol antibody for fluorescent-NU4MNS preparation.

Intranasal immunization

Mice were anesthetized with isoflurane and then placed on their backs with their heads positioned to maximize the residency time for the delivered material to remain on the olfactory surface. Each naris was administered with 568-NU4, NU4MNS or non-immune IgGMNS (5 μl/naris), using a sterile micropipette, slowly over a period of 1 min, keeping the opposite naris and mouth closed to allow complete aspiration of delivered material.

Magnetic Resonance Imaging of Brain Slices

A Falcon tube containing the MNS treated tissue samples was placed in a Bruker PharmaScan 7T animal MRI scanner. Sample were imaged using a T2*-weighted Gradient Echo (GRE) FLASH pulse sequence 3-dimensional acquisition. The total acquisition time (TA) was 6 hr, 25 min. The echo time (TE) was 30ms. The flip angle (FA) was 15 degrees. The image resolution was 0.07mm isotropic, with field-of-view (FOV) 4.62cm/2.52cm/ 0.65cm and image matrix (MTX) 700 pixels/385 pixels/100 pixels. The number of averages (NEX) was 20 to improve the signal-to-noise ratio.

Magnetic Resonance Imaging of Tg and wt mice in vivo

Following intranasal inoculation, the probe was allowed to distribute for 4 hours before MR imaging was performed according to imaging methodology described in Mundt et al. 44 T1
T2, and T2* weighted MR images were acquired on a Bruker BioSpec 7T magnet, using a 25 mm RF quadrature coil. The in-plane resolution was 75 μm with slice thickness 0.4 mm. T1- and T2-weighted images provide anatomical guidance as well as some localization of the NU4MNS and were acquired with a fat suppressed spin echo sequence (Rapid Acquisition with Relaxation Enhancement, RARE) with the following parameters for T1-weighted (TR=1000 ms, TEeff=13.2 ms, rare factor 2, number of excitations, NEX=4) and for T2-weighted (TR=3500 ms, TEeff=58.5 ms, rare factor 4, NEX=4). T2*-weighted imaging provides more of the localization of the NU4MNS as the iron causes local changes in magnetic susceptibility which T2* weighted images can be sensitive to. A gradient echo sequence was used with the following parameters (gradient echo fast imaging, GEFI; TR=1200 ms, TE=5.6 ms, flip angle 35° and NEX=4).

**Supplementary Material**

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. Aβ oligomers bind to neuronal surfaces in saturable, receptor-mediated manner and are distinct from amyloid plaques

a) Binding of FAM-AβOs to neuronal surfaces is saturable. AβO binding to primary hippocampal cells was measured using increasing doses of FAM-AβOs (representative cell images shown in inset). The numbers in the inset and graph correspond to Aβ monomer equivalent concentrations (nM). Fluorescence intensity per length of process is shown as a function of AβO concentration. The data were fit to a sigmoidal dose-response curve with an EC50 value of ~75nM. Saturation is consistent with receptor-mediated binding. b) Sagittal brain sections, 50μm thick, from 8 month old 5xFAD and wt mice were probed with 568-NU4 and counterstained with Thioflavin S. Images were captured from multiple regions of the brain. Top row – 5xFAD cortical region stained with NU4 (red), Thioflavin S (green), and the merged image (right). Bottom row shows the merged images from 5xFAD cerebellum (left), wt cortex (centre), and wt cerebellum (right). Findings demonstrate that NU4 labelling is often associated with, yet distinct from, amyloid plaques. NU4 labelling is more abundant than the ThioS staining. Data supports the notion that NU4 thus affords an excellent targeting antibody for the development of an AβO-specific MRI probe that is distinct from currently available plaque probes. Scale bar = 25μm.
Figure 2. Individual components of the NU4MNS probe

MNS are ligand stabilized and uniform in size. 

- **a)** Structure of carboxylate terminated ligand stabilized MNS and **b)** TEM of the aqueous stabilized MNS. 
- **c)** Relaxivity measurements of MNS at 1.42 T and 60 HZ.
Figure 3. NU4MNS attachment to hippocampal neurons is specific to AβOs
Attachment of NU4MNS to hippocampal neurons is stringently AβO dependent. (Left) Hippocampal cells, treated with FAM-AβOs or vehicle control, were probed with fluorescent NU4MNS. Data show that NU4MNS are highly AβO specific and AβO dependent. (FAM- AβOs - green, NU4MNS - red, and co-localization - yellow). (Right) Dose curves of antibody (NU4) and NU4MNS detection of AβOs shows that NU4 conjugation to MNS causes only minor loss of affinity for AβOs, if at all.
Figure 4. NU4 and NU4MNS discriminate AD human frontal cortex sections from aged controls

Human frontal cortex sections were probed with fluorescently tagged NU4, NU4MNS, or control IgGMNS and imaged. NU4 and NU4MNS detected diffuse plaque-like structures and smaller neuronal deposits, consistent with typical AD pathology. These structures were not seen in control brain and not detected by the non-specific IgGMNS probe, demonstrating that NU4 is capable of discriminating between AD and non-demented controls, and that this specificity is retained after MNS conjugation. Scale bar = 100μm.
Figure 5. NU4 antibody detects dendrite-bound AβOs in fixed tissue and binds its target within 4 hours following intranasal inoculation

a) Mice, 8-month old 5xFAD and wt littermate controls, were inoculated with 568-NU4 intranasally. The probe was allowed to distribute for 4 hours before the animals were sacrificed and their brains removed. Sagittal sections, 50μm thick, were obtained and mounted to slides. Images were captured with an inverted Nikon TE2000 microscope from multiple regions of the brain. The images show 568-NU4 distribution in the CA1 and CA3 hippocampal regions of the 5xFAD mouse on the left and wt mouse on the right. Labelling seen in the 5xFAD mouse is punctate and appears to be more prevalently associated with the somas of the cells. Wt controls showed no 568-NU4 probe distribution in the hippocampus. Data confirm that the 568-NU4 antibody readily distributes to its targets within 4 hours after intranasal administration.

b) Sagittal brain sections, 50μm thick, from fixed 8 month old 5xFAD and wt mice were probed with 568-NU4. Images were captured with a confocal microscope from multiple regions of the brain. Shown, from left to right, are a single plane from Tg hippocampus, the maximum projection of all planes at the same location, and the maximum projection of the hippocampal region from the wt mouse. Images show punctate labelling around the cell bodies of the pyramidal cells in the Tg mouse but not the wt. Scale bars = 15μm.
Figure 6. NU4MNS labelling of human and Tg mouse brain slices gives pronounced, AD-dependent MRI signal that is confirmed in vivo

a) *In vivo* imaging of probe distribution in live mice 4 hours after intranasal inoculation shows labelling by the probe in the hippocampal region of the Tg mice, but not the wt controls.

b) Higher magnification of the hippocampal region of the Tg and wt mice shows probe distribution 4 hours after inoculation and the changes in distribution 96 hours later. While the probe distribution has changed and decreased, there are still detectable levels of probe present.

c) Sagittal sections from Tg, top panel, and wt, bottom panel, 8-month old mice were probed with NU4MNS and layered in agarose for MR imaging. Due to the thinness of the sections, it was necessary to capture cross-sectional images as opposed to traditional “whole slice” images. Using cross-sectional captures, NU4MNS bound to the AβOs in Tg slices (as seen in Figure 1b) produces an MRI signal that appears as linear structures. Cross-sectional imaging allowed us to image both Tg and wt slices at the same time, but does not provide any specific localization information. Representative cross-sectional images were chosen for presentation. Images show a robust signal in the transgenic mice brain sections that is absent from the wt littermate sections.

d) Human brain slices were probed with NU4MNS and examined by cross-sectional MR imaging as described for the mouse brain slices in (c). MR imaging showed that NU4MNS provided a strong signal in the AD samples. Signal was greatly reduced or absent from the age-matched controls. NU4MNS detection of AβOs appeared as linear structures with variable signal intensities along the lines suggesting the non-uniform distribution of AβOs across the section. Representative cross-sectional images were chosen for presentation. Taken together, these data provide proof-of-concept for NU4MNS as an MRI probe of AβO-load in human patients and Tg AD mouse models. Scale bars=5mm.