**In-vitro and in-vivo characterization of CRANAD-2 for multi-spectral optoacoustic tomography and fluorescence imaging of amyloid-beta deposits in Alzheimer mice**

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**ABSTRACT**

The abnormal deposition of fibrillar beta-amyloid (A\textsubscript{\beta}) deposits in the brain is one of the major histopathological hallmarks of Alzheimer’s disease (AD). Here, we characterized curcumin-derivative CRANAD-2 for multi-spectral optoacoustic tomography and fluorescence imaging of brain A\textsubscript{\beta} deposits in the arcA\textsubscript{\beta} mouse model of AD cerebral amyloidosis. CRANAD-2 showed a specific and quantitative detection of A\textsubscript{\beta} fibrils in vivo, even in complex mixtures, and it is capable of distinguishing between monomeric and fibrillar forms of A\textsubscript{\beta}. In vivo epifluorescence microscopy and optoacoustic tomography after intravenous CRANAD-2 administration demonstrated higher cortical retention in arcA\textsubscript{\beta} compared to non-transgenic littermate mice. Immunohistochemistry showed co-localization of CRANAD-2 and A\textsubscript{\beta} deposits in arcA\textsubscript{\beta} mouse brain sections, thus verifying the specificity of the probe. In conclusion, we demonstrate suitability of CRANAD-2 for optical detection of A\textsubscript{\beta} deposits in animal models of AD pathology, which facilitates mechanistic studies and the monitoring of putative treatments targeting A\textsubscript{\beta} deposits.

1. Introduction

The abnormal accumulation and the spread of amyloid-beta (A\textsubscript{\beta}) deposits play a central role in the pathogenesis of Alzheimer’s disease (AD) and leads to downstream pathophysiological events [1,2]. Positron emission tomography (PET) imaging of aberrant A\textsubscript{\beta} deposits has been established as a diagnostic pathological biomarker for AD under clinical setting and been included in the new diagnostic criteria [3]. Three amyloid imaging probes have been approved for clinical usage including 18\textsuperscript{F}-florbetapir [4], 18\textsuperscript{F}-florbetaben [5] and 18\textsuperscript{F}-flumetamol [6]. Higher PET imaging of cortical A\textsubscript{\beta} loads were reported in the brains from patients with AD and mild cognitive impairment compared to healthy controls [7,8]. While PET imaging in small rodents has been used for studying A\textsubscript{\beta}-related disease mechanisms and for developing therapeutic strategies [9-15], it has the disadvantages that it requires extensive infrastructure for the generation of dedicated radiotracers and has a low spatial resolution (1 mm) relative to the mouse brain dimension (10 mm × 8 mm).

Optical imaging techniques such as near-infrared fluorescence (NIRF) imaging and multi-spectral optoacoustic tomography (MSOT) imaging have emerged, enabling the in vivo imaging of physiological, metabolic and molecular function [16-23]. This is mainly due to the fact that optical techniques are cost-effective, do not require an extensive infrastructure and use stable labels that allow validation of imaging findings with histology. Progress is made not only in the development of optical instrumentation and reconstruction algorithms, but also in the design and synthesis of novel A\textsubscript{\beta} imaging probes [24]. For example, several probes have been reported for NIRF imaging including NIAD-4

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2.2. In vitro binding between amyloid probes and recombinant Aβ fibrils

Protection and were approved by the Cantonal Veterinary Office Zurich (vMSOT) has been introduced with added multiplexing and real-time 3D imaging capabilities, thus enabling a wide range of new biomedical applications [39–44]. Previously, intravital optoacoustic imaging of Aβ deposits with intrathecal injection of Congo Red [27] and croconium-derivative [45] in amyloidosis mouse models have been reported.

In the present study, we show that CRANAD-2, previously used for NIRF imaging [33,34], is suitable for MSOT imaging and describe its utility for in vivo whole brain mapping of Aβ deposits in arcAβ mouse model of cerebral amyloidosis [46]. Capitalizing on the fluorescent properties we characterized binding of the probe to Aβ fibrils in vitro using a fluorescence binding assays, in situ with tissue sections and immunohistochemistry and by cross-validation between MSOT and hybrid fluorescence imaging [47,48]. Our results demonstrate suitability of CRANAD-2 for NIRF and MSOT imaging of Aβ deposits.

2. Materials and methods

2.1. Animal model

Seven transgenic arcAβ mice overexpressing the human APP695 transgene containing the Swedish (K670N/M671L) and Arctic (E693G) mutations under the control of prion protein promoter and five age-matched non-transgenic littermates of both sexes were used (18–24 months-of-age) [46]. ArcAβ mice are characterized by a pronounced amyloid deposition, cerebral amyloid angiopathy and vascular dysfunction [43,49–53]. Animals were housed in ventilated cages inside a temperature-controlled room, under a 12 h-dark/light cycle. Pelleted food (3437PXL15, CARIGILL) and water were provided ad-libitum. Paper tissue and red Tecniplast mouse houses® (Tecniplast, Milan, Italy) shelves were placed in cages as environmental enrichments. All experiments were performed in accordance with the Swiss Federal Act on Animal Protection and were approved by the Cantonal Veterinary Office Zurich (permit number: ZH082/18).

2.2. In vitro binding between amyloid probes and recombinant Aβ1–42 fibrils measured by spectrofluorometry

(T4)-[{(1E,5E)-1,7-Bis[4-(dimethylamino)phenyl]-1,6-heptadiene-3,5-dionato-K3}2K2] difluo-roboron (CRANAD-2) [33] was purchased from Sigma-Aldrich AG, Switzerland. The emission spectrum and excitation spectrum were measured by using F-4500 FL Spectrophotometer using CRANAD-2 25 μM in phosphate buffered saline (PBS, pH 7.4). For the measurement of emission spectrum, the excitation is set at 640 nm, emission wavelengths were recorded between 680–900 nm. For the measurement of excitation spectrum, the emission is set at 820 nm, excitation wavelengths were measured between 500–750 nm. Recombinant Aβ1–42 peptides were expressed in E.coli as described previously [54]. Aβ1–42 fibrils were formed by incubating a solution of freshly purified 2 μM Aβ1–42 monomers in phosphate buffer (20 mM sodium phosphate, 0.2 mM ethylenediaminetetraacetic acid, pH 8.0). The aggregation process was monitored by a quantitative fluorescence assay based on the Thioflavin T (ThT) dye [55]. CRANAD-2 stock was dissolved in dimethyl sulfoxide (DMSO, Sigma-Aldrich AG). The in vitro binding between CRANAD-2 (0.1 μM) or ThT (20 μM) with Aβ1–42 fibrils (0–0.2 μM) was measured on a spectrofluorometer (Fluoromax-4, HORIBA Jobin Yvon Technologies, Japan) by recording the emission spectra in the range from 660 nm to 800 nm after excitation at 440 nm, or in the range from 450 nm to 650 nm respectively after the excitation at 440 nm. The lower concentration of CRANAD-2 was selected to avoid precipitation. To investigate whether CRANAD-2 binds selectively Aβ1–42 fibrils, we assessed the binding of CRANAD-2 (0.1 μM) and ThT (20 μM) to other targets that are rich in β-sheet structures including bovine serum albumin, monoclonal antibody, and amyloids formed by lysozyme, α-synuclein and insulin, all at 0.2 μM. Moreover, we assessed whether CRANAD-2 can distinguish between different conformations of Aβ by measuring binding also to Aβ1–42 monomers. Finally, the specificity of CRANAD-2 for Aβ1–42 fibrils was further evaluated by measuring the binding between the probe and Aβ1–42 fibrils (0–0.2 μM) spiked in a cell lysate obtained from E.coli with a total protein concentration of 7 μM.

2.3. MSOT

Cross-sectional MSOT imaging was performed with a commercial inVision 128 small animal scanner (iThera Medical, Germany) as described [56]. Briefly, a tunable (680–980 nm) optical parametric oscillator pumped by a Nd:YAG laser provides 9 ns excitation pulses at a framerate of 10 Hz with a wavelength tuning speed of 10 ms and a peak pulse energy of 100 mJ at 730 nm. Ten arms, each containing an optical fiber bundle, provide even illumination of a ring-shaped light strip with a width of approx. 8 mm. For ultrasound detection, 128 cylindrically focused ultrasound transducers with a center frequency of 5 MHz (60 % bandwidth), organized in a concave array of 270° angular coverage and a curvature radius of 4 cm, were used. Phantom and in vivo MSOT images were acquired at 10 wavelengths, i.e. 680, 685, 690, 695, 700, 715, 730, 760, 800 and 850 nm, coronal section, averages = 10, field-of-view = 20 mm × 20 mm, resolution = 100 μm × 100 μm, a step size = 0.3 mm moving along the axial direction.

For in vivo MSOT, five arcAβ mice and four non-transgenic littermates were imaged with MSOT in vivo. Mice underwent also magnetic resonance imaging (MRI) for co-registration with MSOT data and to facilitate volume-of-interest (VOI) analysis. Mice were anesthetized with an initial dose of 4 % isoflurane (Abbott, Cham, Switzerland) in oxygen/air (100/400 mL/min) mixture and were maintained at 1.5 % isoflurane in oxygen/air (100/400 mL/min). The fur over the head was depilated. The mouse was placed in a mouse holder in prone position. The holder was inserted in an imaging chamber filled with water to keep body temperature within 36.5 ± 0.5 °C. Mice were injected intravenously with CRANAD-2 (2.0 mg/kg, 15 % DMSO + 70 % PBS pH 7.4 + 15 % Kolliphor EL, Sigma-Aldrich AG) through the tail vein. Datasets were recorded before, 20, 40, 60, 90, and 120 min after the injection.

MSOT images were reconstructed using a model-based algorithm, size 20 mm, resolution 100 μm, and filter range from 50 kHz to 7 MHz. The model-based reconstruction incorporates a detailed model of detection geometry that allows for more quantifiable images.

2.4. Hybrid vMSOT-fluorescence imaging

A hybrid vMSOT-fluorescence imaging system [39,57] was used to assess one arcAβ mouse. The MSOT system acquired 2D planar images (with 0.3 mm gap between each scan), with the acquisition window (20 mm × 20 mm) moving along the rostral to caudal axis of the mouse brain. MSOT obtained 3D volumetric images with fixed positioning of the mouse. A short-pulsed (<10 ns) laser was used to provide an approximately uniform illumination profile on the mouse brain surface with optical fluence < 20 mJ/cm². The excited OA responses were collected with a custom-made spherical array (Imasonic SaS, Voray, France) of 512 ultrasound detection elements with 7 MHz central frequency and > 80 % bandwidth. A custom-made optical fiber bundle
(Ceramoptec GmbH, Bonn, Germany) with 4 outputs was used for guiding the laser beam from multiple angles through the lateral apertures of the array. The detected signals were digitized at 40 mega-samples per second with a custom-made data acquisition system (DAQ, Falkenstein Mikrosysteme GmbH, Taufkirchen, Germany) triggered with the Q-switch output of the laser. The pulse repetition frequency of the laser was set to 100 Hz and the laser wavelength tuned between 660 and 850 nm (10 nm step) on a per pulse basis. For the concurrent 2D epi-fluorescence imaging [48], beam from the pulsed OPO laser was similarly used for excitation. The generated fluorescence field was collected by a fibrescopic imaging fiber bundle comprised of 100,000 fibers and then projected onto an EMCCD camera (Andor Luca R, Oxford Instruments, UK). The fibrescope was inserted into the central aperture of the spherical array detector. The system has an overall field-of-view of 15 mm × 15 mm × 15 mm and resolution in the 110 μm range for vMSOT and 44 μm for epi-fluorescence measurements [38,58]. The optoacoustic signals were recorded before injection, 20, 40, 60, 90 and 120 min after injection of CRANAD-2; fluorescence signals were recorded before injection, 22, 42, 62, 92 and 120 min after injection of CRANAD-2. To examine the influence of scalp on the absorbance intensity, the scalp of the mouse was then removed. The mouse head was imaged afterwards using the same setting.

To assess the fluorescence and OA signals of CRANAD-2 binding to recombinant Aβ42, the vMSOT-fluorescence imaging system was used. The spherical array was positioned pointing upwards and filled with agar gel (1.5 %) to guarantee acoustic coupling, which served as a solid platform to place the tubing. One transparent bore polythene tubing (0.28 mm inner diameter, 0.61 mm outer diameter, Smiths Medical) of 15 cm length were fixed on the solid agar gel circular platform (diameter 5 cm) as described earlier [58]. This is to ensure using same condition during measurement for each sample. Uniform illumination of the tubing was achieved by inserting three arms of the fiber bundle in the lateral apertures of the array and a fourth one providing light delivery from the top. The pulse repetition frequency of the laser was set to 100 Hz and the laser wavelength tuned between 550 and 680 nm (5 nm step) on a per pulse basis. Beam from the pulsed OPO laser was similarly used for excitation. The tubing was sequentially filled (using syringe) with CRANAD-2 (1, 3 μM) without and with the presence of Aβ (1, 2 μM). We chose 1–2 μM concentration of Aβ to mimic the accumulation in brain of arcAβ mouse brain based on results from previous MSD and biochemical assays [46,51]. Tubing was flushed with dd water twice between samples. The measurement was repeated three times. All recorded OA signals were normalized with the calibrated wavelength-dependent energy of the laser pulse.

Images were rendered in real-time during the acquisition via fast back-projection-based image reconstruction implemented on a graphics processing unit [59]. A 3D model-based iterative algorithm was used off-line for more accurate reconstruction [60]. Prior to reconstruction, the collected signals were band-pass filtered with cut-off frequencies of 0.1 and 9 MHz. However, acoustic distortions associated to speed of sound heterogeneities, acoustic scattering and attenuation as well as the response of the ultrasound sensing elements of the array are known to additionally play a role in the quality of the images rendered [61].

2.5. MRI

MRI scans were performed on a 7 T small animal MR Pharmascan (Bruker Biospin GmbH, Ettlingen, Germany) with a bore diameter of 16 cm and equipped with an actively shielded gradient capable of switching 760 mT•m with an 80 μs rise time and operated by a ParaVision 6.0 software platform (Bruker Biospin GmbH, Ettlingen, Germany). A circular polarized volume resonator was used for signal transmission, and an actively decoupled mouse brain quadrature surface coil with integrated combiner and preamplifier was used for signal receiving. Mice were anesthetized with an initial dose of 4% isoflurane (Abbott, Cham, Switzerland) in oxygen/air (200–300 mL/min) mixture and were maintained at 1.5% isoflurane in oxygen/air (100–400 mL/min). Mice were next placed in prone position on a water-heated support to keep body temperature within 36.5 ± 0.5 °C, monitored with a rectal temperature probe. In vivo T2-weighted MR images of mouse brain/heart were obtained using a 2D spin echo sequence (Turbo rapid acquisition with refocused echoes) with imaging parameters: RARE factor 8, echo time 36 ms, repetition time 2628 ms, 6 averages, slice thickness 0.7 mm, no slice gap, field of view 20 mm × 20 mm, matrix 512 × 512, giving an in-plane spatial resolution 39 μm × 39 μm, out-of-plane resolution 0.7 mm (slice thickness), within a scan time 12 min 36 s.

2.6. Co-registration with MRI and VOI analysis of MSOT data

Registration between MSOT data and mouse brain MR images can provide a better anatomical reference for regional analysis. The cross-sectional MSOT images were co-registered with T2-weighted MRI data as described [62]. For dynamic data, time course of regional absorbance (a.u.) at 680 nm were plotted and the area-under-curves were calculated. The retention of probe was calculated by averaging values from 60–120 min post-injection.

2.7. Immunohistochemistry and confocal imaging

For immunohistochemistry and confocal microscopy, mice were perfused with 1 × PBS (pH 7.4), under ketamine/xylazine/acepromazine maleate anesthesia (75/10/2 mg/kg body weight, bolus injection) and decapitated. Brains were removed from the skull afterwards, fixed in 4 % paraformaldehyde in 1 × PBS (pH 7.4). Brain tissue were cut horizontally at 5 μm and stained with CRANAD-2, anti-Aβ1-16, antibody 6E10 (Signet Lab, SIG-39320, 1:5000), fibrillar conformation anti-amyloid antibody OC (Merck, AB2286, 1:200), Donkey-anti-Rat Alexa 488 (Jackson, AB-2340686, 1:400), Goat-anti-Rabbit Alexa488 (Invitrogen, A11034, 1:200) and counter-stained using 4,6-diamidino-2-phenylindole (DAPI) for nuclei (Sigma, D9542–10MG, 1:1000). Confocal images of the cortex of non-transgenic littermates and arcAβ mice were obtained using a Leica SP8 confocal microscope (Leica Microsystems GmbH, Germany) at ScopeM ETH Zurich Hörnberg core facility. Sequential images were obtained by using 458 nm, 640 nm lines of the laser respectively. Identical settings were used and images were obtained for the non-transgenic littermates and arcAβ mice at ×20 and ×60 magnification, resolution with Z stack and maximum intensity projection.

2.8. Statistics

Unpaired two-tail student t test with Welch’s correction was used (Graphpad Prism 8.2, Graphpad Software Inc, USA) for comparing values between arcAβ mice and non-transgenic littermates. All data are present as mean ± standard deviation. Significance was set at * p < 0.05.

3. Results

3.1. In vitro characterization of probes on recombinant Aβ1-42 fibrils

We performed a fluorescence assay to assess the binding of CRANAD-2 to pre-formed Aβ1-42 fibrils (Fig. 1) and compared CRANAD-2 with the Thioflavin T (ThT) dye, a small molecule which increases fluorescence signal upon binding to amyloids and currently represents the most common probe for in vitro assay of amyloid formation [63]. As expected, ThT binding to Aβ1-42 fibrils (0–0.2 μM), resulted in a dose-dependent increase in fluorescence intensities at 484 nm (Figs. 1a-c). Upon binding to Aβ1-42 fibrils, CRANAD-2 showed a similar dose-dependent increase in fluorescence intensity at 681 nm (Figs. 1d-f). Linear relations between fluorescence intensity and concentration of 0–0.2 μM and 0–0.012 μM Aβ1-42 fibrils were observed both with CRANAD-2 (Fig. 1e, p = 1 × 10^{-12}, r^2 = 0.991; Fig. 1f, p = 0.0015, r^2 = 0.9831) and ThT (Fig. 1b, p =
4 × 10^{-7}, r^2 = 0.9155; Fig. 1c, p = 0.0136, r^2 = 0.7352), respectively.

In a second set of experiments, we assessed the binding of CRANAD-2 and ThT to other proteins, including monomeric proteins rich in β-sheet structures, amyloid fibrils composed of other proteins and monomeric Aβ42 peptide. ThT showed high intensity upon binding to Aβ42 fibrils and a lower intensity when incubated with α-synuclein, and insulin (Fig. 1g).

CRANAD-2 showed higher intensity signal upon binding to Aβ42 fibrils compared to fibrils composed of α-synuclein, insulin, lysosome, and to the Aβ1-42 monomer, while a non-negligible signal was observed in the presence of bovine serum albumin (Fig. 1h). Compared to CRANAD-2, the signal intensity of ThT upon binding to fibrils of α-synuclein and insulin was higher (Fig. 1h), indicating that the fluorescence signal of CRANAD-2 is more specific to Aβ42 fibrils and a less generic report of the presence of β-sheet structures.

We further investigated the specificity of CRANAD-2 (0.1 μM) for Aβ42 fibrils (0–0.2 μM) by performing the same assay in a complex sample represented by a cell lysate with estimated total protein concentration of 7 μM. Notably, a linear relationship between Aβ42 fibrils and CRANAD-2 fluorescence intensity was observed, even when fibrils were spiked in a cell lysate containing a much higher concentration of a variety of proteins (Fig. 1i).

Overall, these results demonstrate that CRANAD-2 can bind Aβ1-42 fibrils with high affinity and specificity, even in complex mixtures. Importantly, the probe can distinguish between monomeric and fibrillar forms of the peptide.

3.2. In vitro binding of CRANAD-2 to Aβ fibrils increases fluorescence but not OA signal

CRANAD-2 was incubated with Aβ fibrils and measured using an agar phantom with vMSOT and epifluorescence imaging (Fig. 2c, d, SFig. 1). We have extracted the absorbance of CRANAD-2 from the vMSOT measurement and show its absorption spectrum along with the absorbance spectrum of Hb and HbO [54] (Fig. 2a). In addition, we obtained the fluorescence excitation and emission spectrum of CRANAD-2 with a spectrofluorometer (Fig. 2a, b). Analysis of vMSOT images of the phantom did not show a difference in OA signal when CRANAD-2 was co-incubated with Aβ fibrils compared to CRANAD-2 alone (Fig. 2e). In contrast, co-incubation of CRANAD-2 with Aβ fibrils did result in a 50% increase in fluorescence intensity compared to CRANAD-2 alone (Fig. 2f).

3.3. In vivo hybrid vMSOT and epi-fluorescence imaging in arcAβ mouse brain

CRANAD-2 has been shown to cross the blood-brain-barrier and reach the brain parenchyma [33,34]. We explored the ability of OA imaging to detect Aβ deposits in vivo in mouse brain. We used a hybrid system to assess simultaneous 3D vMSOT and planar epi-fluorescence imaging for CRANAD-2 distribution in arcAβ mouse brain. Two arcAβ mouse was scanned in vivo using the hybrid system with intact scalp till 120 min after CRANAD-2 probe injection. A volumetric image is obtained with vMSOT is shown (Figs. 3 and 4). We did not attain actual unmixing of CRANAD-2 and deoxy/oxyhemoglobin (Hb/HbO) signal probably due to the low concentration of probe in the tissue compared to the other two absorbers. We consider endogenous Hb and HbO represent
the OA signal intensity at preinjection (Fig. 4c), which is much abundant compared to the increase in OA signal intensity caused by the injection of CRANAD-2. Changes in MSOT signal (660–850 nm wavelength, acquired with 5 nm gap) in the arcAβ mouse brain were also monitored (Fig. 3). Signal was mainly observed in cortical structures. We observed minor difference between 660 and 680 nm, which fits with the absorbance spectrum of CRANAD-2 with rather flat decline in the absorbance intensity with increase of wavelength.

3.4. MSOT detects Aβ deposits in vivo

Based on the results in section 3.3, we next set out to explore the ability of 3D whole brain Aβ mapping with a commercial MSOT system with 2D image acquisition and 0.3 mm gap between slides. This system cover the spectrum range of 680–850 nm. To allow for accurate regional analysis, we register the MSOT data with MRI data which provides better soft tissue contrast (Fig. 5, SFig. 2). We monitored CRANAD-2 uptake in arcAβ mice and non-transgenic littermates for 90 min post-injection (n = 3–4 each) (Figs. 5a–c). The delta images in relation to the pre-injection images shows high probe uptake in the brain of arcAβ mice, mainly in the cortex (Fig. 5c). Analysis of the dynamics of cortical CRANAD-2 uptake allowed to discriminate arcAβ mice from non-transgenic littermates (Fig. 5a, d). The signal plateaued around 90 min. Higher AUC was observed in the cortical region of arcAβ mice compared to non-transgenic littermates (Fig. 5e).

3.5. Ex vivo staining on mouse brain sections

To validate the specificity of CRANAD-2 binding to Aβ deposits in mouse brain, horizontal brain tissue sections from arcAβ mice and non-transgenic littermates were stained with CRANAD-2 in addition to anti-Aβ antibodies 6E10, which binds any type of Aβ, and OC, which recognizes mature fibrils [65], and were nuclear counterstained with DAPI (Fig. 6). No signal was observed in the cortex from non-transgenic littermates (Figs. 6a, f). CRANAD-2 clearly co-stained with OC or 6E10 stained parenchymal and vessel-associated Aβ deposits in the arcAβ
mouse brain (Fig. 6b-e, g-n). This suggests specific binding of CRANAD-2 to Aβ deposits. We also observed auto-fluorescence (blue) of Aβ plaques in the cortical region of arcAβ mouse brain tissue slice (Fig. 6o), similar to other reports [66].

4. Discussion

Developing tools for non-invasive detection of Aβ deposits at high-resolution in animal models of AD is critical for investigating disease mechanism and translational development of Aβ-targeted therapies. Most probes for Aβ including NIAD-4 [25], AOI-987 [26], BODIPY [27], THK-265 [28], DANIR [29–32], curcumin-derivatives CRANAD series [33,34], luminescent conjugated oligothiophenes [35,36] and DBA-SLOH [37] have been so far been designed for fluorescence imaging application. Nevertheless, Aβ binding fluorescent probes with suitable absorbance spectrum can be employed for OA imaging. We took advantage of the fluorescent property of CRANAD-2 to characterize the suitable of the probe to map cerebral Aβ deposits with NIRF and OA imaging.

The detection sensitivity is higher in PET compared to optical and optoacoustic imaging. Depending on the probe affinity, detection of nanomolar concentration of probes have been reported [67–69]. Whereas in optoacoustic imaging, higher concentrations of probe are needed to achieve detectable signal. The resolution of PET for small animal is usually 0.7–1.5 mm which is almost 10 times lower than that of optoacoustic imaging. In microPET the partial volume effect influence the accuracy of the quantification due to the size of the mouse brain.

CRANAD-2 has an affinity to Aβ aggregates of 0.0387 μM [33], which is much higher than those of BODIPY, AOI-987 [26], similar to DANIR [32] and lower than NIAD-4 [25,32]. The in vitro binding assay demonstrated a linear relationship between fluorescence intensity and concentration of aggregated Aβ_{1-42} fibrils for CRANAD-2. This
properties has been reported for many Aβ binding probes such as AOI-987, THK-265, DANIR and croconium dye [26,28,32,45] and constitutes an advantage for the detection of fluorescence in NIRF imaging. However, when applying such probes for MSOT imaging, the increase in fluorescence quantum yield upon Aβ binding is expected to lead to a decrease in MSOT signal. Despite this feature, two previous studies in a transgenic mouse model of amyloidosis have reported Aβ imaging with other optoacoustic imaging systems using Congo Red [27], and croconium dye [45]. We were also able to clearly detect optoacoustic signals from CRANAD-2 bound to Aβ fibrils in the phantom and in arcAβ mice in vivo. Moreover, there were no differences in OA signal between CRANAD-2 to recombinant Aβ fibrils and CRANAD-2 alone measured in the phantom with MSOT. In addition to an increase in fluorescence intensity, we observed a slight red-shift by 75 nm in fluorescence spectrum upon binding to aggregated Aβ1-42 fibrils as describes in previous reports [33]. Importantly, the probe exhibits little binding to other proteins involved with Aβ plaque formation such as lysozyme [70]. Though, we observed a percentage of binding to bovine serum albumin that is a plasma protein in the circulation and found in Aβ plaques. This is a common phenomenon of Aβ binding probes such as BTA-1, PIB, florbetaben, florbetapir etc [71–74]. Co-staining of CRANAD-2 to brain sections from arcAβ mice with 6E10/OC antibodies of Aβ deposition clearly showed close resemblance in both vascular and parenchymal Aβ deposits.

CRANAD-2 is well-characterized since the development in 2009 [30,33,34]. In our study (Fig. 1), we characterized the binding properties of probe in vitro and showed specific binding to amyloid-β fibril compared to other proteins such as a-synuclein and insulin. There is minor binding to albumin which is common for most of the Aβ probes such as PIB, florbetaben etc. Moreover, we demonstrated in Fig. 6 with immunohistochemistry that CRANAD-2 specifically binds to Aβ plaques. Given the specificity of CRANAD-2 for Aβ deposits and the fact that the probe generates a detectable MSOT signal upon Aβ fibril binding, we explored the capability of in vivo MSOT cerebral Aβ mapping. Probes for MSOT imaging ideally have an absorbance and emission peak at NIR or even NIR II range to allow for sufficient penetration depth and separation from deoxygenated and oxygenated hemoglobin which are abundant in biological tissue, but which have lower absorption in the NIR region of light [19]. This has been considered in the design of most Aβ probes, apart from NIAD-4 and Congo Red which have absorption peaks in the
red part of the spectrum and which require invasive imaging approaches \[25\]. Moreover, some probes, e.g. BODIPY \[27\] derivatives and Congo red \[27\], do not pass across the blood-brain barrier and thus have to be injected intrathecally. CRANAD-2 has a suitable spectrum (excitation maximum 640 nm, emission maximum 685 nm) \[33\]. In previous reports it was shown that CRANAD-2 passes the blood-brain barrier \[33, 34\]. This has motivated us to inject the probe in arcA\(\beta\) mice, which shows parenchymal plaque and cerebral amyloid angiopathy accumulation concomitantly with cognitive impairments from 6 months-of-age, where A\(\beta\) plaque load increases with age \[46\].

The optical absorption and fluorescence contrast provided by CRANAD-2 allowed for cross-validation by simultaneous real-time recording of hybrid 3D vMSOT and 2D epi-fluorescence data \[57\], useful to understand the relation between the fluorescence, absorbance and spectral dependence of the signals generated from probe binding to targets \[75\]. We observed uptake and wash-out of probe with MSOT and epi-fluorescence imaging, though the degree of changes in MSOT signal was lower. The differences may be explained by the fact that epi-fluorescence lacks the depth resolving capacity. Thus effectively signals originating from different cranial compartments (skull and scalp over the head) adds up to provide a surface-weighted signal \[76\]. In contrast, tomographic MSOT imaging allows to map the probe uptake in the brain more accurately in 3D. Thus, epi-fluorescence imaging merely reflects a mixture of wash-out kinetics from different tissues, while MSOT closer represents the actual cerebral kinetics.

We did not attain actual unmixing of CRANAD-2 (probably due to the low concentration of probe in the brain). \[30, 34\] Injecting a higher amount of probe might help but is limited by the rather low solubility of the probe in water. In addition, using more wavelengths could also potentially help during the data acquisition.

To harness the full potential of volumetric imaging, we performed serial MSOT imaging in arcA\(\beta\) and non-transgenic littermates. Co-registration of MSOT data with a \(T_2\)-weighted structural MRI dataset \[44, 62\] allowed to quantify regional probe uptake. We observed a clear difference in brain accumulation of CRANAD-2. In our study, the cortical signals detected by using epi-fluorescence imaging and vMSOT in arcA\(\beta\) mice in this study was in accordance with the known A\(\beta\) distribution, which is highest in dorsal areas like the cortex and decreases ventrally \[43, 46, 49\], and the validation from immunohistochemistry. Several factors influence the image quality of MSOT, such as the modeling accuracy of light propagation in living tissues \[77\]. Further application of regional based fluence correction and non-negative correction in the reconstruction and unmixing steps will enable more accurate quantification \[78, 79\].

In conclusion, we demonstrated the suitability of CRANAD-2 for detecting A\(\beta\) deposits in a mouse model of AD amyloidosis with epi-fluorescence and MSOT imaging. The approach offers further longitudinal monitoring of therapeutics effect targeting at A\(\beta\) and for unveiling the disease mechanism in animal models.
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Availability of data and material

The datasets generated and/or analyzed during the current study are available in the repository DOI zenodo: https://doi.org/10.5281/zenodo.3672403.

Author contributions

RN, AV, PA and JK conceived and designed the study; RN, AV, XD, ZC performed the experiments; RN, AV, XD, ZC, PA analyzed the data; RN, AV, XD, ZC, PA, JK interpreted the results; RN and JK wrote the initial paper draft; all coauthors contributed constructively to writing and editing the manuscript.

Declaration of Competing Interest

The authors declare that there are no conflicts of interest.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi: https://doi.org/10.1016/j.pacs.2021.100285.

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