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Novel multivalent design of a monoclonal antibody improves binding strength to soluble aggregates of amyloid beta

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Abstract

Background: Amyloid- β (A β) immunotherapy is a promising therapeutic strategy in the fight against Alzheimer's disease (AD). A number of monoclonal antibodies have entered clinical trials for AD. Some of them have failed due to the lack of efficacy or side-effects, two antibodies are currently in phase 3, and one has been approved by FDA. The soluble intermediate aggregated species of A β , termed oligomers and protofibrils, are believed to be key pathogenic forms, responsible for synaptic and neuronal degeneration in AD. Therefore, antibodies that can strongly and selectively bind to these soluble intermediate aggregates are of great diagnostic and therapeutic interest.

Methods: We designed and recombinantly produced a hexavalent antibody based on mAb158, an A β protofibril-selective antibody. The humanized version of mAb158, lecanemab (BAN2401), is currently in phase 3 clinical trials for the treatment of AD. The new designs involved recombinantly fusing single-chain fragment variables to the N-terminal ends of mAb158 antibody. Real-time interaction analysis with LigandTracer and surface plasmon resonance were used to evaluate the kinetic binding properties of the generated antibodies to A β protofibrils. Different ELISA setups were applied to demonstrate the binding strength of the hexavalent antibody to A β aggregates of different sizes. Finally, the ability of the antibodies to protect cells from A β -induced effects was evaluated by MTT assay.

Results: Using real-time interaction analysis with LigandTracer, the hexavalent design promoted a 40-times enhanced binding with avidity to protofibrils, and most of the added binding strength was attributed to the reduced rate of dissociation. Furthermore, ELISA experiments demonstrated that the hexavalent design also had strong binding to small oligomers, while retaining weak and intermediate binding to monomers and insoluble fibrils. The hexavalent antibody also reduced cell death induced by a mixture of soluble A β aggregates.

Conclusion: We provide a new antibody design with increased valency to promote binding avidity to an enhanced range of sizes of A β aggregates. This approach should be general and work for any aggregated protein or repetitive target.

Keywords: Multivalent antibodies, Alzheimer's disease, A β , Avidity, Oligomers, Protofibrils

Background

Immunotherapy is one of the fastest growing fields in medical research. The success in drug development is partly due to the capability of antibodies to bind their protein targets with high specificity and affinity. Since IgG antibodies have two identical paratopes for the same

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epitope, they can bind repetitive targets (e.g. oligomeric A β) at multiple sites (epitopes). As the number of connected binding sites on one target increases, the rate of dissociation from the target will decrease. This effect is called avidity, which is defined as the unified strength of multiple interactions between an antibody and its target [1, 2]. The more engaged binding sites, the greater the avidity. For example, IgM antibodies with 10 binding sites can have higher avidity than IgG antibodies, when multiple epitopes on a target are bound simultaneously. It has been shown that antibodies modified into a tetravalent form have higher avidity than bivalent antibodies [3].

Protein aggregation is one of the major pathological hallmarks of several diseases. In Alzheimer's disease (AD), the most common form of neurodegenerative disorders, amyloid beta (A β) forms aggregates, which cause neuronal death and lead to impaired memory [4]. Aggregation of the monomeric A β proceeds through the formation of intermediate soluble oligomers and protofibrils, and eventually lead to formation of insoluble fibrils and plaques, mainly located in the extracellular space in the brain [5–8]. The latter are easily visible in the post-mortem brains from AD patients when labeled with amyloid-binding dyes [9]. However, several studies have shown that the soluble aggregated species (oligomers and protofibrils) are the most toxic forms, since they correlate better with clinical symptoms and have been shown to be the main species responsible for the associated neuronal and synaptic death [7, 10–12].

Passive immunization with anti-A β antibodies as a treatment strategy for AD has been tested in numerous clinical trials [13, 14]. One of the main challenges with AD immunotherapy is to create an antibody with strong binding to toxic species of A β , and less binding to the potentially physiologically relevant monomers or insoluble fibrils. The monomers may exert some neuroprotective effects [15, 16]. It is debated which aggregated species of A β are most harmful. Some studies suggest soluble protofibrils [17], while others suggest smaller oligomers [10, 18] or dimers [19, 20]. A general conclusion is that soluble A β aggregates are toxic to the cells and hence a relevant target. Smaller oligomers of A β are more likely to enter neurons and thereby exert their neurotoxic effects, while larger aggregates like protofibrils might have more indirect toxic effects by for example enhancing neuroinflammation [21].

The binding-selectivity of anti-A β antibodies to aggregated species of A β over monomers is mainly dependent on avidity. Several antibodies can strongly bind to A β aggregates while having a low affinity to monomers via the avidity effect. Examples of such binders include IgM antibodies with 10 binding sites [22, 23], divalent binders [24] and antibodies reaching phase 3 clinical trials

like aducanumab [25] and lecanemab (BAN2401) [26, 27]. Increasing the valency of antibodies could potentially increase the interaction time with the targeted antigen if the additional binding sites are able to bind to the target simultaneously. If one paratope is dissociated, there will still be a second paratope in association with the target, which could also increase the chance of the dissociated paratope to bind again to the target [2]. Efforts have been made to increase the valency of antibodies through designing multimeric formats accompanied with a significant decrease in the dissociation rate constants [28, 29]. However, despite being highly flexible in structure [30], IgG antibodies may not have an avidity effect to small A β oligomers [31] due to the spatial distance between the two paratopes of IgG antibodies, which has been previously determined to be around 100 Å [32]. As illustrated in Additional file 1: Fig. S1, the complementarity-determining regions of an IgG4 antibody (PDB ID 5DK3) in its crystal structure are much larger than a 12-mer A β (PDB ID 2BEG). However, different subclasses of IgG antibodies have different degrees of flexibility, which should be considered when looking at the interactions of specific antibodies with A β [33].

In this study, we set out to develop new functional multivalent antibodies to enhance antibody avidity to soluble aggregates of A β , particularly smaller oligomers, while maintaining a weak binding to monomers. For this reason, we have designed and recombinantly produced antibody formats based on an A β protofibril antibody [26, 27]. This antibody has strong binding to A β protofibrils, moderate binding to insoluble fibrils and weak binding to monomers [34–36]. In this study, we introduced new designs involving engineering additional binding domains (e.g. single-chain fragment variable [scFv]) on the variable domains (binding sites) of the parental antibody, generating tetra- and hexavalent antibodies, and tested their binding to different species of A β aggregates and protective effects on cells.

Materials and methods

Antibody cloning, expression and purification

The heavy and light chains of the different antibodies were cloned into two separate pcDNA3.4 vectors (GeneArt, Regensburg, Germany). The recombinant proteins were expressed as described previously [37]. Briefly, Expi293 cells were transfected with 70% light chain plasmids and 30% heavy chain plasmids using polyethyleneimine as a transfection agent. Seven to 12 days after transfection, the antibodies were purified using an Äkta start system with protein G columns (Cytiva, Uppsala, Sweden). Acetic acid at 0.7% was used as an elution buffer, and absorbance at 280 nm was used to measure the concentrations of purified antibodies. Buffer was

exchanged to phosphate-buffered saline (PBS) using Zeba desalting columns (Pierce biotech, Rockford, IL) and the antibodies were stored at -80°C until further application.

SDS-PAGE analysis

SDS-PAGE analysis was performed to confirm the purity and the size of the purified proteins. The antibodies were mixed with LDS sample buffer (Invitrogen, Waltham, MA) and loaded onto 4%–12% Bis–Tris protein gels (Invitrogen, Waltham, MA) without adding reducing agents. The gel was then stained with PAGE blue protein solution (Thermo Scientific, Waltham, MA) using a pre-stained protein marker (LI-COR biosciences, Bad Homburg, Germany) as a molecular weight standard.

Thermal shift assay

The structural stability of the generated antibodies under thermal stress was evaluated using Tycho nt.6 instrument (NanoTemper Technologies, München, Germany). Equimolar concentrations of the antibodies were heated in a glass capillary where the temperature increased linearly from 35°C to 95°C . Fluorescence intensities at 330 nm and 350 nm were recorded, corresponding to tryptophan fluorescence. The assay was performed under three conditions: immediately after thawing the antibodies, following 2-h incubation at 37°C and following 72-h incubation at 37°C .

Labelling of the antibodies with Iodine-125

The recombinant antibodies were labelled with iodine-125 using Chloramine-T as described previously [38]. Briefly, equal amounts of RmAb158 (molecular weight [MW] 150 kDa), Tetra-RmAb158 (MW 200 kDa) and Hexa-RmAb158 (MW 250 kDa) were mixed with the iodine-125 stock solution (Perkin Elmer Inc, Waltham, MA) and 1 mg/ml of Chloramine-T (Sigma Aldrich, Stockholm, Sweden) in PBS. After 90 s, the reaction was stopped by addition of 1 mg/ml sodium meta-bisulphite (Sigma Aldrich, Stockholm, Sweden). Free and unbound iodine was removed from the labelled antibodies using Zeba mini desalting columns having a MW cut-off of 7 kDa (Pierce biotech, Rockford, IL) and the antibodies were eluted in PBS.

Real-time interaction analysis with LigandTracer

The binding properties of the antibodies to protofibrils were studied using LigandTracer Grey (Ridgeview Instruments AB, Uppsala, Sweden). Briefly, a Petri dish was coated with protofibrils in a defined (target) area and placed on a tilted, rotating support. The area opposite to the target area was defined as the background area. The LigandTracer Grey has a low-energy gamma

detector mounted above the upper part of the dish. When the buffer containing radio-labeled antibodies was added to the dish, the inclination ensures that the liquid was mainly in the lower part of the dish outside the detection area. During each full rotation, the decay corrected signals from the target and the background areas were recorded for 30 s each. Each recording was delayed for 5 s to allow the buffer to drain from the area being detected. The background signal was subtracted from the target signal to represent specific binding of labeled antibodies to protofibrils. To this end, Petri-dishes (Corning Inc., Corning, NY) were coated overnight at 4°C with 300 μl of 500 nM A β 1-42 protofibrils, prepared as described previously [31]. The dishes were blocked with 5% BSA in $1 \times$ PBS for 2 h at room temperature (RT). To establish the affinity and kinetics of the interaction processes, protofibrils were incubated with two antibody concentrations (0.3 and 1 nM in 2 ml of 0.1% BSA/PBS) for 2.5 h and 3 h, respectively. After a total incubation of 5.5 h, the dissociation measurement was initiated by removing the antibody-containing solution and adding 3 ml of 0.1% BSA/PBS buffer to the dish. Data evaluation was performed using TraceDrawer software (Ridgeview Instruments AB, Uppsala, Sweden). The labelling procedure was performed roughly two hours before starting the first incubation.

Surface plasmon resonance (SPR)

The binding strength of the recombinant antibodies to A β protofibrils and monomers was further investigated by SPR using Biacore 8 K instrument (Cytiva, Uppsala, Sweden). Soluble A β 1-42 protofibrils, prepared as described previously [31], were immobilized by amine coupling (NHS/EDC, GE kit #BR100633) on a biacore CM5 sensor chip (Cytiva, Uppsala, Sweden). Three-fold dilution series of the recombinant antibodies (ranging from 100 to 1.23 nM) in PBS-P+buffer (pH 7.4) (Cytiva, Uppsala, Sweden) were injected using a single cycle kinetic method at a flow rate of 30 $\mu\text{l}/\text{min}$ with an association phase of 120 s, followed by a dissociation phase of 7200 s. Injection of buffer was used as a blank control. The binding data were fitted to a 1:1 kinetic model using Biacore Insight Evaluation. The dissociation rate constant was calculated as an average value from 2–5 measurements. Surfaces were regenerated using 3 M MgCl_2 between cycles. For binding to monomers, the recombinant antibodies were immobilized on the mentioned chips at a fixed concentration of 1 $\mu\text{g}/\text{ml}$. A single cycle kinetic method was used to inject a two-fold dilution series of A β 1-40 monomers (ranging from 4000 to 250 nM) (Bachem, H-1194, Bubendorf,

Switzerland). The steady-state kinetic model was used for analysis.

Indirect ELISA to demonstrate the avidity of RmAb158 to A β protofibrils

Ninety-six-well half-area plates (Corning Inc., Corning, NY) were coated overnight with A β 1-42 protofibrils (45 ng/well), prepared as described previously [31], at 4 °C. The plates were blocked the next day with 1% BSA in PBS for 2 h at RT, followed by incubation for an additional 2 h at RT with serial dilutions of RmAb158 (with a starting concentration of 10 nM) and Fab fragment of RmAb158 (with a starting concentration of 1.25 μ M). A polyclonal horse-radish peroxidase (HRP)-conjugated anti-mouse IgG (Sigma-Aldrich, Stockholm, Sweden) was added to the plates and incubated for 1 h at RT, followed by signal development with K-blue aqueous TMB (Neogen Corp, Lexington, KY). Absorbance was measured at 450 nm using a FLUOstar Omega microplate reader (BMG Labtech, Ortenberg, Germany).

Generation of cross-linked stabilized A β 1-42 aggregates and separation through size exclusion chromatography (SEC)

Synthetic A β 1-42 (Bachem, Bubendorf, Switzerland) was dissolved in 10 mM NaOH supplemented with 0.005% Tween-20 to a final concentration of 100 μ M and stored in aliquots at -80 °C. For generation of A β 1-42 aggregates, the stock dilution was diluted in 2 \times PBS, pH 7.4, to a final concentration of 50 μ M. The 50 μ M solution was incubated without shaking for 15 min at 37 °C to generate A β 1-42 aggregates. For stabilization of the metastable A β 1-42 aggregates, the aggregates were stabilized covalently by the Photo-Induced Cross-linking of Unmodified Proteins (PICUP) method according to a protocol described previously [39]. Mechanistically, PICUP involves photo-oxidation of Ru3+ in a tris(bipyridyl)Ru (II) complex (RuBpy) to Ru3+ by irradiation with visible light in the presence of an electron acceptor. Briefly, a typical PICUP reaction was performed in a 50 μ l reaction volume. To the 50 μ M A β 1-42 aggregate solution, 5 μ l of RuBpy (2.5 mM dissolved in water) followed by 5 μ l of ammonium persulfate (APS, 10% (w/v) in water) was added by pipetting (final concentrations of RuBpy and APS in the reaction mixture were 0.25 mM and 1%, respectively). The solution was quickly irradiated for 5 s under a general light bulb on the lab bench. Immediately after irradiation, the reaction was quenched by separating the reaction mixture with Zeba spin desalting column 7 k MWCO equilibrated with PBS supplemented with 0.005% Tween-20 (ThermoFisher, Waltham, MA). The PICUP stabilized A β 1-42 aggregates were heat-treated for 5 min at 95 °C prior to separation

of A β 1-42 aggregates by SEC. A Superdex 200 Increase 3.2/300 column (Cytiva, Uppsala, Sweden) was used for size separation of the first batch of A β 1-42 aggregates (Fig. 6) on a Merck Hitachi D-700 HPLC LaChrom system. The second batch of A β 1-42 aggregates (Additional file 1: Fig. S3a) was separated using a Superdex 75 column (Cytiva, Uppsala, Sweden). The samples were eluted with PBS-Tween, pH 7.4 (50 mM sodium phosphate, 0.15 M NaCl, 0.1% Tween-20, pH 7.4) at a flow rate of 0.08 ml/min and data obtained at 214 nm. Before sample injection, the quenched reaction mixture was mixed 1:1 with a 2 \times mobile-phase buffer (100 mM sodium phosphate, 0.3 M NaCl, 0.2% Tween-20, pH 7.4). Fractions were collected every two minutes and stored at -20 °C for further analysis. To estimate the size of the separated PICUP A β 1-42 fractions, western blot analysis was performed. Samples were mixed with Laemmli sample buffer containing reducing agent and heated for 5 min at 95 °C. The denatured samples were loaded on NuPAGE 12% Bis-Tris gels (ThermoFisher, Waltham, MA), and transferred onto nitrocellulose membranes (Bio-Rad, Hercules, CA). The membranes were blocked with 5% dry milk (Bio-Rad, Hercules, CA) in TBS-Tween buffer, followed by incubation with rabbit anti-A β 42 antibody (Bioarctic AB, Stockholm, Sweden).

Sandwich ELISA to check the binding strength to cross-linked A β 1-42 aggregates and α -synuclein aggregates

The generated antibodies were tested with sandwich ELISA to establish their binding strength to different sizes of the generated cross-linked A β 1-42 aggregates using the N-terminal specific mouse monoclonal antibody 82E1 (IBL/Tecan Trading AG, Mannedorf, Switzerland) as a positive control. Ninety-six-well half-area plates (Corning Inc., Corning, NY) were coated overnight with 1 μ g/ml of the rabbit polyclonal C-terminal-specific A β 1-42 antibody (Invitrogen, Waltham, MA) at 4 °C. On the next day, the plates were blocked with 1% BSA in PBS for 2 h at RT and incubated with 250 pM of the generated cross-linked A β 1-42 aggregates of different sizes for another 2 h. Serial dilutions of the recombinant antibodies were added to the plates and incubated for 2 h at RT, followed by detection for 1 h at RT with HRP-conjugated anti-mouse IgG antibody (Sigma Aldrich, Stockholm, Sweden). Signals were developed with K-blue aqueous TMB (Neogen Corp, Lexington, KY) and the absorbance was measured at 450 nm using a FLUOstar Omega microplate reader (BMG Labtech, Ortenberg, Germany). The wells were washed with ELISA washing buffer (PBS with 0.05% Tween-20) between each step. All serial dilutions were made with ELISA incubation buffer (PBS with 0.1% BSA and 0.05% Tween-20). The absorbance

of the blank (no addition of recombinant antibodies) was subtracted from the absorbance of the samples. For α -synuclein sandwich ELISA, plates were coated overnight with 1 μ g/ml of antibody MJFR-14-6-4-2 (Abcam, Cambridge, United Kingdom) at 4 °C. Following block with 1% BSA in PBS for 2 h at RT, α -synuclein oligomers, prepared as described previously [40], were added at a final concentration of 10 nM and the plates were further incubated for 2 h at RT. Serial dilutions of the recombinant antibodies (RmAb158, Tetra-RmAb158 and Hexa-RmAb158) were added to the plate and incubated for 2 h at RT. The α -synuclein antibody (clone SynO2, recombinantly produced in the lab) was used as a positive control [41]. The signals were developed and detected as described above.

Inhibition ELISA to discriminate the binding between A β monomers, oligomers, protofibrils and fibrils

Inhibition ELISA was performed as described previously [34]. Briefly, 96-well half-area plates (Corning Inc., Corning, NY) were coated overnight with 45 ng/well of A β 1-42 protofibrils at 4 °C followed by blocking with 1% BSA in PBS for 2 h at RT. Serial dilutions of sonicated A β 1-42 insoluble fibrils, prepared as described previously [31], large cross-linked A β 1-42 protofibrils (fraction 1 in SEC chromatogram, Fig. 6), cross-linked A β 1-42 oligomers (fractions 3 and 4 in SEC chromatogram, Fig. 6) and A β 1-40 monomers (Bachem, Bubendorf, Switzerland) were pre-incubated in a non-binding 96-well plate with HexaRmAb158 at a fixed concentration of 250 pM. After 1.5-h incubation, the mixtures were transferred to the A β protofibril-coated plates and further incubated for 15 min at RT. The plates were washed between the steps and the signal was developed and detected as described above.

Cell culture and MTT assay

The mouse neuroblastoma Neuro2a cell line was obtained from ATCC (Manassas, VA) and grown in Minimum Essential Medium (Gibco, Carlsbad, CA) supplemented with 10% fetal bovine serum (Gibco, Carlsbad, CA) at 37 °C with 5% CO₂. The cells were plated in a 96-well plate (Sarstedt cells+, Numbrecht, Germany) at a density of 5000 cells in 100 μ l complete medium per well. To eliminate the effect of serum, the cells were starved 24 h prior to the addition of A β /antibody mixtures by changing to a serum-free medium. Cells were then treated with a heterogenous mixture of cross-linked A β 1-42 aggregates (the sample before being passed through SEC, final concentration 500 nM) either alone or in combination with the antibodies (final concentration 150 nM). Cells incubated at standard conditions for 24 h with PBS were used as a negative control and with

0.005% H₂O₂ as a positive control. After the mentioned incubation time, the treatment medium was discarded and 50 μ l serum-free medium plus 50 μ l MTT reagent (Abcam, Cambridge, United Kingdom) was added in each well and incubated for 3 h at standard conditions, which lead to the formation of formazin proportional to how many cells that are alive. To solubilize the formazin product, MTT solvent (Abcam, Cambridge, United Kingdom) was added and the plates were incubated in the dark for 15 min while shaking. The absorbance was measured at 590 nm using a FLUOstar Omega microplate reader (BMG Labtech, Ortenberg, Germany). Data were obtained from two repetitive experiments where five to six replicates were used for each condition and the absorbance of the blank (cell medium only) was subtracted from the absorbance of the samples.

Results

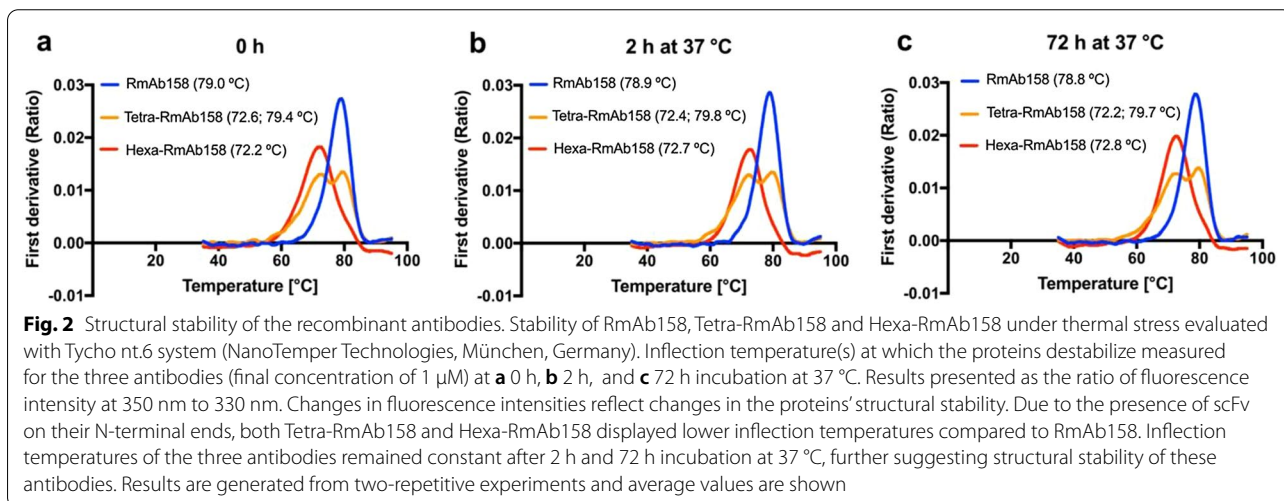
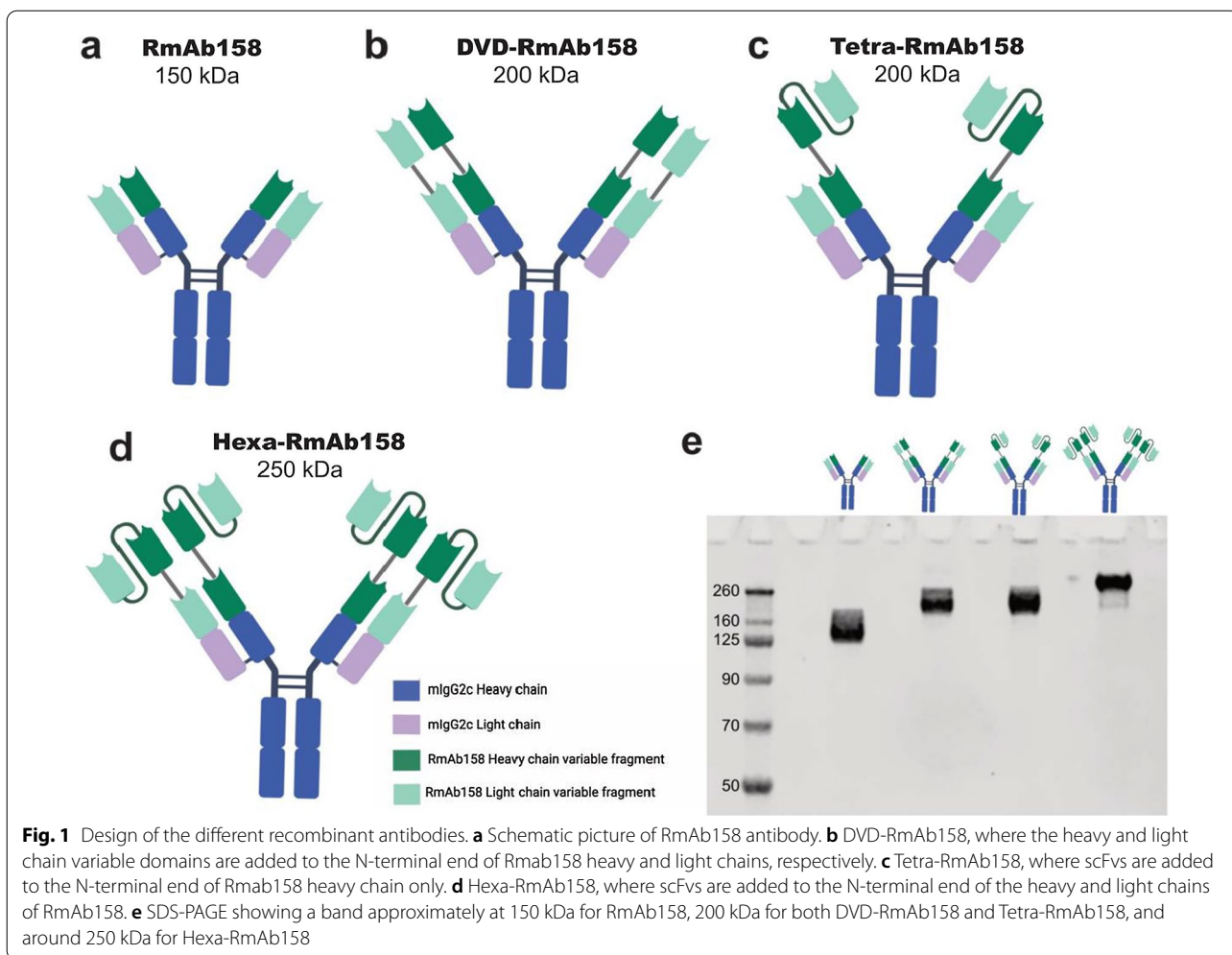
Generation of recombinant multivalent antibodies

A scFv, consisting of the heavy variable domain attached through a glycine-serine rich linker [42, 43] to the light variable domain of RmAb158 (Fig. 1a), was attached to the N-terminal end of the RmAb158 heavy chain to create Tetra-RmAb158 (Fig. 1c), or the N-terminal end of both heavy and light chains of RmAb158 to create Hexa-RmAb158 (Fig. 1d). The scFv was attached with an in-house designed linker where prolines were added to the linker to ensure no alpha helices were formed, glycines added to provide flexibility and serines added to give more hydrophilicity [44]. In addition, a dual variable domain (DVD) antibody was designed, where an additional heavy chain variable domain was attached to the N-terminal end of the heavy chain and an additional light chain variable domain was attached to the N-terminal end of the light chain (Fig. 1b).

The four antibodies were expressed in Expi293 cells [37], giving rise to ~20 mg of RmAb158 or 2–5 mg of DVD, Tetra or Hexa-RmAb158 per liter of transfected cell cultures. After purification with affinity column chromatography, SDS-PAGE analysis showed bands at 150 kDa (RmAb158), 200 kDa (DVD and Tetra-RmAb158) and 250 kDa (Hexa-RmAb158), confirming the size and purity of the produced recombinant proteins (Fig. 1e).

Structural stability of the recombinant antibodies

Structural stability of the generated antibodies under thermal stress was evaluated using the Tycho nt.6 system (NanoTemper Technologies, München, Germany). Inflection temperature which refers to the temperature at which the proteins unfold was determined by measuring fluorescence intensities at 350 and 330 nm. RmAb158 yielded an inflection temperature of 79 °C



(Fig. 2a). Two inflection temperatures were detected for Tetra-RmAb158 (72.6 °C and 79.4 °C) (Fig. 2a). Hexa-RmAb158 displayed a single inflection temperature of 72.2 °C (Fig. 2a). The slightly lower inflection temperatures detected for Tetra- and Hexa-RmAb158 antibodies can be attributed to the presence of scFvs on the N-terminal ends of these antibodies, as scFvs are more susceptible to thermal shifts than full IgG antibodies. The inflection temperatures of the three antibodies remained constant after incubation at 37 °C for 2 h (Fig. 2b) and 72 h (Fig. 2c), further suggesting the structural stability of the generated antibodies.

RmAb158 selectivity for Aβ protofibrils is based on avidity

To demonstrate that RmAb158 utilizes the avidity effect to ensure strong binding to Aβ protofibrils but not monomers, the Fab fragments of the antibody were generated (Fig. 3a). Using an indirect ELISA setup (Fig. 3b), the binding strength to Aβ1-42 protofibrils was examined using the full RmAb158 antibody (MW 150 kDa), where both binding sites are available, and the Fab fragment of RmAb158 (MW 50 kDa), where only one binding site is available. A much stronger binding to Aβ protofibrils was observed for RmAb158 as compared to its Fab fragment alone (Fig. 3c). Hence, the strong binding that RmAb158 has to the protofibrils requires the binding of both arms to the same protofibril. This implicates the importance of avidity as a decisive factor in RmAb158's binding selectivity to Aβ aggregates.

Kinetic evaluation of binding of antibodies to Aβ1-42 protofibrils

The first question on our multivalent antibodies was if they could bind with high avidity to the rather large protofibrils (> 100 kDa in size). The affinity of RmAb158 to Aβ protofibrils has previously been estimated to be approximately 70 pM [35, 45], and the affinity of antibody formats with higher valency was expected to be even higher with a lower rate of dissociation. To verify this, the binding strength of the recombinant antibodies to Aβ1-42 protofibrils was determined with real-time interaction analysis using LigandTracer. For these experiments, iodine-125-labeled antibodies were added to dishes coated with Aβ1-42 protofibrils. The association and dissociation rate constants were obtained by monitoring binding during consecutive incubation steps with two concentrations (300 pM and 1 nM) of the antibodies for 2.5 h and 3 h respectively, followed by a phase where the antibodies were removed to measure dissociation rate. Among the three tested antibodies, Hexa-RmAb158 displayed a much slower dissociation with an almost straight dissociation curve (Fig. 4). Kinetic evaluation was done using a 1:1 curve fit. Here, the association event was detected when the first binding site of an individual antibody binds, while for the appearance of signal reduction for the dissociation phase, all binding interactions of the individual antibody need to be dissociated at the same time and hence the antibody not attached at all. Kinetic evaluation of the binding curve for RmAb158 showed an association rate constant (k_a) of $1.5 \times 10^5 \text{ M}^{-1}\text{s}^{-1}$, and a dissociation rate constant (k_d) of $6.5 \times 10^{-6} \text{ s}^{-1}$. The affinity, K_D , of the antibody to Aβ protofibrils was determined to

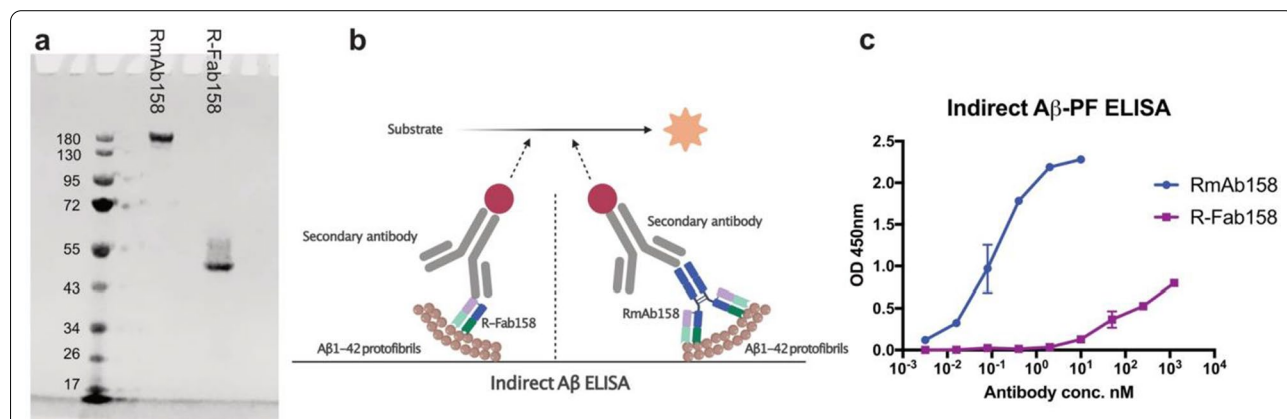
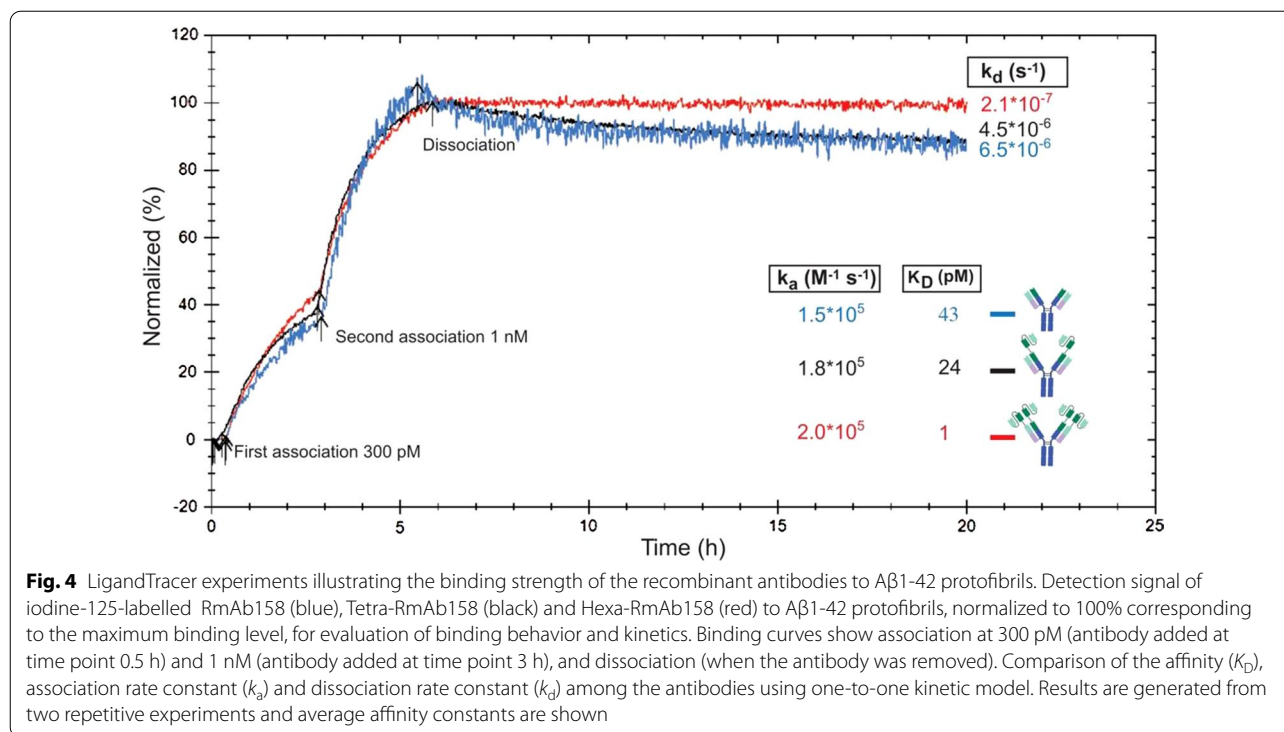


Fig. 3 Studying the avidity of RmAb158 to Aβ protofibrils. **a** SDS-PAGE showing a single band approximately at 150 kDa for RmAb158 and 50 kDa for its Fab fragment, R-Fab158. **b** Schematic representation of indirect Aβ ELISA setup where Aβ1-42 protofibrils are coated on the well surface, followed by binding of the generated RmAb158 or R-Fab158. **c** Indirect ELISA displaying the binding curves of RmAb158 and R-Fab158 to Aβ1-42 protofibrils. Strong binding of RmAb158 to Aβ protofibrils compared to R-Fab158 that demonstrates weak binding. Data are presented as mean ± SD, $n = 2$



be 43 pM. Regarding Tetra-RmAb158 binding to Aβ protofibrils, the antibody displayed an association rate constant of $1.9 \times 10^5 M^{-1}s^{-1}$ and a dissociation rate constant of $4.5 \times 10^{-6} s^{-1}$, with an affinity K_D of 24 pM (Fig. 4). Kinetic evaluation of Hexa-RmAb158 binding to Aβ protofibrils showed an association rate constant of $2.0 \times 10^5 M^{-1}s^{-1}$. For Hexa-RmAb158, the dissociation rate was very low (almost a straight line, see Fig. 4) and for an accurate estimate of the off-rate and affinity, the fitting was performed in two steps where first the dissociation rate was established followed by fitting the association rate constant while using the dissociation rate from the first step. This resulted in a k_d value of $2.1 \times 10^{-7} s^{-1}$, and an affinity K_D of 1 pM (Fig. 4), which were ~40 times lower than the k_d and K_D values of RmAb158. These results indicate that after incubating the antibodies for several hours with a protofibril-coated layer, additional scFv units lead to a more stable and higher-affinity binding of the antibodies.

The binding strength of the recombinant antibodies to Aβ1-42 protofibrils was also determined using SPR (Fig. 5a-d). With SPR, it is more difficult to measure the kinetic properties for as long times as with LigandTracer, which is desired in the case of antibodies with slow rate of dissociation. The curve fit to the dissociation phase was good and there was a large difference in the dissociation rate. RmAb158 demonstrated a k_d of $3.86 \times 10^{-3} s^{-1}$ (Fig. 5a). DVD-RmAb158 demonstrated an almost 10

times slower dissociation with a k_d value of around $2.64 \times 10^{-4} s^{-1}$ (Fig. 5b). Binding of Tetra-RmAb158 and Hexa-RmAb158 to Aβ1-42 protofibrils displayed a k_d value of $2.90 \times 10^{-5} s^{-1}$ and $2.56 \times 10^{-5} s^{-1}$, respectively. These k_d values were more than 100 times lower than that of RmAb158 (Fig. 5c, d).

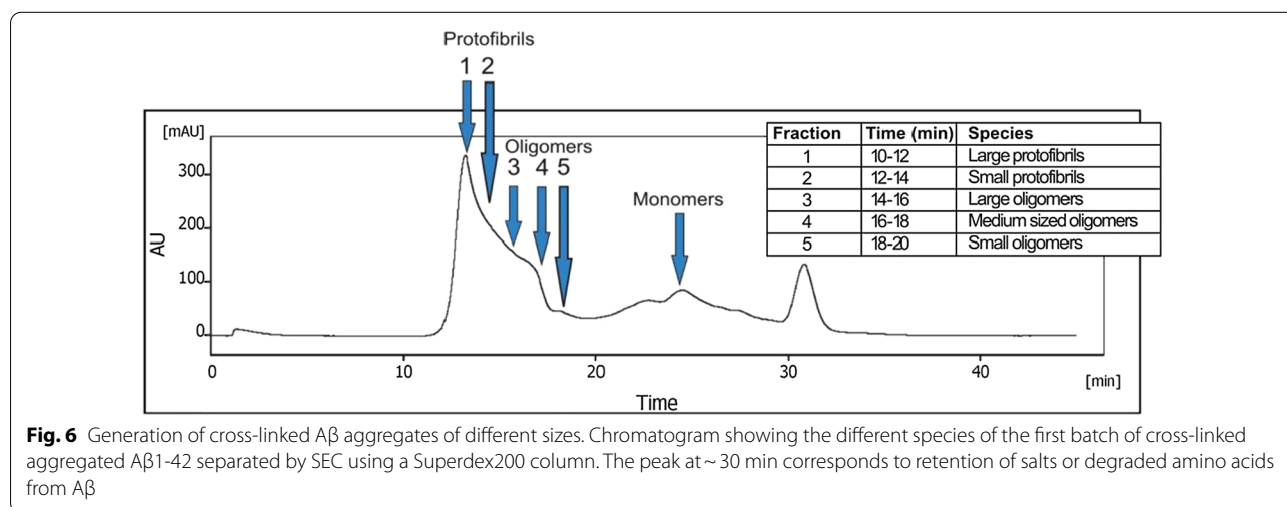
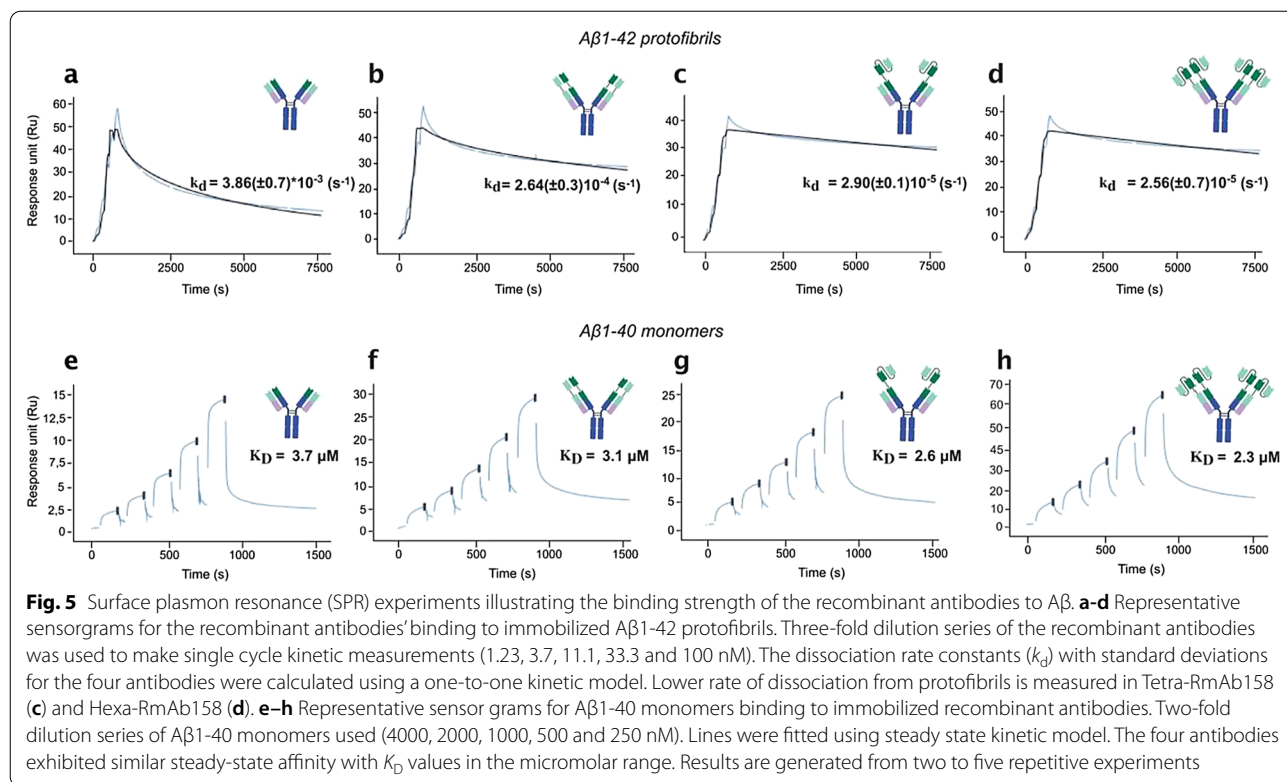
The binding strength of the recombinant antibodies to Aβ1-40 monomers was determined with SPR using the steady-state affinity model. The four antibodies displayed weak binding with a K_D value in the range of 2–4 μM (Fig. 5e-h).

Evaluation of the binding strength of the multivalent antibodies to different sizes of Aβ aggregates

The second part of our study aimed at comparing the binding of the different antibody formats to different sizes of soluble Aβ aggregates.

Generation of cross-linked Aβ aggregates of different sizes

Stable Aβ1-42 aggregates of different sizes were generated using the PICUP method followed by fractionation through SEC using a Superdex 200 column (GE Healthcare). The HPLC-SEC chromatogram confirmed the presence of aggregates of different sizes collected in different fractions, with fraction-1 having the largest size and fraction-5 having the lowest MW (Fig. 6). Synthetic monomers of Aβ had a size of ~4.5 kDa. What is defined as Aβ oligomer and protofibril varies



among studies. In the current study, A β protofibrils were defined as structures with a size of more than 100 kDa that remain soluble after centrifugation at 16,000 \times g, and oligomers as soluble aggregates smaller than this. Therefore, fractions 1 and 2 in the SEC chromatogram were defined as large and small protofibrils

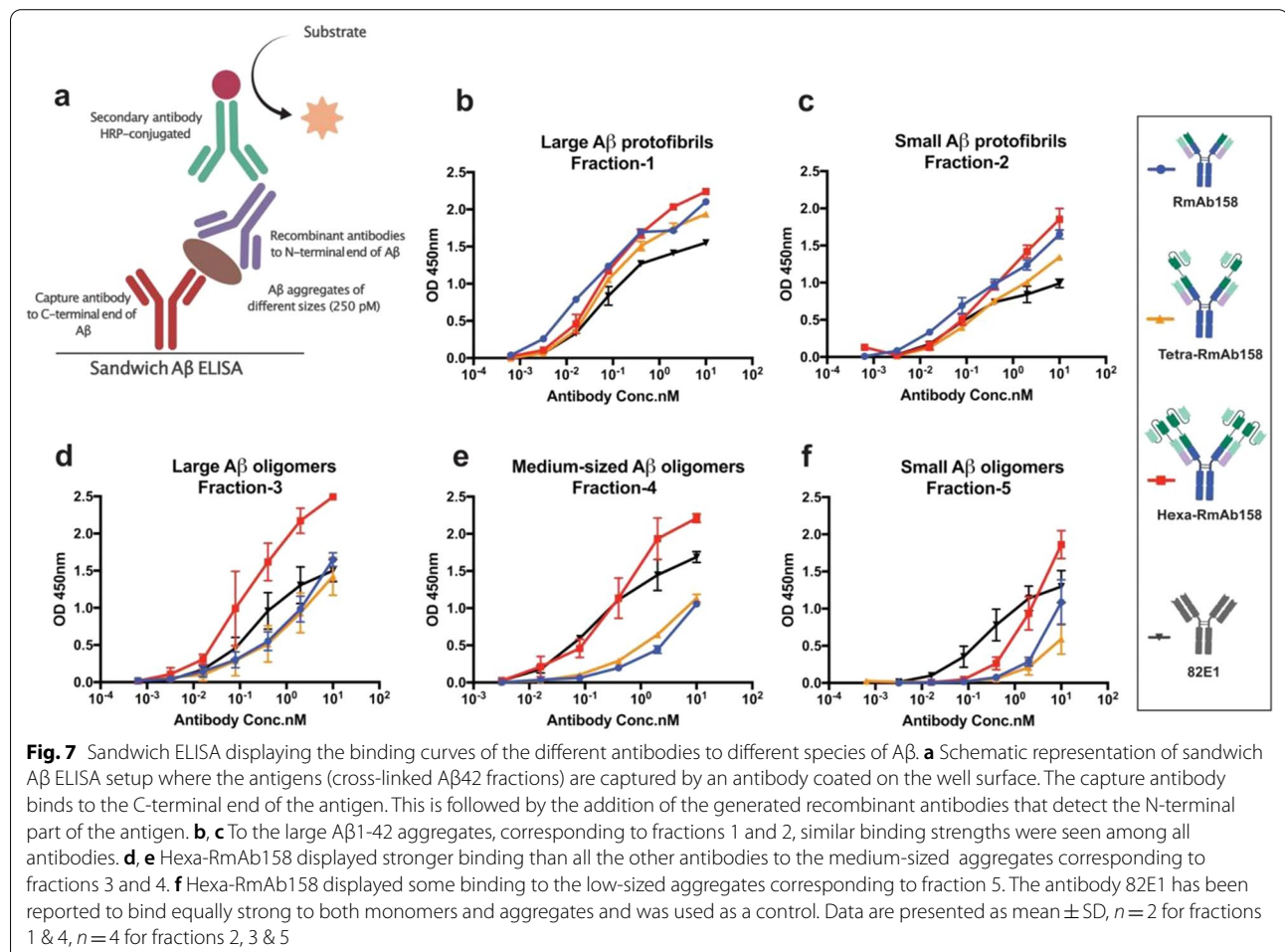
respectively. Fraction 3 was defined as large oligomers, fraction 4 as medium-sized oligomers and fraction 5 as small oligomers (Fig. 6).

Evaluation of the binding strength of the recombinant multivalent antibodies to different sizes of cross-linked Aβ1-42 species with ELISA

Using a sandwich ELISA sparsely coated with an antibody that binds the C-terminal end of Aβ, we evaluated the binding strength of the multivalent antibodies to Aβ aggregates that vary in size. We used this type of sandwich ELISA with two-hour-long incubations to detect binding to differently sized cross-linked Aβ1-42 aggregates (Fig. 7a). Results showed that all antibody formats showed a similar response to the large cross-linked aggregates (protofibrils, fractions 1 and 2, >100 kDa), regardless of the valency (Fig. 7b, c). The method was not sensitive enough to detect differences between very strong binders. However, antibody binding to the smaller aggregates was expected to be substantially reduced and hence differences should be detectable. With such sandwich ELISA setup, we also evaluated the binding strength of the different antibodies to the cross-linked Aβ1-42 fractions 3 and 4 containing the medium-to-large oligomers of around 60–100 kDa. Results showed

that the Hexa-RmAb158 bound stronger than the other recombinant antibodies to fractions 3 and 4 (Fig. 7d, e). The Hexa-RmAb158 also displayed some binding to the small cross-linked oligomers at nanomolar concentrations, while the other recombinant antibodies displayed weaker binding (Fig. 7f). The antibody 82E1 has been reported to bind to Aβ of different sizes with similar binding strengths [46, 47] and was used as a control here. The DVD-RmAb158 antibody showed a very weak binding strength to almost all the cross-linked Aβ1-42 fractions in this experiment. This antibody format was more prone to aggregate and required very sensitive handling and was therefore not included in all our experiments. The binding strength of each individual antibody to the different fractions of Aβ from the same experiment is illustrated in Additional file 1: Fig. S2.

To estimate the size of Aβ oligomers that can best generate an avidity effect by binding Hexa-RmAb158, we generated another batch of cross-linked Aβ1-42 oligomers (Additional file 1: Fig. S3a-b) where we could collect fractions containing even smaller oligomers than those generated before (Fig. 6).



In the same sandwich ELISA setup as described previously, Hexa-RmAb158 displayed the best binding strength to aggregates with a size range of 50–200 kDa (Additional file 1: Fig. S3c). Nonetheless, the avidity of Hexa-RmAb158 to Aβ1-42 decreased in fractions with size <50 kDa (Additional file 1: Fig. S3d–f). These results indicate that oligomers of ~50 kDa and above are the species of Aβ that can be best detected with Hexa-RmAb158. As expected from the previous results, the other tested recombinant antibodies also bound weaker to small aggregates in the concentration range used (Additional file 1: Fig. S3d–f).

To investigate if Hexa-RmAb158 bound stronger to oligomers and protofibrils than to insoluble fibrils and monomers, an inhibition ELISA was performed. In this setup, a pre-incubated Aβ aggregate and Hexa-RmAb158 mixture was added to a protofibril-coated ELISA plate (Fig. 8a). The same setup has previously been used to display strong binding of mAb158 to Aβ protofibrils, while having intermediate and weak bindings to fibrils and monomers respectively [31, 35, 36]. In this ELISA setup, the ability of the different preincubated Aβ aggregates to inhibit Hexa-RmAb158 binding to the protofibrils bound to the plate should be related to the antibodies' binding strength. The Aβ species used in this ELISA setup were sonicated insoluble Aβ1-42 fibrils prepared as described previously [31], cross-linked Aβ1-42 protofibrils (fraction 1 in the SEC chromatogram, Fig. 6), two smaller sizes of cross-linked Aβ1-42 oligomers (fractions 3 and 4 in the SEC chromatogram, Fig. 6) and Aβ1-40 monomers. Because Aβ1-42 is more prone to aggregate, Aβ1-40 was used to assess monomer binding as it is more stable as a soluble monomer [48]. Hexa-RmAb158 displayed a similar strong binding to the three fractions of cross-linked Aβ1-42 (IC50 of 0.9, 1.0 and 2.0 nM, respectively) (Fig. 8b). Importantly, Hexa-RmAb158 exhibited moderate binding strength to sonicated Aβ1-42 fibrils

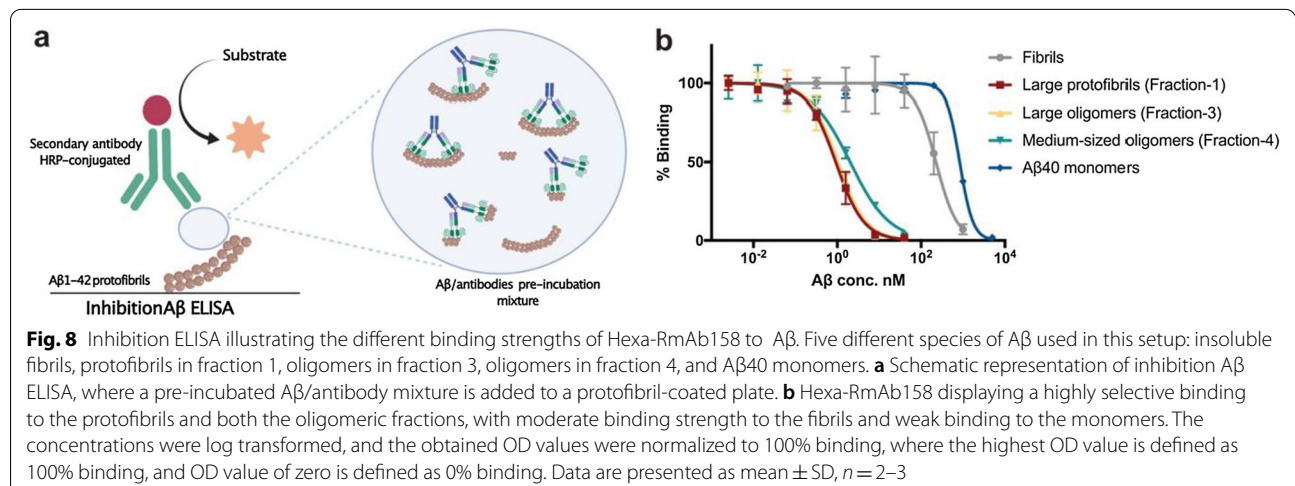
(IC50 227 nM), and weak binding to Aβ1-40 monomers (IC50 834 nM) (Fig. 8b).

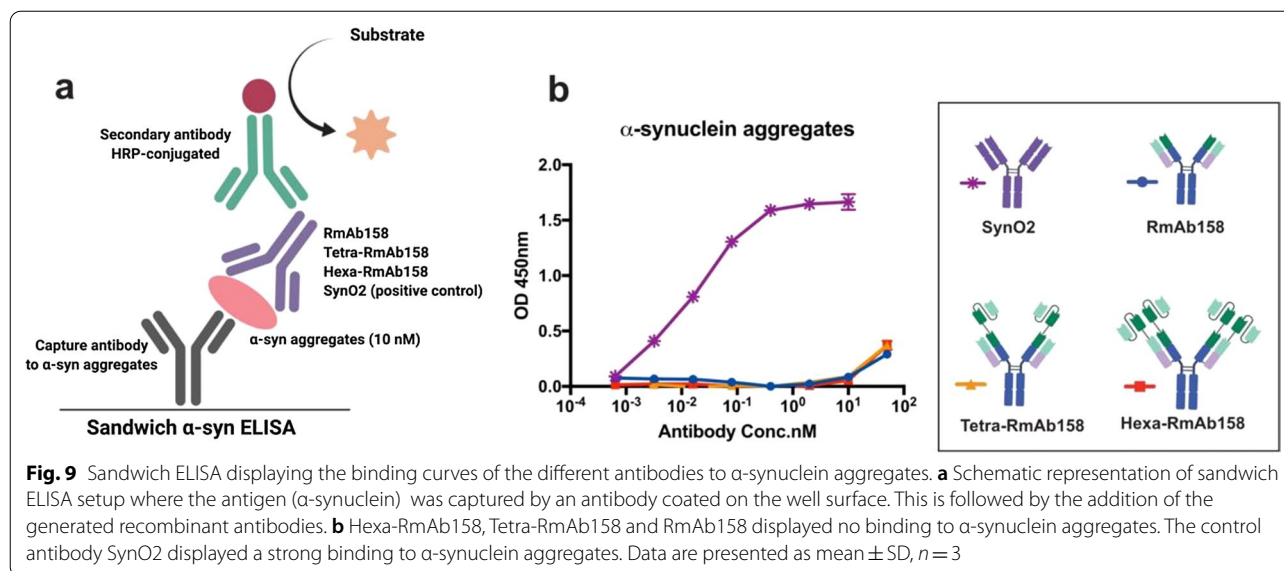
Hexa-RmAb158 and related antibodies demonstrate no binding to protein aggregates other than Aβ

To display that Hexa-RmAb158 does not recognize aggregates of other amyloidogenic proteins, a sandwich ELISA was applied (Fig. 9a) detecting α-synuclein oligomers, the pathological aggregates in Parkinson's disease. The anti α-synuclein antibody (SynO2) was used as a positive control [41]. Hexa-RmAb158, Tetra-RmAb158 and RmAb158 showed no binding to α-synuclein aggregates (Fig. 9b).

The hexavalent antibody protects neuronal cells from the toxic effects of Aβ aggregates

The efficacy of the different antibodies in vitro was studied by investigating their ability to protect mouse neuroblastoma Neuro2a cells from Aβ-induced effects on cell metabolism using MTT assay. Since the concentration of the separated fractions of cross-linked Aβ42 aggregates (Fig. 6) was too low to induce reduction in MTT signal, we performed this experiment with a heterogeneous mixture of cross-linked Aβ1-42 aggregates, which also included some proportion of monomers. After over 24 h of incubation, 500 nM of Aβ1-42 mixture resulted in a reduction of MTT signal by ~53% (MTT signal 47% relative to 100% of PBS-treated cells) (Fig. 10a). The reduced MTT signal was significantly reversed to ~65% by RmAb158 (*P*=0.002), to ~70% by Tetra-RmAb158 (*P*=0.003), and to 74% by Hexa-RmAb158 (*P*<0.001) (Fig. 10a). The control antibody 82E1 that has a high affinity to the non-toxic monomers as well as the aggregates did not significantly reduce the Aβ1-42 induced toxic effects (Fig. 10a).





Discussion

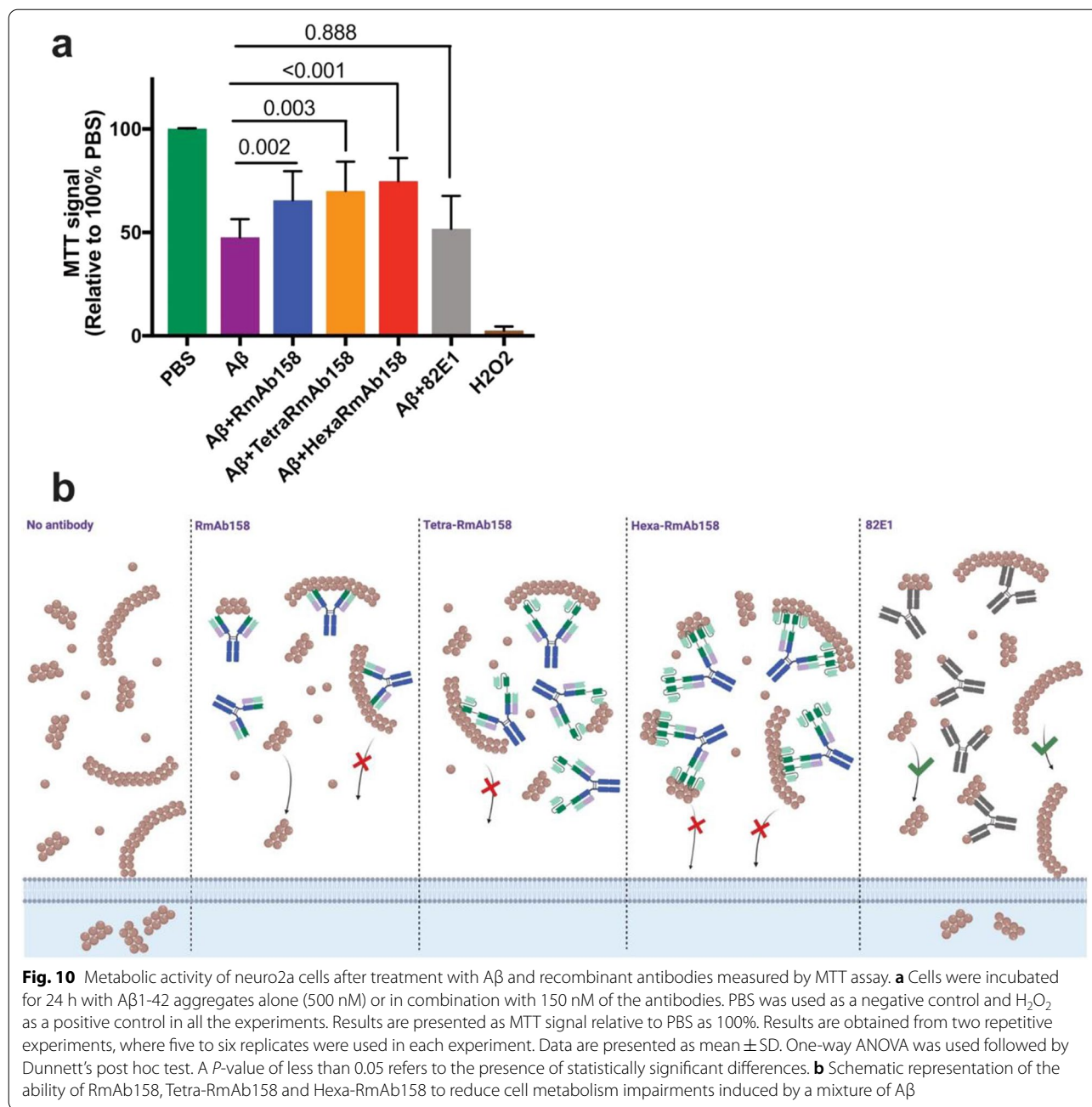
Passive immunotherapies using monoclonal antibodies are in ongoing clinical trials for the treatment of AD. Some of these trials have been discontinued due to the lack of efficacy in slowing cognitive impairment [49–51]. A high incidence of adverse events such as amyloid-related imaging abnormalities with edema (ARIA-E), possibly caused by binding to fibrils in the vessel walls, have been seen in some of the trials [52, 53].

The aim of the current study was to enhance the avidity of monoclonal antibodies through the design of multivalent antibody formats and investigate their binding strength to soluble aggregated species of $A\beta$. These properties are desired because of the high cytotoxicity of the intermediate soluble species of $A\beta$ [10, 12, 54]. In addition, increasing the avidity will also decrease the rate of dissociation from the soluble $A\beta$ aggregates.

To this end, we have designed, produced and evaluated binding characteristics of multivalent antibodies to different aggregated $A\beta$ species, but also to physiologically abundant monomers. The designs were based on RmAb158, an antibody that has previously been shown to have high binding strength to protofibrils due to avidity [31, 34]. The humanized version of RmAb158, lecanemab (BAN2401), is currently in a phase 3 clinical trial with 1795 participating patients with early and prodromal AD. In a phase 2b trial including 856 patients with AD, lecanemab (BAN2401) demonstrated a remarkable biological activity in lowering $A\beta$ levels in the brain with a good safety profile [55].

We utilized the SPR and LigandTracer techniques to compare the binding of the antibody constructs to protofibrils, since these technologies can establish the

affinity and avidity without the necessity of reaching equilibrium in the binding process as with ELISA. For a high avidity binding, as displayed by Hexa-RmAb158 to $A\beta$ protofibrils, 95% of the equilibrium binding will only, theoretically, be reached after incubation for roughly 17 days when having a concentration similar to the apparent affinity for the interaction. Required incubation times are even longer for lower concentrations. The obtained results from both methods revealed a lower rate of dissociation with Hexa-RmAb158, which showed a slower dissociation from $A\beta$ protofibrils when compared to RmAb158 (Figs. 4, 5). This can be explained by the additional binding domains of Hexa-RmAb158 that provide a higher number of antigen–antibody interaction sites. If one of the paratopes dissociates from its interaction site, there are additional paratopes that are still associated with the target in the case of Hexa-RmAb158. In comparison, Tetra-RmAb158 will have fewer available binding sites, and RmAb158 has only one binding site available. Therefore, a complete dissociation of the hexavalent antibody from its target requires very long times. The dissociation rates measured in the SPR are faster than those from the LigandTracer, which could be due to the longer incubation periods enabling more time for multivalent binding and how the protofibrils are connected to the surface. In LigandTracer, unmodified protofibrils are bound directly to the plate, while in the SPR, the protofibrils are bound to the sensorchip with amine coupling. Since the N-terminal end of $A\beta$ 1–42 is the epitope for mAb158 [31], it is likely that this is the cause for the weaker binding detected in the SPR, but the relative difference in avidity should still be rather correct. By comparing the measured dissociation process



and the one estimated by the 1:1 fit in SPR, it can be seen that the actual dissociation is more heterogeneous with a fraction that dissociates more rapidly, leaving more stably bound fraction behind that dissociates more slowly. This heterogeneity might be related to a variation in multivalent binding on the SPR sensorchip, where some antibodies do not manage to bind with all possible binding sites. The advantage of real-time interaction analysis with LigandTracer over SPR is that it allows monitoring of binding to coated layer of Aβ protofibrils while having

longer incubation times to allow reaching of a binding equilibrium.

One of the complications in designing Aβ oligomer antibodies is the cross-reactivity with insoluble fibrils and monomers. Several antibodies have been designed to target Aβ oligomers, but they have been shown to also bind to fibrillar and monomeric species. Strong binding of antibodies to Aβ fibrils is likely to be associated with an increased risk of ARIA-E, which is a frequently observed adverse event in AD clinical trials

with monoclonal antibodies [56]. In addition, the monomeric A β might play a physiological role and exist in higher amounts than oligomers [15, 57]. Binding to A β monomers in the periphery could also interfere with the ability of the antibodies to reach their intra-brain target. Therefore, antibodies that selectively bind to the oligomers and protofibrils, with low binding to fibrils and monomers, are desired. Here, we showed with inhibition ELISA that the Hexa-RmAb158 has a much stronger binding to the oligomers and protofibrils, which is ~200 times stronger than its binding to the insoluble fibrils, and 800 times stronger than its binding to the monomers. This has previously been demonstrated with mAb158 as well using similar inhibition ELISA setup [31, 35]. Furthermore, our ELISA experiments displayed no binding of Hexa-RmAb158 and related antibodies to α -synuclein aggregates, further confirming the previous report of no binding of the parental mAb158 antibody to protein aggregates other than A β [31]. This suggests that binding of Hexa-RmAb158 is specific to the N-terminal of A β and not directed towards a structural element common for different amyloidogenic protein aggregates. Being able to strongly bind the soluble oligomeric aggregates of A β could be of therapeutic benefit, since it has been shown that the soluble A β aggregates are toxic to neurons and associated with enhanced release of inflammatory cytokines [58, 59]. This can be explained by their colocalization with several pre- and post-synaptic markers, faster induction of apoptotic changes and activation of the mitochondrial death pathway. In addition, previous research has shown that the small soluble A β 1-42 aggregates have a higher permeability to cell membranes [21]. In our study, Hexa-RmAb158 was efficient in reducing cell metabolism impairment caused by A β of different sizes (Fig. 10a), which could be attributed to the selectivity and low dissociation rates of this antibody to the soluble toxic aggregates of A β .

Our approach to designing multivalent antibodies could also be applied for other diseases caused by aggregated proteins or repetitive targets. Multivalent antibody designs could provide higher chances for their paratopes to rebind to their targets with decreased dissociation rates, which in turn, could aid in keeping of antibodies in areas around the target. Strong and continuous occupancy of targets could be of particular importance in cancer immunotherapy, where studies have displayed heterogenous and non-tumor specific distribution of antibodies [1]. It is however worth mentioning that enhancing the avidity of antibodies is also dependent on the nature and the surface size of the targeted antigen.

Conclusion

In conclusion, we have developed a hexavalent antibody that can bind with high avidity to soluble aggregates of A β including small oligomers, while having low binding to the physiological monomers. Due to the decrease in the rate of dissociation, the binding strength of the hexavalent antibody to protofibrils is enhanced by 40 times when measured using real-time interaction analysis with LigandTracer. The binding of the antibody to different A β species and the ability to reduce cell death from the toxic effects of A β illustrate the potential of such multivalent designs for the generation of diagnostic and therapeutic interventions in the future. Reforming antibodies to customize the binding profile to targets is possible for many antibodies with extensive use beyond AD.

Abbreviations

A β : Amyloid- β ; AD: Alzheimer's disease; ARIA-E: Amyloid-related imaging abnormalities with edema; DVD: Dual variable domain; PICUP: Photo-induced cross-linking method; scFv: Single chain fragment variable; SPR: Surface plasmon resonance.

Supplementary Information

The online version contains supplementary material available at <https://doi.org/10.1186/s40035-021-00258-x>.

Additional file 1. Fig. S1. IgG antibody and 12-mer A β oligomer binding. **Fig. S2.** Sandwich ELISA displaying the binding properties of each antibody to different cross-linked fractions of A β 1-42 generated in Fig. 6. **Fig. S3.** Sandwich ELISA displaying the binding curves of the different antibodies to another batch of cross-linked A β 1-42 fractions.

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Schematic illustrations were created with BioRender.com.

Authors' contributions

G.H designed the project; G.H and F.R designed the constructs; F.R, R.F, K.H and G.H generated the recombinant proteins; F.R, J.B and T.G performed and analyzed radiochemistry and LigandTracer experiments; R.F and K.H performed and analyzed SPR experiments; L.S, L.L and A.M.L generated the cross-linked A β fractions; F.R, D.S, G.H designed and performed ELISA experiments; F.R and N.G.M performed structural stability and cell experiments; F.R and G.H wrote the manuscript with valuable inputs from all the co-authors. All authors read and approved the final manuscript.

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Availability of data and materials

The datasets used and/or analysed during the current study are available from the corresponding author on reasonable request.

Declarations

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Competing interests

R.F.; K.H.; A.M.L. and L.S. are employees of BioArctic AB, Stockholm, Sweden. L.L. is cofounder of BioArctic AB, Stockholm, Sweden. J.B. is CEO of Ridgeview Instruments AB, Uppsala, Sweden. All the other authors declare that they have no competing interests.

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