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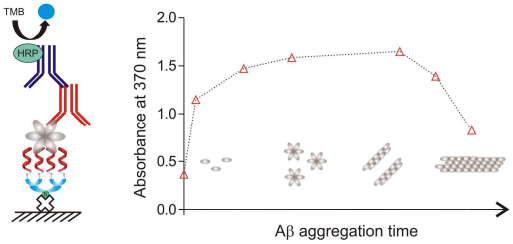

Multivalent foldamer-based affinity assay for selective recognition of Aβ oligomers

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Abstract

Mimicking the molecular recognition functionality of antibodies is a great challenge. Foldamers are attractive candidates because of their relatively small size and designable interaction surface. This paper describes a sandwich type enzyme-linked immunoassay with a tetravalent β-peptide foldamer helix array as capture element and enzyme labeled tracer antibodies. The assay was found to be selective to β-amyloid oligomeric species with surface features transiently present in ongoing aggregation. In optimized conditions, with special emphasis on the foldamer immobilization, a detection limit of 5 pM was achieved with a linear range of 10 - 500 pM. These results suggest that protein mimetic foldamers can be useful tools in biosensors and affinity assays.

Keywords: foldamers; β-amyloid oligomers; bioaffinity assay; molecular recognition; antibody mimetics

1. Introduction

While antibodies are still the gold standards for high affinity and selective recognition of molecular targets, their limitations, including antigenicity, poor pharmacokinetic properties and costly time-consuming production, called for the development of artificial antibody mimetics with more favorable properties in

therapeutic and diagnostic applications. To date, several antibody mimetics such as affibodies, anticalins, DARPins, nanofitins, fynomers and avimers have been developed, having various advantages over conventional antibodies in respect of stability, solubility, tissue penetration and also production costs[1-3]. In this respect peptidic foldamers (artificial oligopeptides capable of folding into well-defined conformations) provide an advanced approach for the design of selective synthetic receptors. The molecular conformation that enables selective recognition is not directly formed during chemical synthesis but the selective interaction surface is created by a subsequent folding process. Folding is determined by the primary structure of the foldameric sequence, the solvent and the interaction partner acting as a template. Thus, foldamers are able to form biomimetic shapes (helices, sheets) in a controlled manner[4-6] enabling these structures to form extended surface patches with anchor points in designed spatial positions, necessary for interaction with the target protein. This property makes foldamers potential alternatives of antibodies in therapeutic and diagnostic applications. To date different protein surfaces have been successfully targeted with foldamers, such as: Somatostatin receptor[7] GLP-1 receptor,[8] PTHR1,[9] the p53-hDM2,[10] the BH3-Bcl-x_L[11, 12] and the VEGF-VEGFR1[13] interactions, the gp41 virus cell infusion protein assembly[14], the γ-secretase enzyme[15] and amyloid aggregation.[16] Whilst biomimetic molecular recognition can be achieved with foldamers, their application in therapy, diagnostics and as analytical tools is still a major challenge. [17] The different isoforms of β-amyloid is regarded as one of the most important factors in the onset of Alzheimer's disease (AD), [18][ref] and the selective detection of oligomeric Aβ species is a crucial task in developing diagnostic tools for AD, as the concentration of oligomeric forms in the cerebrospinal fluid is regarded as the most relevant biomarker for the onset of the disease.[19] Multiple approaches have been published for capturing/detecting soluble aggregated forms of Aβ: Aβ oligomer specific antibodies; [20-24] simultaneous application of multiple N-terminal specific antibodies;[25-28] a generic aggregation-sensitive peptide,[29] and Aβ self-recognition via seeded polymerization.[30, 31] Advanced detection methods have also been utilized for Aβ oligomer sensing, [32] including DNA biobarcode amplification [21], localized surface plasmon resonance [20] and electrochemical techniques. [33-35] It has been pointed out that heterophilic antibodies in biofluids may cause false positives in the antibody-based Aβ capture schemes, therefore alternative molecular recognition elements are sought. [22]

In an attempt to neutralize synaptotoxic β-Amyloid oligomers we have previously introduced the β-peptide foldamer helix ACHC-β^3hArg-ACHC-ACHC-β^3hAsp-ACHC-Gly-Gly-Cys (**1,** Figure 1) (ACHC: (1*S*,2*S*)-2-amino-cyclohexanecarboxylic acid), which is linked by a maleimide-functionalized zero-generation (G0) poly(amidoamine) (PAMAM) dendrimer (**4**) to form a foldamer-dendrimer tetravalent helixarray (**7**).[36] The conjugate provided an interaction surface capable of capturing Aβ oligomers, which play a central role in the pathogenesis AD, and showed promising molecular recognition properties including a two-stage interaction with Aβ oligomers with low nanomolar and submicromolar affinities toward the target in solution. The first, high affinity binding stage required all four recognition segments, whereas the second low affinity binding involved only two arms. Here we explored the feasibility of implementing a foldameric recognition element, i.e. a *de novo* designed foldamer conjugate (biot-**8**), as an alternative to a capture antibody in an affinity assay for high sensitivity quantitation of Aβ oligomers and possibly to indicate the state of an ongoing aggregation. This study shows the applicability of foldamers as recognition elements in standard biochemical assays, and their potential use in diagnostic applications.

2. Materials and Methods

2.1 Synthesis of compounds 1-3.

Peptidic foldamers were synthesized by Fmoc-based solid phase peptide synthesis on Tentagel R RAM resin (0,19 mmol g^{-1}) with 1-[Bis(dimethylamino)-methylene]-1H-1,2,3-triazolo[4,5-b]pyridinium-3-oxid hexafluorophosphate (HATU) used as a coupling reagent in the presence of N,N-diisopropylethylamine (DIPEA). Biotinylation was carried out using N-biotinyl-6-aminohexanoic acid (Sigma Aldrich) and a Gly-Gly linker was used in the synthesis of **2**. For **3**, the acetylation of the N-terminal was carried out on solid phase with acetic anhydride (10% v/v) and DIEA (5% v/v) in DCM for 30 min at room temperature. The peptide sequences were cleaved from the resin using TFA/H_2O/ DTT/TIS (90:5:2.5:2.5) at room temperature for 3 h. The TFA was removed in vacuo and the peptide was precipitated in dried diethyl ether, filtered off, dissolved in 10% aqueous acetic acid and lyophilized. The crude foldamer was dissolved in 150 µL concentrated acetic acid and diluted with 9 mL water then purified by RP-HPLC on a Phenomenex Luna C18 (250 mm x 21.20 mm, 100 Å, 10 µm) column. The solvent system consisted of 0.1% TFA in water (A), and 0.1% TFA in 80% acetonitrile (B). The default gradient was 0%-40% B during 30 min and then 40%-60% during 80 min at a flow rate of 3 mL min^{-1}, with detection at 206 nm. Purity was confirmed with HPLC-MS and HPLC-UV measurements.

2.2 Synthesis of compounds 4-6.

For the synthesis of **4**, G0-PAMAM dendrimer solution in methanol (Sigma Aldrich) was dried in vacuo and dissolved in DMF. 3-maleimidopropionic acid was coupled to the free amino groups with HATU/DIPEA activation. The sample was diluted with water and purified by RP-HPLC on a Phenomenex Luna C18 (250 mm x 21.20 mm, 100 Å, 10 μm) column. Oligolysin-dendrons **5** and **6** were synthesized by Fmoc-based solid phase peptide synthesis on Rink-amide resin (0.3 mmol g^{-1}), applying HATU as coupling reagent in the presence of DIPEA. Biotinylation of the templates was carried out by coupling an Fmoc-L-Lys(Biotin)-OH (Iris Biotech) amino acid as an initial building block. 3-maleimidopropionic acid was coupled to the N-terminus of the scaffold on the solid phase, with HATU/DIPEA activation. The dendrimers were cleaved from the resin using the same method as the peptide foldamers. The lyophilized maleimidopropionyl-lysine-dendrimer was dissolved in a mixture of ACN/H_2O at a final volume of 10 mL and injected onto a Phenomenex Luna C18 (250 x 21,20 mm, 100 Å, 10 μm) column, at a flow rate of 3 mL min^{-1}. Gradients were 10-40% B over 120 min in case of compound **5** and 0-50% B over 100 min in case of the compound **6**.

2.3 Synthesis of compounds 7-10.

The maleimide-functionalized dendrimer was dissolved in 4 mL 50mM NaH_2PO_4/Na_2HPO_4 buffer (pH=7.0) solution. 8 or 16 equivalents of the foldamer peptide was dissolved in 1 mL of the same buffer, for **5** and **6** respectively, then added drop wise to the dendrimer under constant stirring. The reaction was stirred overnight at ambient temperature and the following day, the mixture was injected directly onto a Phenomenex Jupiter C4 (250 x 10 mm, 300 Å, 10 μm) semipreparative HPLC column applying different gradient elution for each conjugate at 3 mL min^{-1} flow rate.

2.4 Preparation of the Aβ samples.

Recombinant Aβ1-42 was purchased from rPeptide (Bogart, GA, USA). Amyloid was dissolved in deionized water (conc. 1 mg ml^{-1}) with the pH set to 11 using 100 mM NaOH. The solution was sonicated for 3 minutes then incubated for 2 hours at room temperature. The solution was diluted to 50 μM final amyloid concentration with 26.67 mM PBS and the pH was set to 7.4 using 1 M HCl. The sample was incubated at 37°C for the required time intervals (typically 3 hours). 1% BSA and 0.05% TWEEN20 was added after the incubation. Dilution to the required concentrations were made with a probing buffer (20 mM PBS, containing 1% BSA and

0.05% TWEEN20, TPBS). The samples were characterized by TEM as reported in (17). For the selectivity test against the fibrillar form, 50 μM Aβ was incubated for 72 h at pH 4.0 and diluted with the washing buffer to the appropriate concentrations.

2.5 Isothermal calorimetric titrations

Isothermal calorimetric titrations (ITC) were performed with a Microcal VP-ITC microcalorimeter in pH 7.4 PBS buffer solution. In individual titrations, 10 μL ligand containing solution was injected from the computer-controlled 300-mL microsyringe at intervals of 300 s into the Aβ oligomer solution, dissolved in the same buffer as the ligand. All measurements were carried out at 285 K. The Aβ concentration in the cell was 100 μM and the total ligand concentration was set in the syringe so that the titration stopped when the precipitation became excessive. Control experiments were performed by injecting the ligand into a cell containing buffer with no target, and the heats of dilution were subtracted from those measured in the presence of Aβ. The experimental data were fitted to the two independent site binding model by using a nonlinear least-squares procedure, with ΔHb, ΔHb', K_d, K_d' (association constants), n and n' (number of binding sites for monomer) as adjustable parameters.

2.6 ELISA experiments.

PIERCE (Rockford, IL, USA) avidin (125 pmol/well or 60 pmol/well) coated plates were used. The capture molecule was dissolved in PBS (conc. 10 μg ml^{-1}) and 100 μl capture molecule solution was pipetted in each well and incubated for 2 hours at room temperature. The plate was washed with 3 × 200 μl TPBS (20 mM PBS containing 1% BSA and 0.1% TWEEN20) and incubated with 100 μl diluted amyloid solution under shaking (overnight at 4 °C). After washing the plate with 3 × 200 μl TPBS, the primary antibody (6E10, Covance, Leeds, UK) was diluted with the washing buffer (1:10000 dilution) and 100 μl diluted primary antibody solution was pipetted into each well. The sample was incubated for 1 hour at room temperature. The plate was washed with 3 × 200 μl TPBS and 100 μl of the secondary antibody (Histols-M (Histopatology Ltd., Pécs, Hungary) in 250x dilution or anti-mouse IgG HRP (Dako, Glostrup, Denmark) 1:10000 dilution) was pipetted to each well. After 1 hour incubation at room temperature, the plate was washed with 2 × 200 μl TPBS (the first washing step has the TPBS solution left in the plate for 30 minutes). Development was carried out with 100 μl 3,3',5,5'-

tetramethylbenzidine (TMB) solution pipetted into each well and the absorbance was measured with a plate reader (NOVOstar OPTIMA, BMG Labtech, Offenburg, Germany) in plate mode, for approximately 1.5 hours.

For validation, Innotest® β-Amyloid$_{(1-42)}$ (Innogenetics, Gent, Belgium) assay was performed according to the manufacturer's instructions. The selectivity tests were carried out in artificial cerebrospinal fluid (ACSF), made of Dulbecco's Modified Eagle Medium (DMEM) containg 1% fetal bovine serum (FBS), both purchased from Sigma-Aldrich. 0.1% Tween20 was added to prevent aspecific binding.

2.7 SPRi measurements.

A HORIBA SPRi-Plex II (HORIBA Jobin Yvon S.A.S. Palaiseau, France) surface plasmon resonance imaging (SPRi) system was used for multiplexed SPR measurements at a fixed working angle selected for optimal response in the PBS running buffer (10 mM phosphate, 137 mM NaCl, 2.7 mM KCl). The sensitivity across the active area of the chip was normalized using the refractive index dependent signal change for 180 mM NaCl containing PBS. The foldamer interactions were measured in PBS running buffer at a flow rate of 50 µL min^{-1} and 25.00 °C. The signal change recorded for control (Figure S7) was used as a negative control and subtracted from the signals recorded in the various foldamer spots. The injected volume for each analyte and regeneration solution was 180 µL. 5 mM NaOH solution was used (3.6 min, flow rate 50 µL min^{-1}) to regenerate the foldamer-modified surfaces after each interaction. Kinetic evaluation of binding interactions was performed with global analysis using first order kinetics with Scrubber 2 (GenOptics version, BiaLogic Sofware, Campbell, Australia). Detailed procedure for spotting SPR sensor chips is found in the supporting text.

3. Results and Discussion

3.1 Design of the affinity assay.

In the sandwich assays, the capture antibody is immobilized on the well plate surface by coating hydrophobic polystyrene surfaces using passive adsorption. The recognition interface of the Fv region remains mostly accessible due to the large size of the immunoglobulin molecule (MW = 150 kDa). However, for the low molecular weight foldameric capture element (MW = 5.2 kDa), the oriented immobilization is crucial to make the recognition segments available for analyte binding. Therefore the foldamer sequences were custom-designed and biotin-tagged to enable their oriented immobilization to streptavidin-coated well plates (Figure

2). The subsequent capture of Aβ species was detected optically with a primary monoclonal mouse antibody specific to the free N-terminus of Aβ chain (6E10) and a horseradish peroxidase-conjugated secondary anti-mouse antibody.

Two biotinylation strategies were followed. First, the centrally symmetric PAMAM-foldamer was tagged at the foldamer termini (biot-**7,** Figure 1C). Second, the biotin-tag was placed in the focal point of a redesigned template so the helical units may freely point away from the surface. The centrally symmetric PAMAM dendrimer is not suitable for this biotinylation strategy and thus, was replaced with a functionalized oligolysine dendron having focal symmetry (**5**). The biotinylation was carried out at the root of the dendron by solid-state coupling of ε-biotinyl-Lys. The capture ligand was synthetized by maleimide-thiol coupling in non-tagged (**8**) and tagged forms (biot-**8**). The binding affinity was tested in the solvent phase by ITC. For **8**, a two-stage enthalpogram was observed, similar to **7**, with dissociation constants of 4.1 ± 2.5 nM (n = 0.05), and 374 ± 102 nM (n = 0.25) (Figure 3C). This finding confirmed that the Aβ binding features were carried by the foldamer recognition segments, and the function of the dendritic linker was tethering.

The ELISA experiment performed with biot-**8** as capture element revealed marked affinity increase when compared with biot-**7** (Figure 3A). The EC_{50} value for biot-**8** was 0.97 ± 0.04 nM indicating the presence of the high-affinity interaction. By contrast, biot-**7** has an EC_{50} value of 648.5 ± 11.2 nM, which is in line with the second-stage binding event observed in solution for **7**. There can be two reasons for the lower affinity: (i) the biotinylation removes the N-terminal amino group, which is potentially critical for the recognition mechanism, (ii) and the geometry of biot-**7** on the surface prevented projecting all its foldamer helices thus impeding the high-affinity binding (Figure 3B). The role of the N-termini was tested by the acetylation of the foldamer helices of biot-**8** (biot-**9**). The ELISA experiment indicated an EC_{50} value of 30.16 ± 2.27 nM for biot-**9** (Figure 3A), which confirmed the involvement of the free N-termini in high-affinity binding. The acetylation of N-termini however did not fully account for the lower affinity observed for biot-**7** and thus, the accessibility of the foldamer segment in the immobilized ligand appears to be essential in order to obtain low nM dissociation constants.

3.2 Multivalency Effects

It has been previously shown that the divalent PAMAM-foldamer conjugate does not display low nanomolar affinity[36] and spurred on by the good performance of biot-**8**, the effects of increasing the number of arms on

the affinity were tested. An octavalent oligolysin dendron template was synthesized (**6**) and the foldameric recognition segments were coupled through maleimide-thiol ligation (**10**). ITC experiment revealed that there was no change in the initial gradient of the curve and only the foldamer conjugate/Aβ ratio decreased in accord with the octavalent design (Figure S2). The curve could not be evaluated quantitatively by assuming the two binding site model *a priori*. Independent quantitative data on the affinity was obtained through SPR experiments with capture elements biot-**8** and biot-**10** attached to the streptavidin coated gold surface. There were no difference between the affinities of the two derivatives and only an increase in the binding levels was found (Figure 4A). The same phenomenon relating to affinity was observed in ELISA experiments where no difference was found between the EC_{50} values of biot-**8** and biot-**10** (Figure S5). Sensitivity improvement in ELISA could not be detected possibly due to steric crowding in the detection step (Figure S5). From these findings, it can be concluded that the high-affinity binding does not require more than four foldameric capture segments. This is indicative of an interaction with a specific interface displayed by the Aβ oligomers rather than capturing repeating features of the cross-β-sheet surface of the aggregated forms.

3.3 Detection of Aβ oligomers by ELISA

The sensitivity in detecting Aβ species can be crucial because their concentration in body fluids is in the picomolar range or less.[37, 38] Using a HRP-polymer tagged secondary antibody (Histols-M) increased the sensitivity of the system due to the multiple copies of HRP (Figure 4B). Noting that steric hindrance may prevent efficient detection, further improvements to sensitivity were attempted by decreasing the surface crowding on the well surface. This was tested by comparing the ELISA results obtained by using streptavidin precoated plates with surface loads of 60 pmol and 125 pmol per well. The EC_{50} value did not change with the surface load but the signal intensity was higher for the 60 pmol plate (Figure 4B) in agreement with the steric hindrance hypothesis. Utilizing the improved capture ligand and the optimized protocol, the limit of detection (3 σ) was estimated to 5 pM (n=12), and linear dependence was obtained over the concentration range 10 - 500 pM (R^2=0.9974).

The effect of ligand multivalency suggested that foldamer-based capture element shows selectivity for the Aβ oligomers and this was tested using both predominantly monomeric and oligomeric Aβ samples. The Aβ solutions with different aggregation state were prepared according to literature protocols.[36, 39] For the ELISA performed with the capture element biot-**8**, concentration-dependent (0 - 200 pM) signal was observed

for the oligomeric sample (Figure 5A). The fresh monomeric Aβ did not yield signal (LOD estimated to 3600 pM). The monomeric Aβ contents of the samples were checked in parallel by using a commercially available Aβ monomer-selective ELISA kit (Innotest®) commonly utilized in clinical studies. It was obvious that the monomer-sensitive commercial sandwich ELISA kit produced concentration-dependent signal only for the monomeric Aβ sample (Figure 5B). Low intensity response was detected above 100 pM for the aggregated Aβ sample, which can be attributed to the residual monomeric Aβ content.

These results revealed that the capture ligand biot-**8** is selective for the Aβ oligomers, and the foldamer-based sandwich assay gives complementary response to the monomer Aβ selective kit utilizing an Aβ C-terminal selective antibody as capture element. The applicability of the assay as a potential diagnostic tool requires the detection of the analyte in a complex biological matrix. No signal was observed for an an artificial cerebrospinal fluid (DMEM cell culture media containing 1% fetal bovine serum). The responses recorded for oligomeric Aβ in an this matrix showed no significant difference compared with those obtained for the same concentrations of Aβ in a buffer solution. This confirmed the selectivity of the assay against the rich variety of proteins in fetal bovine serum (Figure S8).

3.4 Monitoring time-dependent aggregation of Aβ

It was also known from earlier studies that the foldamer conjugate **7** cannot bind fibrillar Aβ with high affinity in the solution phase, suggesting that this newly devised ELISA method should be capable of monitoring the aggregation state of an Aβ solution by solely detecting the oligomer content. A standard Aβ aggregation procedure (incubation of a freshly disaggregated Aβ monomer sample at the concentration of 50 μM, 37°C) was followed with the stock solution being sampled at regular intervals. Samples were diluted to a total Aβ concentration of 500 pM, then applied onto the ELISA plate. The intially recorded absorbances increased with time, then plateaued after 3 hours (Figure 5C) and tapered off after 18 hours. TEM analysis was carried out in parallel, confirming that Aβ oligomer specii were formed in the first 3 h and were captured by biot-**8**. Towards the end of the experimental run (18 h), aggregation transformed the oligomers and residual monomers to fibrils (Figure S6) which did not show affinity to biot-**8**. These results strongly suggested that this system is able to indicate the state of an ongoing aggregation in a pM sample by detecting transient Aβ surface features with high affinity to our foldamer-based capture element. It must be noted however that quantitative

measurements with fibrillar Aβ at pM concentrations are not possible due to its disaggregation into oligomeric and monomeric forms. [40] Therefore selectivity of our foldamer-ELISA assay against the fibrillar form can not be concluded from these data.

4. Conclusions

Foldamers are attractive candidates as molecular recognition elements due to their programmable molecular surface, attained with short chain lengths. In this study, a hexameric β-peptide 14-helix was utilized with a surface engineered to bind to Aβ aggregates. Two-stage binding was observed in the solution phase when the foldameric recognition segments were attached to a tetravalent oligo-lysine dendron template (**8**). The first stage has a low nanomolar affinity toward Aβ oligomers with fractional stoichiometry indicating that multiple copies of aggregated Aβ chains are necessary to form a surface patch to be recognized by the tetravalent ligand. The second, lower affinity step proved to be stoichiometric with a ratio of 1:4 for the **8**: Aβ chain ratio. The foldamer-ELISA assay was designed by using the biotinylated derivative of the multivalent foldamer **8** attached to streptavidin precoated plate surface as a capture element. We found that the free N-termini and the accessibility of the four foldameric arms are essential for the high-affinity interaction with Aβ oligomers. The optimized foldamer-ELISA was sensitive to the Aβ oligomers in the picomolar range. Aggregation time-dependent tests with monomeric, oligomeric and fibrillar Aβ proved that this system is selective to Aβ surface patterns transiently present during the ongoing aggregation process.[41] These results point to the utility of protein mimetic foldamers in biochemical assays and sensors, where they can functionally mimic the molecular recognition properties of antibodies.

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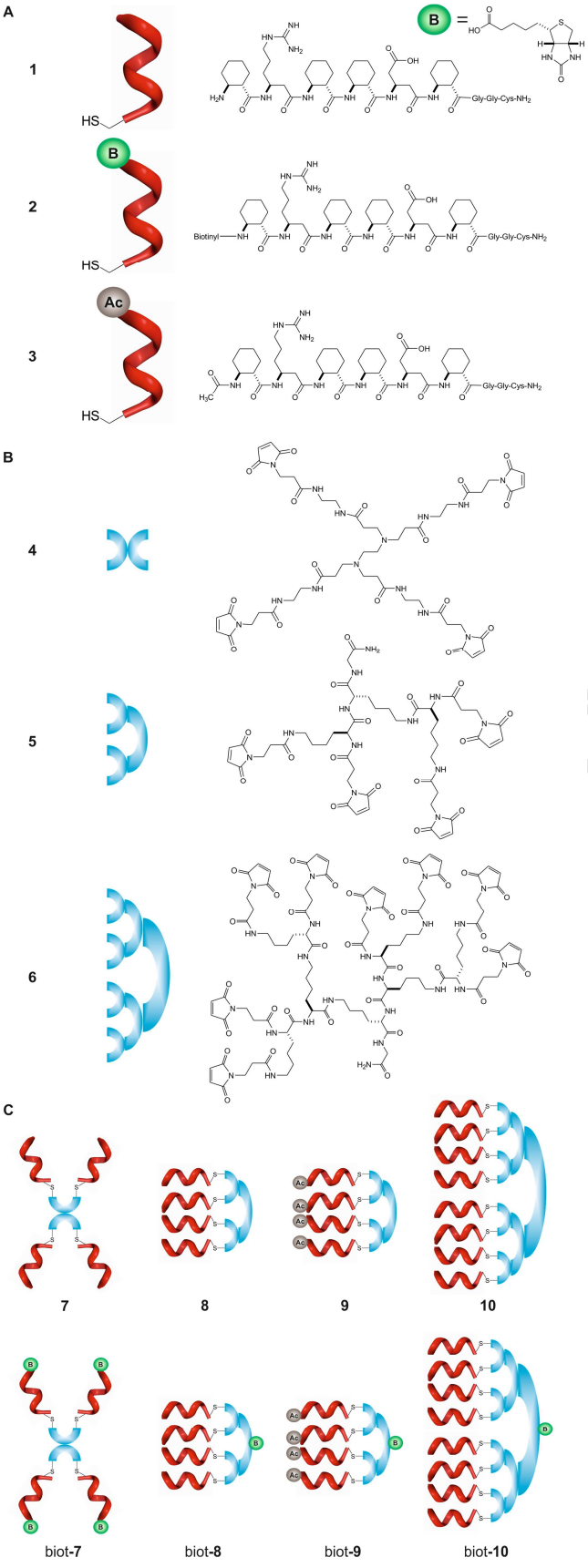
A
1
HS
B =
Gly-Gly-Cys-NH2
2
B
HS
Biotinyl
Gly-Gly-Cys-NH2
3
Ac
HS
Gly-Gly-Cys-NH2
B
4
5
6
C
7
8
9
10
biot-7
biot-8
biot-9
biot-10

Figure 1. Structure and schematic representation of (A) foldamer recognition elements, (B) templates (with biotin-tag (ε-biotinyl-Lys) or Gly at the root of the dendron), (C) multivalent foldamer conjugates.

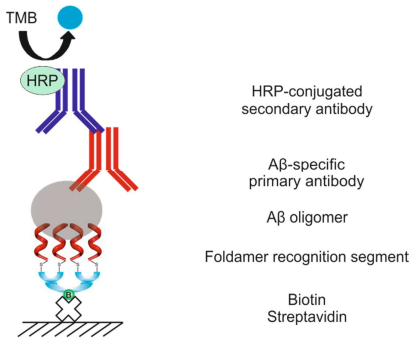

Figure 2. Design of the foldamer-based immunoassay platform. HRP designates horseradish peroxidase and TMB indicates 3,3′,5,5′-tetramethylbenzidine.

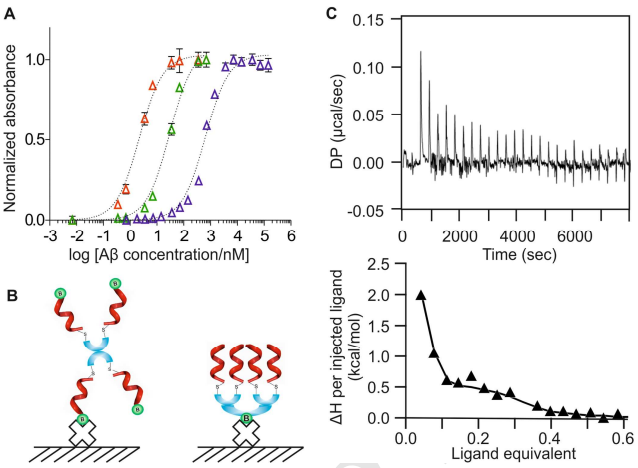

Figure 3. A) Comparison of the immunoassay affinities observed for biot-**7** (blue), biot-**8** (red) and biot-**9** (green) as capture elements. Absorbances were normalized to the absorbance maximum in each measurement. B) Schematic representation of biot-**7** and biot-**8** surface geometry. C) ITC enthalpogram and thermogram for the titration of Aβ oligomers with **8.**

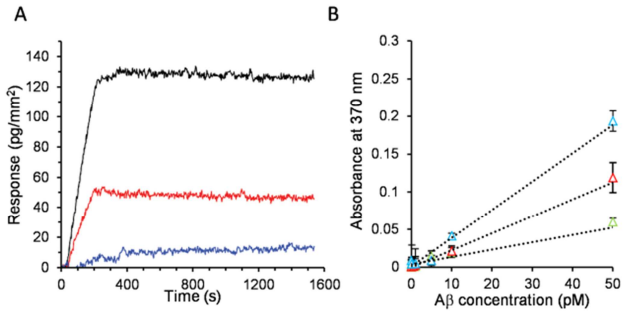

Figure 4. (A) Representative SPR sensorgram of 2000 nM amyloid oligomers binding to 10 µM spotted biot-**10** (black), biot-**8** (red), and **2** (blue). See details in the Supplementary Information (Table S1, Figure S3 and S4). (B) Optimization of the ELISA setup. Recorded absorbances with original conditions (green), after applying Histols-M (red), and after lowering the surface load to 60 pmol (blue).

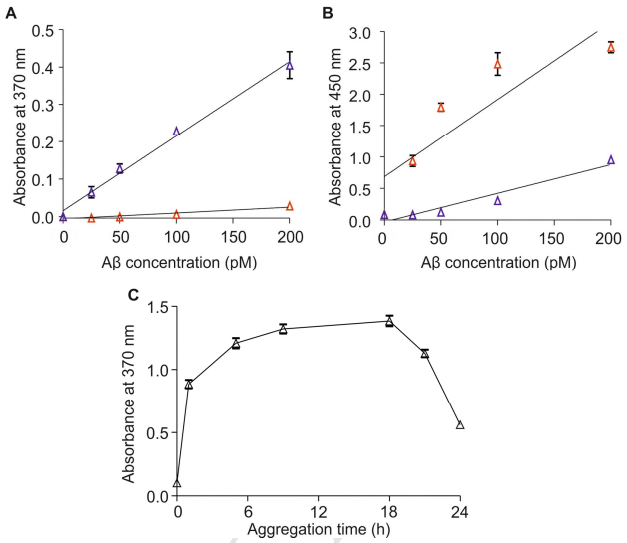

Figure 5. (A) Recorded absorbances in ELISA with the foldamer-based capture element for monomeric (red) and oligomeric (blue) Aβ. (B) Recorded absorbances with the Innotest® kit for the same Aβ samples. (C) Aggregation time-dependence of the ELISA signals.

Highlights

- A sandwich-type immunoassay is developed to detect β-amyloid.
- The assay utilizes foldamer helices as recognition elements.
- The assay is selective to β-amyloid oligomers against monomeric forms.
- β-amyloid oligomers can be detected at pM levels.