Chapter 8

Transmigration of amyloid-β specific heavy chain antibody fragments across the *in vitro* blood-brain barrier

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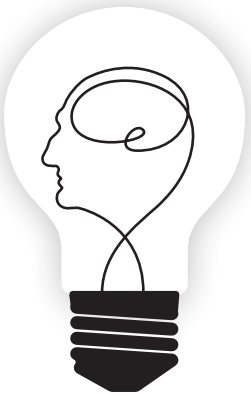

Abstract

Previously selected amyloid-β recognizing V_HH affinity binders derived from the Camelid heavy chain antibody repertoire were tested for their propensity to cross the blood-brain barrier (BBB) using an established *in vitro* BBB co-culture system. Of all tested V_HH, ni3A showed highest transmigration efficiency which is, in part, facilitated by a 3 amino acid substitution in its N-terminal domain. Additional studies indicated that the mechanism of transcellular passage of ni3A is by active transport. As V_HH ni3A combines the ability to recognize amyloid-β and to cross the BBB, it has potential as a tool for non-invasive *in vivo* imaging and as efficient local drug targeting moiety in patients suffering from cerebral amyloidosis such as Alzheimer's disease and cerebral amyloid angiopathy.

Introduction

Alzheimer's disease (AD) is the most prevalent form of dementia and clinically characterized by an irreversible process of cognitive decline.[1] Cerebral amyloid angiopathy (CAA), is the main cause of non-hypertensive intracerebral hemorrhage in the elderly.[2] AD and CAA share amyloid beta (Aβ) accumulation and aggregation as one of their primary neuropathologic characteristics.[3,4] Furthermore, a definitive diagnose can only be made with certainty by post-mortem histological analysis of brain tissue. Ideally, treatment of AD and CAA patients has to be started before significant cognitive loss has occurred. However, this requires novel diagnostic methods, ideally non-invasive, allowing the early detection of these diseases. Therefore, several classical antibodies, targeted at Aβ, have been developed for detection and treatment of cerebral amyloidosis, but their utilization *in vivo* has been hampered by their size which prevents them from crossing the blood-brain barrier (BBB) effectively.

Camelidae co-express unusually shaped antibodies which are devoid of light chains next to their conventional immunoglobulin repertoire. These antibodies, referred to as heavy chain antibodies (HCAb), are composed of two identical heavy chains. Their antigen-binding properties are therefore only defined by the variable domains of these heavy chains (V_HH). Interestingly, these V_HH by themselves are fully capable of antigen binding.[5]

V_HH have a molecular mass of ~15 kDa with affinities similar to those achieved with conventional antibodies.[6] V_HH rapidly pass the renal filter, resulting in a rapid tissue penetration and fast blood clearance.[7] Moreover, it was demonstrated that they can cross the BBB: V_HH FC5 binds to human cerebromicrovascular endothelial cells (HCEC) and transmigrates across an *in vitro* human BBB model.[8] Therefore, V_HH are considered to have potential as tools for early non-invasive diagnosis using targeted contrast agents and delivery of therapeutic agents in patients with neurodegenerative disorders.

Targeted drug delivery and non-invasive early diagnosis using targeted contrast agents in neurodegenerative disorders are complicated by the regulated interface created by the BBB. The BBB's primary function is to maintain homeostasis of the brain and protect it against undesirable compounds and cells. For the identification of compounds that can cross the BBB the availability of *in vitro* BBB (co)-culture systems is imperative. Previously, we reported on an *in vitro* BBB co-culture system consisting of bovine brain capillary endothelial cells (BCEC) and newborn rat astrocytes. This established BBB model has been studied thoroughly and was shown to mimic the BBB *in vitro*, having small paracellular permeability, expression of various transporters and a relatively high transendothelial electrical resistance (TEER).[9]

In a previous study, we described a panel of V_HH which we selected against Aβ by phage display.[10] As a first step toward *in vivo* application, we here analyzed their propensity to cross the BBB *in vitro* and the influence of three specific amino acid substitutions on this crossing ability. The combination of both properties, that is, the ability to cross the BBB and to recognize Aβ, would render these V_HH promising tools for non-invasive *in vivo* imaging and efficient local drug targeting in patients with AD or CAA.

Materials and Methods

V_HH used in this study

The V_HH used in this study were described previously.[10] V_HH ni3A, ni8B and va2E are selected against Aβ. V_HH ni3A and ni8B are derived from a non-immune library; va2E is selected from an immune library created after immunisation with post-mortem cerebral blood vessels of a Hereditary Cerebral Hemorrhage With Amyloidosis – Duchtype (HCHWA-D) patient. V_HH FC5 encoding mRNA and amino acid sequences are deposited in the GenBank no. AF441486 and no. AAL58846, respectively and was produced synthetically.

Subcloning and Production

The V_HH genes were subcloned into the pUR5850VSV production vector.[11] Thereafter, the V_HH were produced in *E. coli* and purified from the periplasmic supernatants as described earlier.[12] To test the necessity of the presence of three atypical amino acids in the N-terminus (framework 1; FR1) of ni3A for its ability to cross the BBB, a chimearic V_HH 3A2E was constructed by PCR amplification of FR1 of ni3A and the region starting from complementarity determining region 1 (CDR1) until FR4 of va2E, and subsequent cloning of the chimearic PCR product into the pUR5071myc and pUR5850VSV vectors. All vectors were sequence verified (LGTC, Leiden, Netherlands). Production was performed as described previously.[12]

The open reading frame of the similarly sized control protein alpha-synuclein (SNCA) was PCR amplified from human brain cDNA, cloned, sequence verified and its encoded product was produced in the pET28 production system (Novagen, Madison, WI, USA). Primer sequences are available upon request. All proteins were purified using Talon metal affinity resin (Clontech, Palo Alto, CA, USA) according to the instructions of the manufacturer.

Immunohistochemistry

Frozen brain tissue sections (8 µm) from the neocortex of neuropathologically confirmed HCHWA-D, AD patients and non-demented controls were rinsed in PBS, fixed with ice-cold acetone for 10 min, incubated with peroxidase blocking reagent (Dako Cytomation) for 20 min, washed in PBS and incubated with the anti-Aβ V_HH (20 ng/µl) in 1% BSA/PBS overnight in a wet chamber. Thereafter, the sections were rinsed with PBS and incubated with mouse-anti-VSV for 1 hour and EnVision$^{+®}$ system labelled Polymer-Hrp anti-Mouse (Dako Cytomation) for 30 min. Detection was performed with Liquid DAB$^+$ Substrate Chromogen System (Dako Cytomation). In addition, hematoxylin counterstaining was performed and the sections were dehydrated and mounted in micromount mounting medium (Surgipath, Richmond, IL, USA). 6E10 (Covance, Princeton, New Jersey, USA) was used as a positive control for Aβ staining.

In vitro blood-brain barrier system

The *in vitro* BBB model was prepared as described before.[9,13] Briefly, brain capillaries were isolated from cortices of bovine brain, acquired at a slaughterhouse (de Boer, Nieuwerkerk a/d IJssel, The Netherlands). The capillary fraction was prepared by homogenization, captured by percolation with nylon meshes and subsequently digested by enzymes. Astrocytes were isolated from cortices of brains of newborn Wistar rats (Harlan B.V., Zeist, the Netherlands) and used

for co-culture purposes and the preparation of astrocyte conditioned medium. Brain capillaries were cultured on collagen and fibronectin coated culture flasks in 50% astrocyte conditioned medium to brain capillary endothelial cells (BCEC). The astrocytes were seeded on the bottom of the filter. Two days later the BCEC were passaged on collagen coated Transwell polycarbonate filters (surface area: 0.33cm^2, pore size: 0.4 µm, Corning Costar, Cambridge, MA, USA) and cultured to tight monolayers in 50% astrocyte conditioned medium in 5 days.

***In vitro* blood-brain barrier transport**

Transport studies were performed after 10 days of seeding and subsequently 5 days of seeding on the filters in DMEM+ 10% fetal calf serum (Bio Whittaker Europe, Verviers, Belgium) at 37°C. Transport studies were initiated by adding 10 µg V_HH to the upper chamber of the *in vitro* BBB system. 60 µl aliquots were taken from the bottom chamber at 5 – 15 – 30 – 60 and 90 min. As a control a protein with the same size as a V_HH (SNCA) was used. Membrane integrity was monitored with transendothelial electrical resistance (TEER) measurements, which was shown before to be a reliable and sensitive method to rule out paracellular BBB permeability.[9] The threshold was set at a resistance of 400 Ohm x cm^2. After 120 min the cells were washed 3 times 300µl DMEM+S, harvested and lysed with MPER (Pierce Biotechnology, Rockford, IL, USA). For determination of the amount of V_HH/protein that transmigrated across the model, 25 µl aliquots collected from the bottom chamber were immobilized on a Ni-NTA HisSorb 96-well plate (QIAGEN Benelux, Venlo, The Netherlands) diluted in 1% BSA with a final volume of 200 µl, at 4°C overnight. Plates were washed 4 times with PBS 0.05% Tween (PBST) and incubated with anti-c-*myc* / anti-VSV monoclonal antibody diluted in 1% BSA for 1 hour at room temperature. After washing, plates were incubated with polyclonal Rabbit anti-Mouse Immuno globulins conjugated with HRP (DakoCytomation). Plates were washed and detection was performed by adding 100 µl OPD (3,7 mM o-phenyldiamine, 50 mM $Na_2HPO_4.H_2O$, 25 mM citric acid) supplemented with 0.01% H_2O_2 to the wells. When the reaction was clearly visible, 50 µl 1M H_2SO_4/well was added to stop the reaction. The extinction at 490 nm was measured utilizing a Biotec synergy HT plate reader. Statistical analyses were performed by unpaired 2 tailed t-test comparing target V_HH to control for each time point (**Figure 8.3**) or 2-way ANOVA followed by a Bonferonni post hoc test (**Figure 8.4**) using GraphPad Prism version 5.00 for Windows (GraphPad Software, San Diego California USA). Threshold value for statistical significant differences was set at 5%.

Results

Subcloning and purification of specific V_HH

Two independent V_HH clones, ni3A and ni8B respectively (**Figure 8.1B**), were chosen for this study.[10] These V_HH were subcloned into the pUR5850-VSV production vector. Ni3A has an unexpected intrinsic efficiency in crossing the BBB. (see below, **Figure 8.3**) In order to investigate the necessity of the presence of three, for V_HH unusual amino acid residues in the N-terminus of ni3A, [R15-D-G-D], with respect to crossing the BBB, chimaeric V_HH 3A2E was constructed. 3A2E consists of the N-terminus from ni3A fused, at the boundary between FR1 and CDR1, with

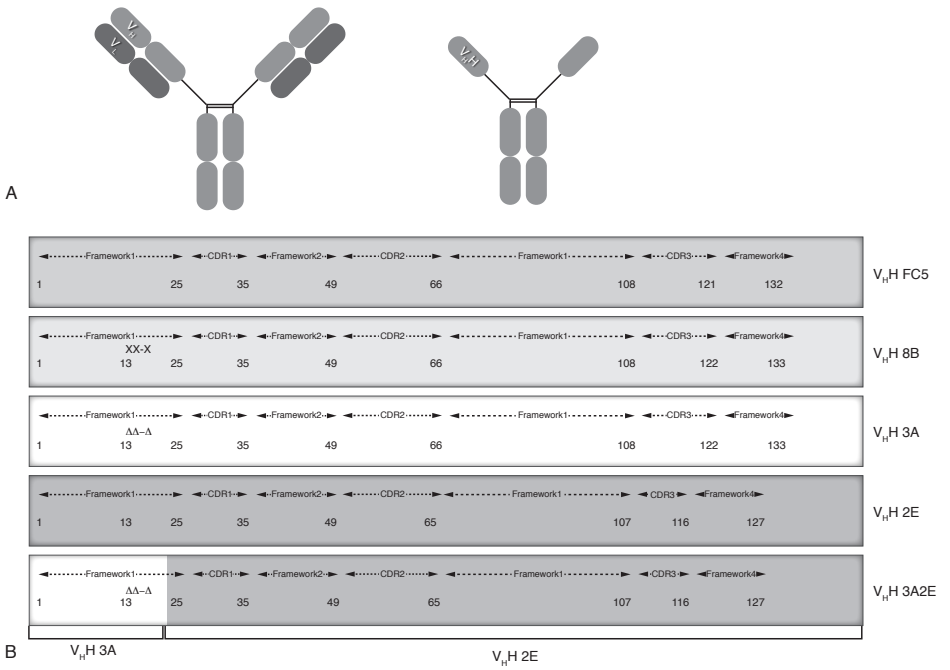

Figure 8.1 Overview single and chimaeric V_HH constructs
(**A**) Schematic representation of a conventional IgG with two heavy and two light chains (*left*) and a Camelid heavy chain IgG (*right*). The variable domains, including the V_HH domains used in this study are indicated. (**B**) Schematic representation of the V_HHs used for the different experiments. First (top to bottom) the monovalent V_HHs FC5 (*grey*), 8B (*less grey*), 3A (*white*), and 2E (*dark grey*) are shown. 8B and 3A differ 3 amino acids in framework 1, 13, 14 and 16, depicted with XX-X and ΔΔ-Δ, respectively. 2E is an unrelated V_HH that differs from ni3A and ni8B. At the bottom the chimaeric V_HH 3A2E, used to investigate the influence of the three unusual amino acids (ΔΔ-Δ) of 3A on the transport, is depicted. 3A2E consists of amino acids 1-16 of 3A and amino acids 17-127 of 2E.

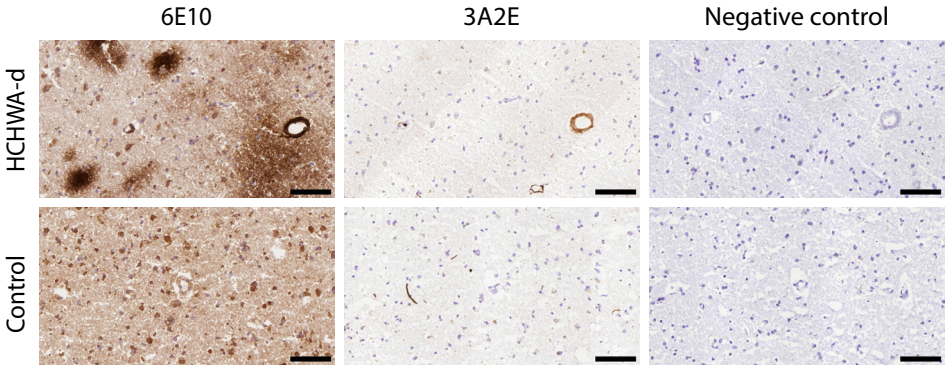

Figure 8.2 Immunohistochemistry using chimaeric V_HH 3A2E
Shown are adjacent neocortical cryosections of confirmed AD, HCHWA-D and control subjects stained with a commercial anti-Aβ antibody (6E10), chimaeric 3A2E or its negative control. 3A2E retains its functionality of recognizing preferentially vascular Aβ. On adjacent sections 6E10 showed the presence of large parenchymal deposits, only partially detected by 3A2E within the AD subject and completely lacking upon HCHWA-D tissue. Both control and negative control staining showed no non-specific staining patterns. Scale bar = 100 μm.

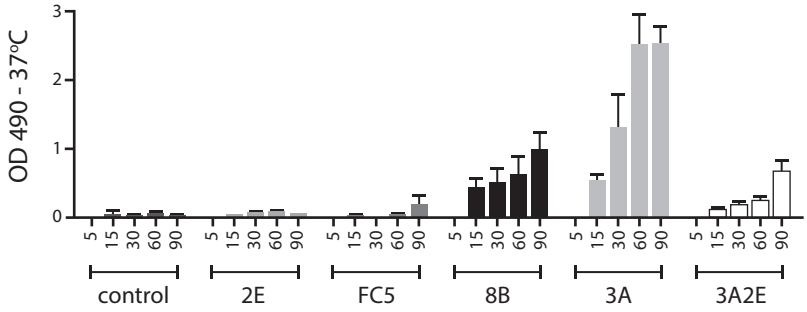

Figure 8.3 Transmigration of V_HHs across the in vitro BBB system
Transmigration of the control (SNCA) protein and V_HHs 2E, FC5, 8B, 3A and the chimaeric V_HH 3A2E at 37°C. Measurements were taken 5 – 15 – 30 – 60 – 90 minutes after addition of the V_HH samples to the system. Data is represented as OD490 at consecutive time points. *=p<0.05 compared to the control, trending p values are noted in the graph. V_HH 8B and V_HH 3A demonstrate significant transport across the *in vitro* BBB. In addition, the chimaeric V_HH 3A2E shows transport relative to the control and native 2E V_HH.

the C-terminus of va2E and therefore also contains these unusual amino acid residues. V_HH va2E also recognizes Aβ but was selected from a different phage display library.[10] After production in *E. coli* the pure V_HH were isolated from the periplasmic fraction and purified by immobilized metal affinity chromatography on Talon beads. An overview of the single and chimaeric V_HH constructs is shown in **Figure 8.1**.

Immunohistochemistry

Frozen brain tissue sections of AD, DS and HCHWA-D patients showed specific Aβ staining of 3A2E indicating that the Aβ recognizing propensity of va2E was preserved in this chimaeric molecule (HCHWA-D and control sections are shown in **Figure 8.2**). The specificity of the Aβ staining was confirmed by the absence of staining in control brain tissue sections and an identical staining pattern was obtained with, the commercially available Aβ antibody, 6E10 in all patients. In contrast to 6E10, only vascular staining was observed with 3A2E, equal to ni3A and ni8B, as previously described.[10]

In vitro blood-brain barrier transport

V_HH ni3A, ni8B and FC5 were assessed for their ability to transmigrate across the BBB *in vitro*. While FC5 was previously described to cross the BBB as it was selected for this propensity, we didn't anticipate that ni3A showed higher transmigration velocity than FC5.[8] Yet, the transmigration rate of ni3A and ni8B was significantly higher compared to FC5 (respectively p<0.01 and p=0.02). (**Figure 8.3**)
Although V_HH ni3A differs by 3 amino acids in its N- terminus from ni8B but has furthermore identical CDRs, a significantly higher transmigration velocity was observed by ni3A (p<0.05). In order to elucidate the capacity of ni3A to cross the BBB system with high efficiency, two additional V_HH were tested: V_HH va2E and chimaeric V_HH 3A2E. The difference in crossing efficiency between va2E and the unrelated control protein of similar molecular weight (SNCA) did not reach statistical significance (p=0.22). Chimaeric V_HH 3A2E has the same FR1 as ni3A

and the 2E-specific C-terminus. Chimaeric V_HH 3A2E displayed a significantly higher transmigration rate compared to 2E (p=0.002), indicating that the 3 amino acids subtracted from ni3A facilitate the crossing efficiency of V_HH. At 4°C there was no significant passage of the V_HH ni3A, indicating that the transmigration of ni3A is by active transport. (**Figure 8.4**) Time as well as temperature was a significant determinant of transport speed, as was determined by 2-way ANOVA (time p=0.01 and temperature p<0.01, respectively), confirming the temperature dependency of the transport of V_HH ni3A. In all experiments TEER was measured to rule out paracellular BBB permeability.[9]

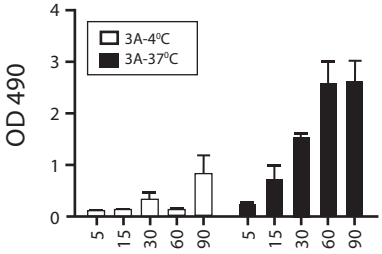

Figure 8.4 Energy-dependence transmigration of ni3A across the in vitro BBB
Measurements were taken 5 – 15 – 30 – 60 – 90 minutes after addition of the samples to the system at 4°C and 37°C. Data is represented as OD490 at consecutive time points. *=p<0.05 compared to the control, trending p values are noted in the graph. Transport of 3A is time and temperature dependent, as determined by 2-way ANOVA. Significance of the separate time points was determined by Bonferonni post hoc test.

Discussion

The availability of effective treatment for neurodegenerative disorders like AD and CAA is currently lacking but dearly needed. This lack of available therapy is partly based on our poor understanding of the pathogenesis of these diseases. The absence of reliable and sensitive biomarkers that permit studying cerebral amyloidosis in its early phases has certainly contributed to this situation. Cerebral Aβ accumulation and aggregation is one of the primary neuropathologic characteristics of AD and CAA and is assumed to play a key role in the pathogenesis of these diseases. Consequently Aβ is considered to be an important biomarker of cerebral amyloidosis.[3,4] Recently, molecular imaging techniques have been developed that permit detection of brain amyloidosis *in vivo*. Currently, positron emission tomography (PET) using amyloid-binding radiotracer compounds are being used for detecting cerebral amyloidosis in patients worldwide.[14-16] However, these agents detect both vascular and parenchymal Aβ, and are thus not well suited to differentially recognize AD and CAA. Therefore, the development of imaging moieties that can be used to differentiate between vascular and parenchymal amyloid in living patients could help detecting CAA and/or AD *in vivo*.

Previously we reported V_HH which differentially recognize vascular and parenchymal Aβ deposition.[10] In this study we assessed the ability of these recently selected V_HH for their ability to serve as imaging moieties and vehicles for tissue targeting. These V_HH have the potential to serve as delivery vectors for efficient local drug targeting and for diagnostic purposes. It is believed that V_HH have advantages to serve as imaging agents and/or transport moieties behind the BBB as they may overcome some of the current hurdles to transmigrate over the regulated interface created by the BBB.[7,17] In this study, an established *in vitro* co-culture model of the

BBB was used to test different Aβ recognizing V_HH for their ability to cross the BBB. Previously it was shown that in order to acquire a high quality *in vitro* co-culture model of the BBB one needs an optimal isolation method of bovine brain capillaries, and specific culture procedures for primary bovine BCEC and rat-astrocytes.[9] The co-culture systems used in this study are superior to other transport systems with respect to paracellular permeability and the maintenance of influx and efflux.[18]

As V_HH FC5 was previously reported to cross the BBB *in vitro*, we compared the crossing efficiency of FC5 to V_HH ni3A selected against Aβ. To our great surprise, ni3A showed higher transmigration efficiency than FC5 at 37°C. It was reported previously that transport of FC5 across the *in vitro* BBB was by uptake and transcytosis via clathrin-coated vesicles, via a temperature and energy dependent receptor-mediated endocytosis (RME).[19] The first step towards elucidation of the mechanism behind the ni3A transport was to test the energy dependence of this transport. An experimental observation that would support active transport is temperature dependence because energy consumption and active transport processes are minimal at 0–4°C while functioning normally at 37°C.[20] Although energy dependence can also be observed for passive diffusion[21,22], the temperature dependency by which a compound will diffuse through a medium is neglectable according to the formula:

$D=D_o e^{-EA/RT}$ [8.1]

(where *D* is the diffusion coefficient, *Do* is the "frequency factor," EA is the activation energy, R is the gas constant and T is thermodynamic, or absolute, temperature (i.e. in Kelvins)).[23] Thus the influence of temperature on diffusion is minimal since the difference between 4°C (=277 kelvin) and 37°C (=310 kelvin) has a neglect influence on the diffusion coefficient of compounds. Therefore transport of ni3A at 4°C and 37°C was compared to investigate temperature and energy dependence. At 4°C there was barely passage of ni3A. This indicates temperature and energy dependence and suggests that the mechanism of transcellular passage of ni3A is by active transport.

Ni3A passes the system with the highest efficiency compared to all other V_HH tested. Because ni3A is only three amino acids different from ni8B in FR1, and they present different crossing efficiencies, we hypothesized that those amino acid residues could potentially be key for the crossing propensity. It is interesting to note that these three amino acid diversion of ni3A is a special feature that is not present in any germline V_HH gene. In order to test this hypothesis, V_HH 3A2E was constructed, a chimaeric V_HH in which we combined FR1 of ni3A with the carboxyterminus (CDR1-FR4) of va2E. This affinity binder va2E was selected against Aβ from an immune library[10] and showed no crossing ability.

In vitro BBB passage of va2E was significantly and positively facilitated by the replacement of the three ni3A-specific amino acids. Although the unique amino acids in ni3A are not sufficient for efficient transmigration we conclude that they positively affect the transmigration rate. Apparently, a combination of N-terminal amino acid substitutions and other ni3A and ni8B-specific downstream sequences allow ni3A to outperform other V_HH in its transmigration efficacy. In support, chimaeric 3A2E and ni8B, both having only one of these features, show intermediate transmigration efficiencies. The reason for the effect of the three amino acids remains to be elucidated. From numerous studies on enhancement of drug transport, it is known that positively charged amino acids can increase the BBB permeability and transport

into the brain.[24] Ni3A has 18 positively charged amino acids and ni8B 17, this could be a reason for the enhanced transport. Further analysis of the tertiary structure and the interaction between the different molecules is necessary to determine the exact nature of the higher transport rate of ni3A.

In conclusion, in this study V_HH ni3A shows in addition to a high affinity for Aβ, the ability to cross the BBB *in vitro*. This transport is temperature sensitive and facilitated by three unique amino acids. Ni3A has potential as *in vivo* imaging agent and local drug targeting moiety. Further studies will need to focus on its mechanism of transmigration and its application *in vivo*.

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PART TWO | Development of Molecular Imaging strategies

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