Supporting information

Polymeric complements to the Alzheimers disease biomarker β-amyloid isoforms Aβ1−40 and Aβ1−42 for blood serum analysis under denaturing conditions

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Experimental Section

Chemicals

The peptides MVGGVV ($A\beta_{35-40}$), GGVVIA ($A\beta_{37-42}$), GGVVIA ($A\beta_{37-42}$), GLMVGGVV ($A\beta_{33-40}$), GLMVGGVVIA ($A\beta_{33-42}$) were purchased from Genscript (Piscataway, NJ, USA). The synthetic Aβ-peptides (1–38), (1–40) and (1–42) used as standards for the PAGE analysis were purchased from Bachem (Weil am Rhein, Germany), whereas the isoforms (1–37) and (1–39) came from Biosynthan (Berlin, Germany). The synthetic Aβ-peptides (1–40) and (1–42) used to spike the serum, tetrabutylammonium hydroxide (1M in MeOH), methacrylic acid (1), methacrylamide (2), 2-trifluoromethacrylic acid (3), N,N-diethylaminoethylmethacrylate (4), 2-hydroxyethyl methacrylate (5), 4-vinylpyridine (6), N-*t*-butylmethacrylate (7) pentaerythritol triacrylate (PETA), divinylbenzene (DVB), and ethylene glycol dimethacrylate (EDMA) were purchased from Sigma-Aldrich (Steinheim, Germany) whereas functional monomer (8) (N-(2-aminoethyl)methacrylamide hydrochloride) was purchased from Polysciences Inc (Eppelheim, Germany). The monomers were purified prior to use as follows: Monomers 1 and 5 were distilled under reduced pressure; EDMA was washed consecutively with 10% NaOH, water and brine, then dried over $MgSO_4$, filtered and distilled under reduced pressure. All the other monomers were used without further purification.

The crosslinker N,O-bismethacryloyl ethanolamine (NOBE),[S1] and functional monomer 10 (N-3,5-bis(trifluoromethyl)-phenyl-N'-4-vinylphenylurea)[17] were synthesized as reported in the literature. The initiatior *N*,*N*′-Azo-bis-(2,4-dimethyl)valeronitrile (ABDV) was purchased from Wako Chemicals (Neuss, Germany). Acetic anhydride analysis grade, was purchased from Merk KGaA (Darmstadt, Germany). The base 1,2,2,6,6-pentamethylpiperidine (PMP) was purchased from Fluka (Buchs, Switzerland) Anhydrous dimethylsulfoxide (DMSO), anhydrous acetonitrile (ACN), methanol (MeOH) of HPLC grade and ACN of HPLC grade, were purchased from Acros (Geel, Belgium).

Template synthesis

The peptide, ($A\beta_{37-42}$ or $A\beta_{35-40}$) (100 mg) was dissolved in the minimum amount of DMSO by adding aliquots of 100 µl. After the addition of NH_4HCO_3 to a concentration of 50 mM, 1.2 equivalents of acetic anhydride in MeOH were added drop-wise and the mixture stirred for 24 h. The reaction was monitored with the ninhydrin test in solution and by reversed phase HPLC (vide infra). After solvent evaporation, the product was dried under vacuum, washed with small fractions of cold water and lyophilized to offer the product as a yellow ($A\beta_{37-42}$) or white ($A\beta_{35-40}$) solid of ca 95% purity as estimated by HPLC. To the acetylated hexapeptide product ($AcA\beta_{35-40}$ or $AcA\beta_{37-42}$) an equimolar amount of tetrabutylammonium hydroxide (TBAOH) (1M) in MeOH was added followed by isolation of the salt by drying under vacuum at room temperature.

Combinatorial MIP library

The functional monomers shown in Figure 1 were used to create MIP/NIP libraries according to the design layed out in Tables S1 and S2 following a previously reported procedure.[16] The composition of each MIP was: template (0.8 µmol); functional monomer (80 µmol); crosslinker (400 µmol); initiator (ABDV) (4.8µmol) and porogen (210µL). Nonimprinted polymers (NIPs) were prepared leaving out the template. Library 1 (Table S1) was prepared using the acetylated epitope AcGGVVIA (T1) as template and DMSO as porogen, whereas in Library 2 (Table S2) the template consisted of the TBA salt of T1 (T2) using ACN/DMSO (65/35) as porogen. In Library 1 (Table S1) the base, PMP was added in columns 8, 9 and 10 (1 equivalent of the template). In Library 2 (Table S2) the urea monomer (10) was added in all the wells (1 equivalent of the template). Stock solutions of the components (monomers, initiator, template) were transfered into 96-well PTFE microtitre plate using a 4-port liquid sample handler LISSY from Zinsser Analytic (Frankfurt, Germany). Monomers 9 and 10 displaying limited solubility were transferred as solids to the respective wells. Prior to pipetting the solutions were purged with nitrogen for 5 min. Each pipetting step was accompanied by purging the well with Argon for 5 s. The plates were sealed with a PTFE coated closures obtained from Radleys (Shire Hill, Saffron Walden, Essex, UK). The polymerization was then initiated thermally at 40°C and after 24h the polymers were cured at 60°C for a

further 24h. The plates were then placed in the vacuum oven at 50°C for 48h to evaporate most of the porogen. The polymers were then weighed, crushed, and transferred into a 96 well Captiva 0.45µm filter plate (Varian, NL) and washed using the automated pipetting station with consecutive steps of mixture of MeOH, MeOH with TFA (1%) and water.

Prior to the peptide incubation experiment, the library was washed with water and conditioned with the buffer used for the test. A solution (800 µl) of Aβ37-42 (H-GGVVIA-OH) (100 µM) in Hepes buffer (1 mM, pH=7) was added to each well and the plate left equilibrating for 24h. The load of peptide (100 µM) corresponds to 10% of the maximum concentration of binding sites assuming quantitative template removal. After filtration, the concentration of free nonbound peptide was determined by reversed phase HPLC analysis, using MeOH/water (35/65) (0.1% TFA) as mobile phase, injecting 100 µl of sample with a DAD set at 205 nm. The amount of peptide adsorbed by the polymeric particles was determined by the following formula:

$$q = \frac{(C_0 - C)v}{m} \quad (1)$$

where q (µmol of peptide/g of polymer) is the amount of peptide adsorbed per gram of polymer, C_0 (mM) is the initial peptide concentration, C (mM) is the final protein concentration in the supernatant, V (mL) is the total volume of the adsorption mixture, and m is the mass of polymer in each well. The imprinting factor (IF) was calculated as the ratio $IF=q_{MIP}/q_{NIP}$. After the first binding experiment the plate was washed with MeOH/water/AcOH (80/5/15) and MeOH/water (50/50), until the template could no longer be detected in the washing solution, by reversed phase-HPLC. The plates were thenerafter reused in a subsequent binding experiment.

Synthesis of MIP Aβ42 and MIP Aβ40

The MIP complementary to Aβ42 was prepared in the following manner. Ac-GGVVIA (8 mg), N-3,5-bis(trifluoromethyl)-phenyl-N'-4-vinylphenylurea (10) (5 mg), tetrabutylammonium (TBA) hydroxide (1M) in methanol (14µL) and the HCl salt of N-(2-aminoethyl)methacrylamide hydrochloride (8), (235mg), were dissolved in DMSO

(600 µL) and ACN (900µL). Divinylbenzene (DVB) (930mg) and the free radical initiator ABDV (12 mg) were then added. After dissolution, the solution was transferred to a glass tube, cooled to 0 °C and purged with nitrogen for 10 min. The glass tube was then sealed and polymerisation initiated thermally by placing the tube in a water bath set at 50°C. Polymerisation was allowed to proceed at this temperature for 48 h. The tube was then broken and the MIP monolith crushed into smaller fragments. The template molecule was removed through the following sequential washing steps: MeOH (100 mL), MeOH/water (0.1MHCl) (90/10, v/v) (100 mL) and finally MeOH (100 mL). The MIP particles were allowed to equilibrate for *ca.* 6 h with each washing solution, after which the wash solution was decanted off. Thereafter, the resulting bulk polymers were ground and sieved to a final size range of 25-50 µm. Prior to use, they were sedimented using MeOH/water (80/20, v/v) in order to remove fine particles. A non-imprinted polymer was prepared in the same manner, but in the absence of the template molecule. A MIP for the Aβ40 was prepared analogously but using the N-acetylated hexapeptide AcMVGGVV as template.

Analysis of binding affinity and capacity

The polymer particles (10mg) were suspended in GuHCl buffer (4M) (1mL) containing different amounts of Aß 33-42 and Aß 33-40 (0.005–0.2 mM) followed by incubation of the mixtures for 24 h at room temperature. After incubation, the supernatant was collected and analysed by reversed phase HPLC as indicated below to determine the free (F) concentration and the q-values according to equation 1. The binding experiments were carried out in triplicate. Sigma Plot was used for non-linear fitting of the resulting binding isotherms to the Freundlich isotherm model.

Optimized solid phase extraction (SPE) procedure

Solid-phase extraction cartridges (1mL, Varian, Spain), were packed with 20 mg of the Aβ imprinted or the corresponding nonimprinted polymers. The cartridges were equilibrated with 5 mL of Guanidine Hydrochloride (GuHCl 4M) solution, and the sample containing the peptide, dissolved in buffer (GuHCl 4M), was percolated at a constant flow rate of 1 mL min^{-1} with the aid of a peristaltic pump. The cartridges were

washed with 0.5 mL of water/AcN (95:5, v/v) to elute the non specifically retained compounds. Finally the peptides were eluted with 0.3 mL of a solution of 5% of TFA in MeOH. The cartridges were reequilibrated with 10 mL of buffer (GuHCl 4M) before reuse. The elution fractions from the SPE column were directly analysed by reversed phase HPLC.

Analysis of blood serum samples

Serum samples (Sigma-Aldrich, Steinheim, Germany) were fortified with the peptide analytes. Then the samples were diluted with 9 volumes of GuHCl (4M). Diluted samples were incubated 30 minutes at room temperature, prior to solid-phase extraction following the optimized procedure. Non-fortified serum samples were preconcentrated and spiked with the peptide stock solutions for calibration purposes. All the analysis were carried out in triplicate. Absence of carry over effects were verified by blank runs using nonspiked serum samples. The eluted fractions were analysed by reversed phase HPLC (Aβ33-40, Aβ33-42) or by urea-SDS-PAGE/immunoblot analysis (Aβ1-40, Aβ1-42).

Reversed phase HPLC

Chromatographic data were acquired with an HPLC 1100 system from Agilent Technologies that consisted of a quaternary pump, an autosampler and a diode array detector. Chromatographic separations of the peptides were performed using a Luna C18 (155 mm× 4.6mm I.D., 5µm) HPLC column protected by a C-18 guard column (4.0mm×3.0mm I.D., 5µm), both from Phenomenex (Torrance, CA,USA) in the gradient mode with a mobile phase composition resulting from combining solvent A (Methanol:water (0.1% TFA) 20:80) and solvent B (Methanol: water (0.1% TFA) 80:20) as follows: 30% B, (2min), 30–60% B, (8 min), 60% B, (15min). The flow rate was 1 mL/min and the column temperature was kept at room temperature 25° C. The injection volume was 100µL, and all the compounds eluted within 16 min. The diode array detector wavelength was set at 205nm for all peptides. Quantification was performed using external calibration peak area measurements. Linear calibration graphs were obtained in the 0.5–50 mg/L range for all the peptides ($r^2 > 0.999$).

Urea-SDS-PAGE/immunoblot analysis

The separation was performed by urea–SDS-PAGE on 10% T/5% C gels as described before.[19,S2] In brief: gels were subjected to semi-dry blotting onto Immobilon-P PVDF membranes (Millipore) at 1 mA/cm^2 for 45 min. The PVDF membranes were then boiled for 3 min in PBS in a microwave oven to improve antibody binding . For immunostaining the monoclonal Aβ-antibody 1E8 (Nanotools, Germany) was used. The blots were finally developed with ECL-plusTM chemiluminescent substrate according to the manufacturer's instructions (GE Healthcare) and visualized with an Intas Imager (Intas, Göttingen, Germany). The amounts of the Aβ-peptides on the blots were estimated with the Quantity One Software 4.6.5 (BioRad, Munich, Germany) in comparison with a five-step dilution series of a synthetic Aß peptide mix.

References

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Table S1. Design of 96-well plate Library 1 using T1 as template and DMSO as solvent for finding polymers with affinity for the C-terminus of Aβ1-42.

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P1	1	2	3	4	5	6	7	8	9	10	11	12
A	EDMA M1	EDMA M2	EDMA M3	EDMA M4	EDMA M5	EDMA M6	EDMA M7	EDMA M8-PMP	EDMA M9-PMP	EDMA M10-PMP	EDMA M1	EDMA M1-M5
B	NOBE M1	NOBE M2	NOBE M3	NOBE M4	NOBE M5	NOBE M6	NOBE M7	NOBE M8-PMP	NOBE M9-PMP	NOBE M10-PMP	NOBE M1	NOBE M1-M5
C	PETRA M1	PETRA M2	PETRA M3	PETRA M4	PETRA M5	PETRA M6	PETRA M7	PETRA M8-PMP	PETRA M9-PMP	PETRA M10-PMP	PETRA M1	PETRA M1-M5
D	DVB M1	DVB M2	DVB M3	DVB M4	DVB M5	DVB M6	DVB M7	DVB M8-PMP	DVB M9-PMP	DVB M10-PMP	DVB M1	DVB M1-M5
E	EDMA M1	EDMA M2	EDMA M3	EDMA M4	EDMA M5	EDMA M6	EDMA M7	EDMA M8-PMP	EDMA M9-PMP	EDMA M10-PMP	EDMA M1	EDMA M1-M5
F	NOBE M1	NOBE M2	NOBE M3	NOBE M4	NOBE M5	NOBE M6	NOBE M7	NOBE M8-PMP	NOBE M9-PMP	NOBE M10-PMP	NOBE M1	NOBE M1-M5
G	PETRA M1	PETRA M2	PETRA M3	PETRA M4	PETRA M5	PETRA M6	PETRA M7	PETRA M8-PMP	PETRA M9-PMP	PETRA M10-PMP	PETRA M1	PETRA M1-M5
H	DVB M1	DVB M2	DVB M3	DVB M4	DVB M5	DVB M6	DVB M7	DVB M8-PMP	DVB M9-PMP	DVB M10-PMP	DVB M1	DVB M1-M5

Rows A-D correspond to MIPs and rows E-F to NIPs. Columns 1 to 10 correspond to polymers prepared using the different monomers 1 to 10, column 11 to polymers prepared using monomer 1 with 41% cross-linker and column 12 to polymers prepared using a mixture of monomers 1 and 5.

Table S2. Design of 96-well plate Library 2 using T2 as template, ACN/DMSO (65/35) as solvent and in presence of urea monomer 10

P2	1	2	3	4	5	6	7	8	9	10	11	12
A	EDMA M1-M10	EDMA M2-M10	EDMA M3-M10	EDMA M4-M10	EDMA M5-M10	EDMA M6-M10	EDMA M7-M10	EDMA M8-M10	EDMA M9-M10	EDMA M10	EDMA M1-M10	EDMA M1-M5-M10
B	NOBE M1-M10	NOBE M2-M10	NOBE M3-M10	NOBE M4-M10	NOBE M5-M10	NOBE M6-M10	NOBE M7-M10	NOBE M8-M10	NOBE M9-M10	NOBE M10	NOBE M1-M10	NOBE M1-M5-M10
C	PETRA M1-M10	PETRA M2-M10	PETRA M3-M10	PETRA M4-M10	PETRA M5-M10	PETRA M6-M10	PETRA M7-M10	PETRA M8-M10	PETRA M9-M10	PETRA M10	PETRA M1-M10	PETRA M1-M5-M10
D	DVB M1-M10	DVB M2-M10	DVB M3-M10	DVB M4-M10	DVB M5-M10	DVB M6-M10	DVB M7-M10	DVB M8-M10	DVB M9-M10	DVB M10	DVB M1-M10	DVB M1-M5-M10
E	EDMA M1-M10	EDMA M2-M10	EDMA M3-M10	EDMA M4-M10	EDMA M5-M10	EDMA M6-M10	EDMA M7-M10	EDMA M8-M10	EDMA M9-M10	EDMA M10	EDMA M1-M10	EDMA M1-M5-M10
F	NOBE M1-M10	NOBE M2-M10	NOBE M3-M10	NOBE M4-M10	NOBE M5-M10	NOBE M6-M10	NOBE M7-M10	NOBE M8-M10	NOBE M9-M10	NOBE M10	NOBE M1-M10	NOBE M1-M5-M10
G	PETRA M1-M10	PETRA M2-M10	PETRA M3-M10	PETRA M4-M10	PETRA M5-M10	PETRA M6-M10	PETRA M7-M10	PETRA M8-M10	PETRA M9-M10	PETRA M10	PETRA M1-M10	PETRA M1-M5-M10
H	DVB M1-10	DVB M2-M10	DVB M3-M10	DVB M4-M10	DVB M5-M10	DVB M6-M10	DVB M7-M10	DVB M8-M10	DVB M9-M10	DVB M10	DVB M1-M10	DVB M1-M5-M10

Table S3. Fitting parameters, weighted average affinity (K) and total number of binding sites (Q) obtained by fitting the binding isotherms in Figure S5 to the Freundlich model.

	Guest	*K* (mM^{-1})	Q ($\mu mol\ g^{-1}$)	*n*	r^2
MIP Aß42	Aβ33-42	89 ± 6	4.7 ± 0.2	0.65 ± 0.04	0.994
NIP	Aβ33-42	70 ± 5	1.8 ± 0.1	0.66 ± 0.03	0.998
MIP Aß40	Aβ33-40	154 ± 9	4.8 ± 0.4	0.82 ± 0.05	0.994
NIP	Aβ33-40	94 ± 6	0.9 ± 0.1	0.92 ± 0.1	0.990

n= heterogeneity index, r^2= regression coefficient

Table S4. Physicochemical properties of the epitope imprinted polymers

Polymer	Elemental compositions[a]			S[b] (m^2/g)	Swelling[c] (v/v)	Swelling[c] (v/v)
	%C	%H	%N		ACN:GuHCl (5:95)	DMSO
MIPAβ42	79.15	7.59	2.69	0.4	1.42	1.33
MIPAβ40	79.07	7.70	2.67	0.7	1.41	1.30
NIP	78.48	7.89	2.55	3.6	1.36	1.25

a) The elemental composition of the polymers assuming full incorporation of the monomers are: C: 83.80%, H: 7.92%, N: 2.41%.

b) The Brunauer-Emmett-Teller (BET) specific surface area.

c) Volume swelling factor

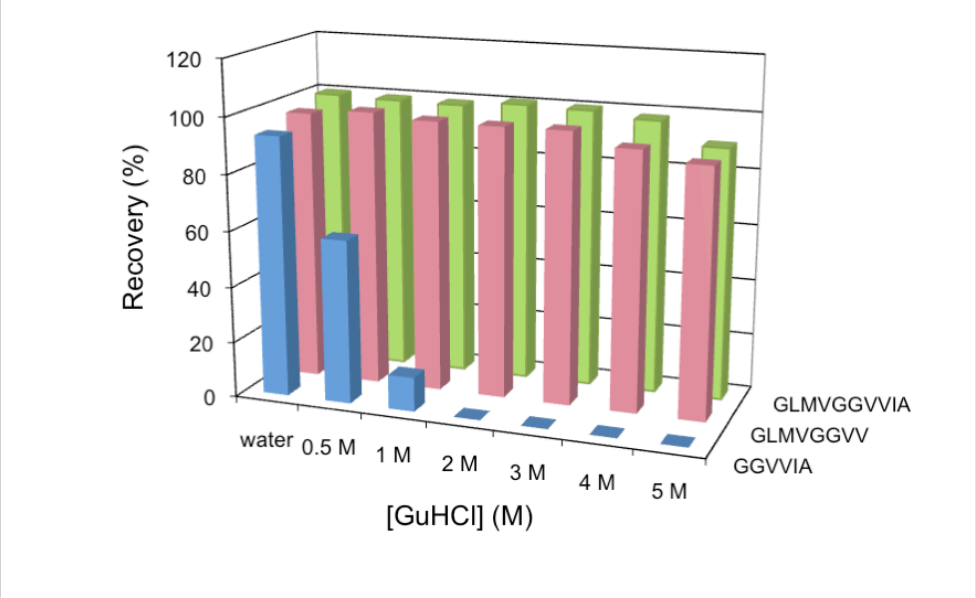


Figure S1. Recoveries (n=3) obtained for the epitope sequences on MIP Aβ42 (P8) using a solution of guanidine hydrochloride (GuHCl) at the indicated concentrations as loading solvent. Conditions otherwise as in Figure 2c,d with no intermediate washing step.

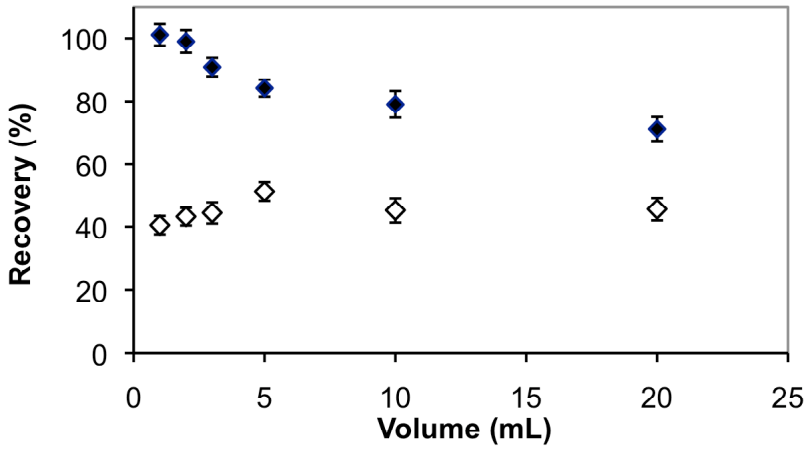
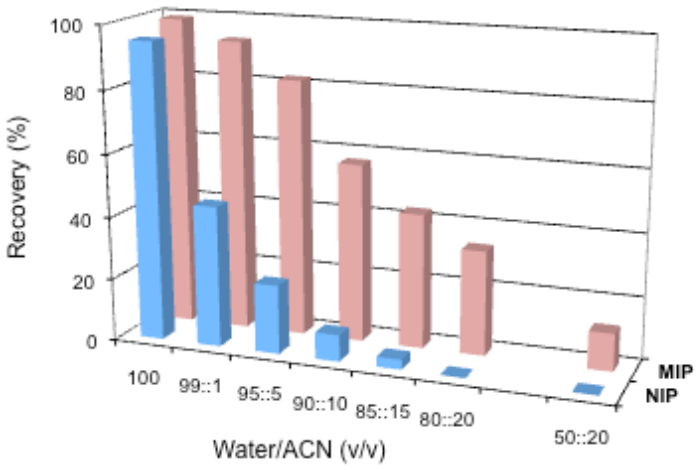


Figure S2. Recovery versus load volume of GMLVGGVVIA (closed symbols) and GMLVGGVV (open symbols) (100 µg/L) dissolved in GuHCl (4M) buffer after percolation through MIP Aβ42 (20 mg).

A)



B)

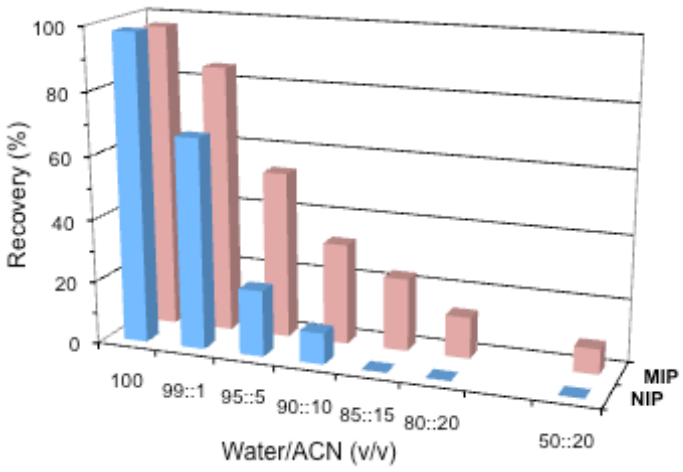


Figure S3. Recoveries obtained on MIP Aβ42 (P8) and the corresponding NIP for (a) GLMVGGVVIA and (b) GLMVGGVV after percolation of 5 mL of a peptide mixture (1mg/L of each peptide) in GuHCl (4M) followed by one single washing step (0.5mL) with the indicated solvent mixture. Elution was performed using 0.3mL of MeOH (5% TFA). The samples were analyzed in triplicate.

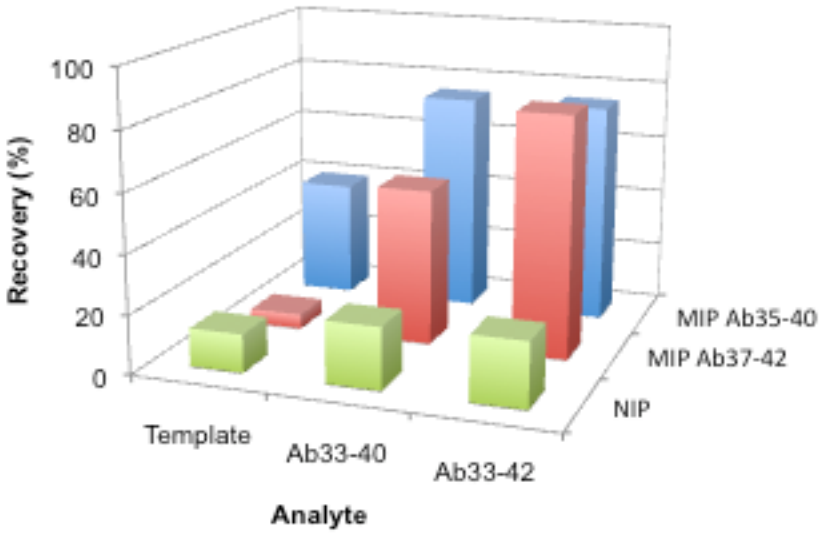
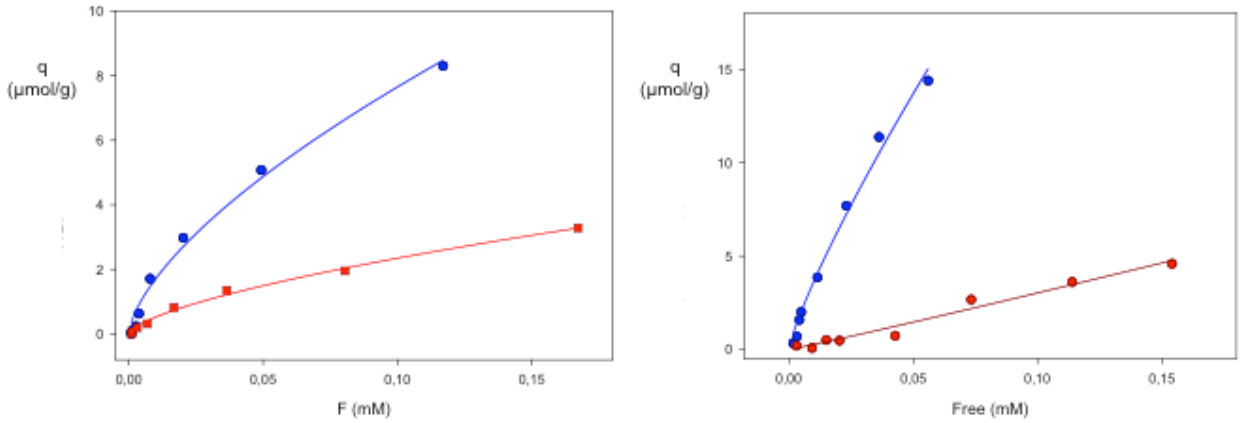


Figure S4. Recoveries obtained on MIP Aβ42, MIP Aβ40 and the corresponding NIP after percolation of 5 mL of a peptide mixture (1mg/L of each peptide) containing the template (GGVVIA), Aβ33-40 (GLMVGGVV) and Aβ33-42 (GLMVGGVVIA) (a) GLMVGGVVIA in buffer GuHCl (4M)/ACN: 95/5 (v/v) and one single wash with 0.5mL of GuHCl (4M)/ACN: 95/5 (v/v). The samples were analyzed in triplicate**.**



A. B.

Figure S5. Equilibrium binding isotherms for the uptake of Aß33-42 (A) and Aß33-40 (B) by MIPs (blue line) (using Aß 37-42 (A) and Aß 35-40 (B) as templates) and NIP (red line) in GuHCl (4M)/ACN: 95/5 (v/v). F=concentration of the free solute, q=specific amount of bound solute. The binding constants (K) and specific number of binding sites (Q) were obtained from Freundlich fitting (Table S3). Each data represents the average of two replicate measurements with a coefficient of variation in the range of 4.2-6.6%.

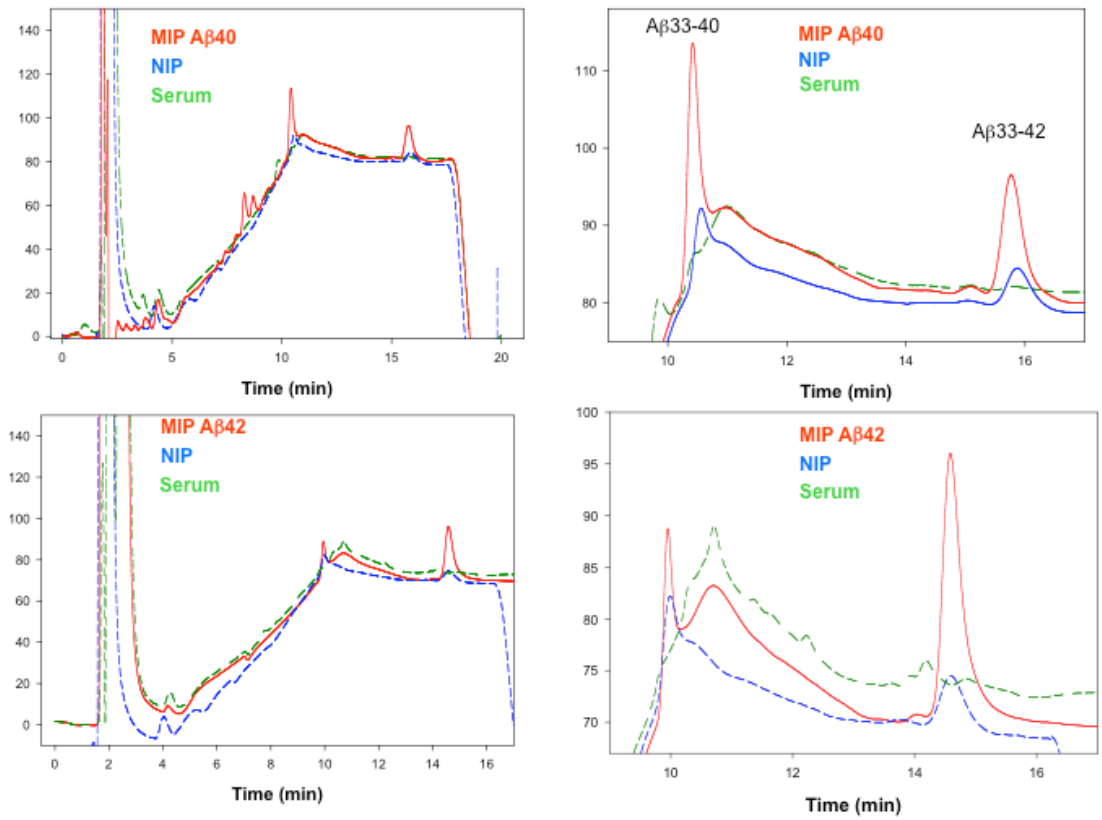


Figure S6. HPLC-UV chromatograms of elution fractions after SPE of Aβ33-40 (GLMVGGVV) (peak at ca 10min) and Aβ33-42 (GLMVGGVVIA) (peak at ca 15min) from spiked serum samples (2.5µg/mL) on MIP (red trace) and NIP (blue trace) as indicated. The green profile represents a blank serum sample. The analysis was performed in triplicate.

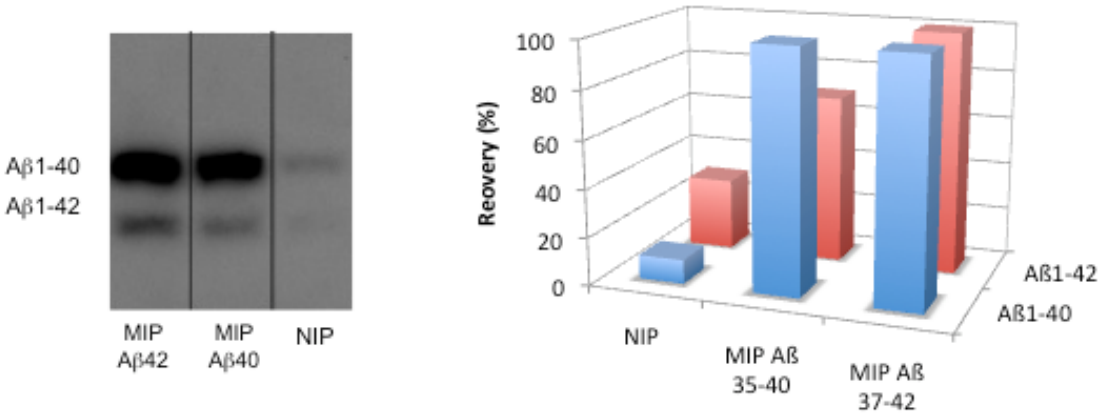


Figure S7. Results from urea SDS-PAGE/immunoblot analysis of elution fractions after SPE of Aß1-40 (0.5mg/L) and Aß1-42 (0.1mg/L) spiked in GuHCl (4M) buffer.