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Polymeric Complements to the Alzheimer's Disease Biomarker β-Amyloid Isoforms Aβ1−40 and Aβ1−42 for Blood Serum Analysis under Denaturing Conditions

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Supporting Information

ABSTRACT: Treatment of Alzheimer's diesease (AD) is plagued by a lack of practical and reliable methods allowing early diagnosis of the disease. We here demonstrate that robust receptors prepared by molecular imprinting successfully address current limitations of biologically derived receptors in displaying affinity for hydrophobic peptide biomarkers for AD under denaturing conditions. C-terminal epitope-imprinted polymers showing enhanced binding affinity for Aβ1−42 were first identified from a 96-polymer combinatorial library. This information was then used to synthesize molecularly imprinted polymers for both of the β-amyloid (Aβ) isoforms and a corresponding nonimprinted polymer. A solid-phase extraction method was developed to be compatible with sample loading under conditions of complete protein denaturation. This resulted in a method capable of quantitatively and selectively enriching a shorter C-terminal peptide corresponding to the sequences Aβ33−40 and Aβ33−42 as well as the full-length sequence Aβ1−40 and Aβ1−42 from a 4 M guanidinum chloride solution. Application of the method to serum allowed selective, high-recovery extraction of both biomarkers at spiking levels marginally higher than clinically relevant concentrations found in cerebrospinal fluid.

Protein detection is heavily dependent on the use of biologically derived affinity reagents (e.g., antibodies or antibody fragments, receptors, or aptamers), mainly because of their ability to effectively recognize and bind their targets in competitive media.[1] In spite of these benefits, these binders either can be hard to obtain because of high costs or poor availability or may not be amenable to robust analytics because of limited stability. Such problems have partly hampered the development of diagnostic methods of neurodegenerative disorders such as Alzheimer's disease (AD).[2,3] Among the identified biomarkers that possibly could reliably predict the disease, the various forms of the peptide β-amyloid (Aβ), and in particular the ratio of the two isoforms, Aβ42/Aβ40, has been found to have the highest diagnostic potential.[4] However, in contrast to cerebrospinal fluid (CSF), the accurate quantification of Aβ1−42 in human biological fluids such as blood is difficult because of very low concentrations (ca. 30 pg/mL) and the masking of Aβ1−42 epitopes with blood plasma proteins or by Aβ oligomerization.[5] Hence enzyme-linked immunosorbent assays (ELISAs) for total Aβ require a predilution step that apart from lowering the target concentration may induce further aggregation, resulting in underestimation of the Aβ level. We here demonstrate a new approach to overcome this problem that makes use of robust, nonbiologically derived receptors in the form of molecularly imprinted polymers (MIPs).[6] The latter are unaffected by antibody-destroying chaotropic agents, allowing affinity enrichment of the amyloid peptides in a fully dissociated form.

Molecular imprinting is used to an increasing extent in the production of synthetic receptors for peptides and proteins.[7−9] Recent promising examples have demonstrated effective mimics of antibodies[10] capable of performing in a biological setting in vivo.[11] The most viable approaches for producing such MIPs are based on the use of epitopes as templates (i.e., a short peptide corresponding to a solvent-exposed, usually terminal sequence of the protein target).[12−14] In contrast to previously published examples involving hydrophilic peptide sequences as templates, the imprinting of the hydrophobic and strongly self-aggregating amyloid peptides have posed new challenges. In order to prepare MIPs for discrimination of the two C-termini of Aβ1−40 and Aβ1−42, we selected the corresponding N-acetylated C-terminal hexapeptide sequences as templates. The C-terminal sequence Aβ34−42 and shorter C-termini exhibit notoriously poor solubility in a variety of solvents, and they precipitate in water to form fibers of aggregated peptide in a stable antiparallel β-sheet conformation.[15] Poor solubility was also confirmed for the acetylated templates, and only a few solvents [e.g., dimethyl sulfoxide (DMSO), *N*,*N*-dimethylformamide (DMF), formic acid] were capable of dissolving the peptides at sufficiently high concentrations for molecular imprinting. DMSO was therefore chosen as the base solvent in the preparation of the first set of MIPs using AcGGVVIA (T1) as the template. Transforming T1 into its tetrabutylammonium salt T2 ($AcGGVVIA^-TBA^+$) enhanced the solubility significantly, and polymerization could then be performed in less competitive solvents, including up to 65% acetonitrile. This was used to prepare a second set of MIPs. Combinatorial imprinting was then used to identify monomer combinations leading to MIPs displaying Aβ affinity.[16] Two

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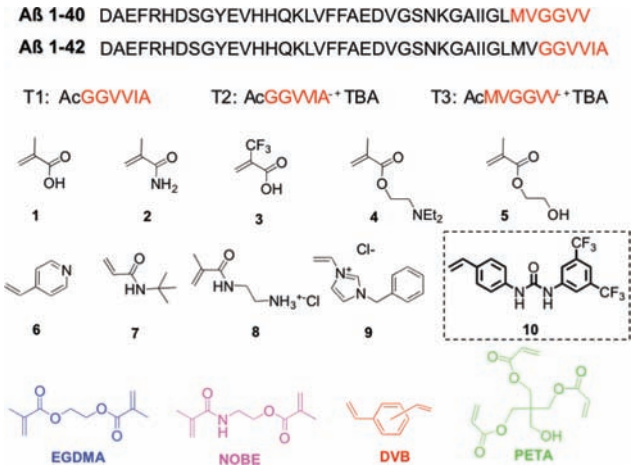

Figure 1. Templates T1−T3, functional monomers, and cross-linkers used to create libraries of Aβ MIPs.

libraries of polymers in the 96-well-plate format were prepared from a number of acidic, basic, positively charged, and neutral functional monomers combined with four different cross-linkers (Figure 1 and Tables S1 and S2 in the Supporting Information). Either T1 or T2 was used as the template, the latter in combination with the 1,3-diarylurea host monomer **10**,[17] which is capable of forming a twofold hydrogen bond to the template oxyanion.

The polymers were freed from the template by washing in methanol and acidified methanol and then subjected to a template-rebinding experiment (template added in free, nonacetylated form) in HEPES buffer (pH 7.5). The diagrams representing the quantity of bound peptide on MIPs versus nonimprinted polymers (NIPs) (Figure 2) revealed the general performance of the individual polymers of the libraries. The polymers prepared using the hydrophobic cross-linker divinylbenzene (DVB) displayed the highest template binding among the cross-linkers tested. For the first library, which was prepared using T1, the binding, although high, appeared to be mainly nonspecific in nature, as shown by the similar Q values for the MIP and the NIP (Figure 2a). This contrasted with the results for the library prepared from T2, where numerous polymers exhibited imprinting factors (IF = Q_{MIP}/Q_{NIP}) exceeding unity (Figure 2b). This is reasonable in view of the fact that these polymers were prepared in a solvent that would compete less effectively with monomer−template interactions and in the presence of the designed host monomer **10**. The six most promising polymers were then carried over to a solid-phase extraction (SPE) cartridge for further comparison of their affinities and selectivities. SPE consists of a loading step, a washing step, and an elution step followed by measurement of the amount of peptide in each fraction. Figure 2c,d shows the recovery of the template peptide (Aβ37−42) as well as the longer sequences Aβ33−42 and Aβ33−40 in the elution step after sample loading in HEPES buffer (pH 7.5) in the absence or presence of 1 M guanidinium chloride (GuHCl).

In absence of GuHCl, all of the polymers retained significant amounts of the peptides, with a slight preference for the longer sequences containing the Aβ42 C-terminus. Whereas the binding of the shorter hexapeptide GGVVIA to all of the polymers was completely suppressed in the presence of GuHCl, one of the MIPs (P8) stood out by retaining nearly all of the longer sequences Aβ33−40 and Aβ33−42 under these conditions. This

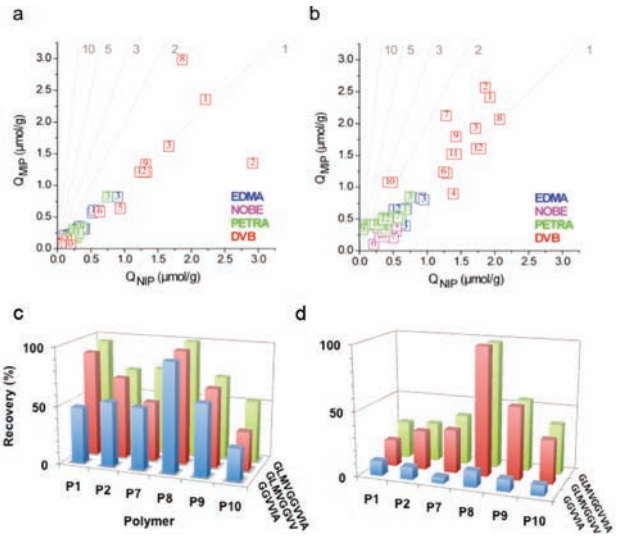

Figure 2. (a, b) Amounts of peptide (Q) bound to polymers from the libraries prepared using (a) T1 and (b) T2 after incubation of the polymers in a solution of H-GGVVIA-OH (100 μM) in HEPES buffer (0.1 M, pH 7.5). The diagonal lines represent the theoretical IF values of 1, 2, 3, 5, and 10). The MIP/NIP couples are represented by numbers 1−12 corresponding to the functional monomers and colors representing the cross-linkers in Figure 1. (c, d) SPE recoveries (n = 3) obtained after percolation of 1 mL of a mixture of the three peptides Aβ33−42 (GMLVGGVVIA), Aβ33−40 (GMLVGGVV), and Aβ37−42 (GGVVIA) (1 mg/L each) through the indicated MIP cartridges followed by elution with MeOH containing 5% trifluoroacetic acid. The peptides were dissolved in HEPES buffer (0.1 M, pH 7.5) in the absence (c) and presence (d) of 1 M GuHCl. No washing step was applied.

MIP was prepared using DVB as the cross-linking monomer and charged monomer **8** combined with urea monomer **10** as functional monomers. The latter monomers can effectively complement the negatively charged template to provide a potentially potent binding site for the C-terminal sequence (Figure 3).

In order to probe whether P8 would bind the peptide under strongly denaturing conditions, the peptides were loaded in the presence of increasing concentrations of GuHCl, after which the recovery upon elution was measured. This showed that the polymer retained the longer-epitope sequences (Aβ33−40 and Aβ33−42) nearly quantitatively at GuHCl concentrations of up to 4 M [recovery values: Aβ33−40, 92% (3% RSD); Aβ33−42, 98% (4% RSD)], whereas binding of the shorter-template sequence (Aβ37−42) was gradually inhibited and 100% disrupted at 2 M GuHCl (Figure S1 in the Supporting Information), the latter most likely due to its more polar character.

Although the recovery of Aβ33−42 slightly exceeded that of Aβ33−40, the binding was dominated by nonspecific contributions. Nevertheless, optimization of the loading and elution volumes (Figure S2) and an intermediate washing step resulted in significantly lower recoveries of the nontemplated peptides [e.g. washing with 5% ACN in water resulted in the following recoveries: Aβ33−40, MIP 53% (5% RSD) and NIP, 21% (7% RSD); Aβ33−42, MIP 82% (6% RSD) and NIP 22% (4% RSD); n = 3] (Figure S3). The fact that the binding preference was reversed for a MIP prepared using template T3 (Figure 1) complementary to the Aβ1−40 C-terminus (Figure S4) provided unequivocal evidence that the polymers contained binding sites complementary to the two C-termini. The binding isotherms recorded in 4 M GuHCl are informative in regard to the affinity of those sites under denaturing

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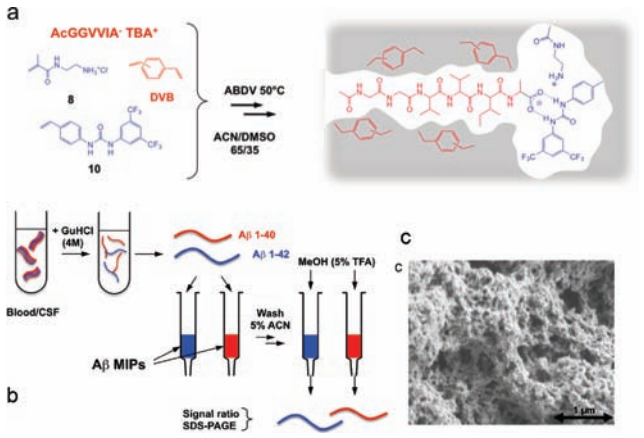

Figure 3. (a) Procedure for preparing P8 for binding of the C-terminus of Aβ33−42 and the hypothetical structure of the imprinted binding site. (b) Principle of affinity SPE under denaturing conditions. (c) Scanning electron microscopy image of MIP Aβ42 (P8).

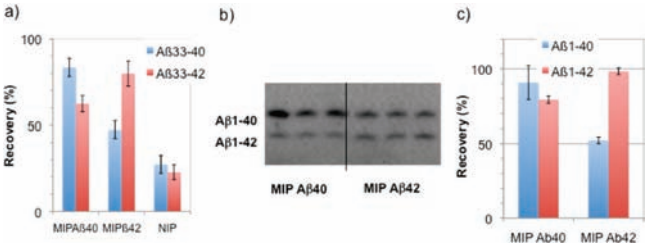

Figure 4. (a) Recovery of the C-terminal epitopes of Aβ in the elution fractions after SPE of peptide-spiked blood serum samples (2.5 μg/mL) on the two complementary MIPs and the NIP. The analysis was performed in triplicate with the RSD indicated. (b) Stained gel from urea-SDS-PAGE/immunoblot analysis of elution fractions from SPE of a blood serum sample spiked with Aβ1−40 (5 ng/mL) and Aβ1−42 (1 ng/mL). (c) Recoveries estimated from spot intensities of the gel in (b) and the associated RSDs.

conditions (Figure S5). These displayed a MIP-characteristic curvature best fitted with continuum models such as that of Freundlich, from which an average binding constant (K) and a specific capacity (Q) can be estimated (Table S3).[18] Thus, both MIPs contain sites with affinities exceeding 10^5 M^{-1}, albeit in different abundances (MIP Aβ42, $Q > 2$ μmol/g; MIP Aβ40, $Q > 4$ μmol/g). The presence of sites exhibiting even higher affinities would be reflected in the ability of the MIP to extract low concentrations of the target peptides (see below).

With this information in hand, the final SPE procedure (Figure 3b) was established and subsequently applied to denatured blood serum samples. Serum samples were thus fortified with each peptide at 2.5 μg/mL, diluted 10 × with GuHCl 4M, incubated, and loaded onto the MIP or NIP cartridges. The elution fractions analyzed by reversed-phase HPLC are shown in Figure S6, and the associated recoveries are plotted in Figure 4a. The recovery of Aβ33−42 was markedly higher on MIP Aβ42 than on the NIP, but more striking is the lower recovery of Aβ33−40 on this MIP versus the NIP. The reverse behavior was observed when the MIP Aβ40 was tested, although with less-pronounced selectivity. These results show that a shorter sequence corresponding to the Aβ1−42 C-terminus can be selectively extracted with high recovery using an epitope-complementary polymer.

In order to probe whether these sites can be accessed by the full-length β-amyloid peptide, we fortified a serum sample with synthetic Aβ1−40 (5 ng/mL) and Aβ1−42 (1 ng/mL) at relative concentrations reflecting those typically found in CSF samples from patients. These samples were subjected to a sample pretreatment identical to that in the preceding example, whereafter the elution fractions were analyzed for the peptides by urea-SDS-PAGE/immunoblot.[19] It was gratifying to observe that the intensity of the stains was correlated with the expected relative enrichments of the peptides (Figure 4b,c). Hence, the elution fraction after SPE on MIP Aβ40 enriched Aβ40 with respect to Aβ42, whereas Aβ42 was quantitatively recovered after percolation through MIP Aβ42 with only 50% of the Aβ40 recovered in this fraction. Much lower recoveries were obtained after percolation through a reference NIP (Figure S7).

Although at the borderline of the detection limit of the PAGE method, similar relative intensities were observed for serum samples spiked at clinically relevant concentrations. The discrimination of the nontemplate sequence is not complete, but the performance is promising given the high recoveries obtained. This effective binding cannot be ascribed solely to a correct placement of complementary functional groups at the templated sites (Figure 3a) but must also depend on the macroporous morphology of the polymer (Figure 3c and Table S4). With most pores exceeding 100 nm, the ca. 4.5 kDa protein can easily access the inner pore system of the MIP particles.

In summary, the combinatorial MIPs identified in this work are potent receptors for both the C-terminal end and the full-length sequence of the β-amyloid isoforms Aβ1−40 and Aβ1−42, distingushing them as the first synthetic polymers capable of this level of discrimination. Their ability to bind these two biomarkers in a programmed way under denaturing conditions is promising with respect to both fundamental studies of peptide aggregation and AD diagnostics.

■ ASSOCIATED CONTENT

Supporting Information. Experimental section reporting procedures for polymer synthesis and characterization; associated figures and tables; and complete refs 1, 3, and 4. This material is available free of charge via the Internet at http://pubs.acs.org.

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