University
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**Faculty
of Chemical Technology**

Development of the magnetic bioaffinity carrier for the anti-amyloid *beta* 1–42 antibodies detection

Marcela Slováková[1]*, Pavla Murasová[1], Věra Vozandychová[1],
Jiří Palarčík[2], Veronika Podzemná[3], and Zuzana Bílková[1]

[1] *Department of Biological and Biochemical Sciences,*
The University of Pardubice, CZ–532 10 Pardubice, Czech Republic

[2] *Institute of Environmental and Chemical Engineering,*
The University of Pardubice, CZ–532 10 Pardubice, Czech Republic

[3] *Center of Materials and Nanotechnologies – CEMNAT,*
The University of Pardubice, CZ–530 02 Pardubice, Czech Republic

In this paper, the development of a new affinity carrier was the key point for the anti-amyloid β 1–42 antibodies detection in human serum, as the potential marker of the Alzheimer's disease. Magnetic solid-phase preparation comprised the amyloid β 1–42 peptide immobilization on the superparamagnetic carboxyl beads that had been modified by bisamino-polyethylene glycol spacer to gain terminal amine groups and subsequently analyzed by the zeta potential measurement. The following immobilization of the amyloid β 1–42 peptide onto the modified superparamagnetic beads was performed by the carbodiimide chemistry. Nonspecific sorption of serum contaminants was prevented by using of bovine serum albumin. The developed magnetic carrier was analyzed by scanning electron microscopy. The amyloid β 1–42 peptide bioaffinity carrier was applied for detection of natural anti-amyloid β 1–42 antibodies in human serum. A comparison with spacer-free bioaffinity carrier has confirmed a divergent optical density values for control serum sample. The new bioaffinity carrier was applied to the detection of anti-amyloid β 1–42 antibodies in control serum and in sera of patients with neurodegenerative disorders.

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* Corresponding author, ✉ marcela.slovakova@upce.cz

Introduction

Development of efficient methods for fast, simple, and selective isolation of specific antibodies of clinical or diagnostic significance still represents a great challenge for researchers. The determination of anti-amyloid β antibodies (anti-Aβ Abs) in biological material is typically carried out by the universal and highly specific enzyme-linked immunosorbent assay (ELISA), where ligand – a specific antigen, is bound onto a surface of microtiter plates [1,2], or by the newly developed thiophilic chromatography [3]. In the recent years, the use of magnetic spherical carrier as the solid phase has also gained popularity in the ELISA systems. The magnetic ELISA brings a high comfort of fast and simple magnetic separation during the individual steps of the respective methods. An increase in the sensitivity of the magnetic ELISA method is directly related to the magnification of specific surface of the spherical particles [4–6]. The selection of suitable magnetic particles for the individual applications depends on their magnetic properties. Thus, the materials from which they are made as well as their size are essential [7]. Magnetic ELISA has been evaluated as accurate, less time-consuming and more sensitive than conventional ELISA [8,9]. Magnetic ELISA combines advantages of using the immunomagnetic beads with classic method ELISA having potential to increase the accuracy and specificity in comparison with usual immunotests [9].

Recently, more and more studies deals with the role of autoimmunity in the pathogenesis of Alzheimer's disease (AD). It has been found that human IgG repertoire contains naturally occurring antibodies to amyloid beta (Aβ), which may play a role in the pathogenesis, as well as in the treatment of AD [10–12]. Aβ peptide is about 4 kDa large molecule and being generated by proteolytic cleavage of amyloid precursor protein in the plasma membrane by β and γ-secretase [13]. This cleavage gives rise to several isoforms of Aβ of which 40/42 hydrophobic amino acid peptide tends to polymerize and form the plaque [14]. Specifically, the N-terminal anti-Aβ Abs were effective in removing plaques because only N-terminal parts could be easily accessible to antibodies [15–18]. Therefore, immunochemical detection of natural anti-Aβ antibodies (nAβ-Abs) in human serum is connected to the therapeutic potential strategy of the Aβ-Abs found in intravenous immunoglobulin (IVIg) for the treatment of patients with AD [11,19–21].

Based on these facts, we had prepared a magnetic bioaffinity carrier composed of amine containing superparamagnetic beads with immobilized Aβ 1–42 peptide and applied the resultant bioaffinity carrier in the pilot experiment for the detection of the N-terminal Aβ-Abs antibodies in human sera.

Materials and Methods

Reagents, chemicals and magnetic microspheres

1-Ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC), the sodium salt of N-hydroxysulfosuccinimide (sulfo-NHS), 2-(N-morpholino)ethanesulfonic acid (MES), bovine serum albumin (BSA) and poly(ethylene glycol) bis(3-aminopropyl) terminated (NH_2-PEG-NH_2) spacer (MW 1,5 kDa) were purchased from Sigma-Aldrich (St. Louis, MO, USA). All the other chemicals were of reagent grade and obtained from Penta (Chrudim, Czech Republic). Water used for preparation of buffers was filtered through a TKA Smart2Pure system (Thermo Scientific TKA, Niederelbert, Germany). Peptide Aβ 1–42 (MW 4,514 kDa) was purchased from Apronex (Vestec, Czech Republic). Human patient sera were provided from the National Institute of Mental Health (Klecany, Czech Republic). All patients, whose samples had been analyzed, signed the informed consent. Control sera were provided by healthy donors – volunteers from the University of Pardubice.

ProMag™ 1 Series COOH Surfactant Free (further denoted as Promag, 0.88 μm) were purchased from Bangs Laboratories (Fishers, IN, USA). Magnetic suspension agitations and separations were performed using a Grant Bio PTR-30 rotator (Wolf-Laboratories, Pocklington, York, UK) and a Dynal MPC-S magnetic separator (Biotech, Prague, Czech Republic).

Preparation of the magnetic immunosorbents

To prepare the immunosorbent, Aβ 1–42 peptide was immobilized to the carboxyl superparamagnetic microparticles by I) two-step carbodiimide method or II) one-step carbodiimide method [22] with the aid of polyethyleneglycol ($NH2$-PEG-$NH2$) as the homobifunctional linker. In both cases, magnetic particles (1 mg) were washed 4 times with a 0.1M MES (pH 4.5) and activated with EDC in 0.25 mL (0.0391 mol L^{-1} in 0.01M MES; pH 4.5) and sulfo-NHS in 0.5 mL (5.76·10^{-3} mol L^{-1} in 0.01M MES; pH 4.5) and incubated 10 min. at room temperature under rotation. Subsequently, magnetic carrier was washed with 1 mL of 0.1M MES (pH 4.5), in case (I) Aβ 1–42 was added (50 μg), filled up to 1 mL with 0.01M MES (pH 4.5), and incubated overnight at 4 °C on a rotator. After overnight incubation, the functionalized particles were blocked with 1 mL 1% BSA. In case (II) $NH2$-PEG-$NH2$ (0.003 mg) was added, filled up to 1 mL with 0.01M MES (pH 4.5), and incubated overnight at 4 °C on a rotator. After overnight incubation particles modified with PEG were washed with 1 mL of 0.1M MES (pH 4.5) and activated again with EDC and sulfo-NHS as described above. Subsequently, magnetic carrier was washed with 1 mL of 0.1M MES (pH 4.5), Aβ 1–42 was added (50 μg) and filled up to 1 mL with 0.01M MES (pH 4.5). After

overnight incubation at 4 °C on a rotator, the functionalized particles were blocked with 1% BSA (1 h, RT, rotation) and washed 10 times with 0.1M PBS buffer (pH 7.4). Storage conditions were as follows: 4 °C, with addition of 0.01% NaN_3.

SEM analysis of magnetic particles

Superparamagnetic microparticles were dried at a temperature of 37 °C and imaged by the SEM instrument JEOL JSM 7500F (JEOL, Tokyo, Japan). To produce an electrically conductive surface for SEM, specimens were sputter-coated with thin gold film (~10 nm) using Leica EM ACE 200 sputter (Leica, Wetzlar, Germany).

Zeta potential measurement

A zeta potential analyzer (Malvern Instruments, Worcestershire, UK) was used in the zeta measurememt protocols. The zeta potential of the beads was measured in a phosphate buffer made 10 and 100 mM in concentration and pH adjusted from 3 to 11 using either HCl or NaOH. The total volume for all the measurements was 1 mL, and the concentration of the beads chosen as $3 \cdot 10^{-3}$ %. Each measurement included 2×12 cycles of uniform measurements and one mixing step to prevent magnetic particles from settling and sedimentation.

Detection of anti-Aβ Abs in serum using the magnetic bioaffinity carrier

100 µL of diluted sera (1:100) was added to 0.05 mg of magnetic particles functionalized with the Aβ 1–42 peptide and the resultant mixture incubated for 1 hour in 37 °C under shaking. Afterwards, the particles were washed 3 times with 1 ml 5% BSA in PBS-T buffer. 100 µL of goat anti-human IgG labeled with HRP and diluted 1:16 000 was added and incubated with particles another one hour at 37°C under shaking. Subsequently, the particles were washed three times with 5% BSA in PBS-T buffer and twice with PBS buffer. 100 µL of substrate solution – 0.1M acetate buffer (pH 5.5), 0.03% H_2O_2 with 0.3 mM 3,3',5,5'-tetramethylbenzidine (TMB; 6 mg mL^{-1} in DMSO) acting as a chromogen was added and incubated for 15 min at 37 °C in dark. The reaction was stopped by adding 50 µL 1M H_2SO_4 and the absorbance measured using a Multiscan RC ELISA reader (Labsystems, Helsinki; Finsko) at a wavelength of 492 nm. The results were obtained in three replicates. All the samples and all three measurements of each sample were used to evaluate the data.

The averages were calculated from the values measured as the replicates. Data showed a violation of data normality, and Kruskal–Wallis test was used for statistical evaluation; P-values ≤0.05 being considered statistically significant.

Results and discussion

Immobilization of the amyloid β 1–42 peptide to magnetic beads

Naturally occurring Aβ-antibodies recognize the Aβ epitope at its N-terminus, amino acids 1–15 [18]. To detect free natural N-terminal Aβ Abs, we immobilized the peptide Aβ 1–42 by the C-terminus to the magnetic carrier. First, the ProMag carboxyl beads were modified by the bisamino-polyethylene glycol (NH_2-PEG-NH_2) spacer, which was followed by the Aβ 1–42 peptide immobilization. The scheme of the Aβ 1–42 peptide immobilized to superparamagnetic beads is in the Fig. 1. C-terminus Amyloid β 1–42 peptide bioaffinity carrier will allow one to detect specifically N-terminal Aβ Abs from human serum. Another great advantage of using a spacer before the peptide immobilization was formation of an affinity ligand binding space near the beads.

Fig. 1 Schema of the Aβ 1–42 peptide immobilization using of the bisamino-polyethylene glycol spacer to the magnetic carboxyl beads

NH_2-PEG-NH_2, bisamino-polyethylene glycol

To prevent nonspecific interaction of the magnetic beads after amine modification, the concentration of the bisamino-polyethylene glycol spacer was optimized. Zeta potential, as a measure of the magnitude of the repulsion or attraction between beads, was measured for each concentration chosen. Its measurement provided insight into the dispersion mechanism.

The results in Fig. 2 showed that the spacer did not significantly reduce the stability of the Promag magnetic particles. Beads modified with the lowest amount of spacer (3 µg) are still well stable and least susceptible to an aggregation from all other bindings of bisamino-polyethylene glycol spacer.
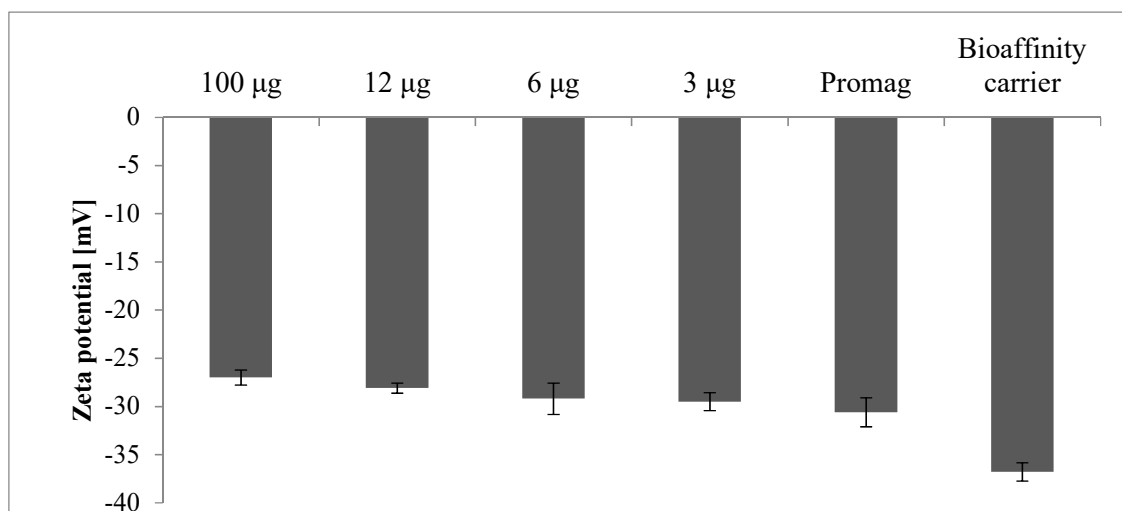

Fig. 2   Zeta potential analysis of Promag beads with different amounts of the bisamino-polyethylene glycol spacer and the Aβ 1–42 peptide bioaffinity carrier in 0.01M PBS buffer pH 7.4

Average of at least 12 repetitions

The magnetic beads functionalization with bisamino-polyethylene glycol spacer was followed by the immobilization of Aβ 1–42 peptide via C terminus by two-step carbodiimide chemistry. Tricine SDS-PAGE analysis confirmed binding efficiency of the Aβ 1–42 peptide to the amine modified magnetic beads (not shown). Further, the bead surface and bead size of the prepared magnetic affinity carrier were analyzed by the scanning electron microscopy to ensure unchanged particle size for use in magnetic ELISA method. Beads featured in Fig. 3A confirmed a spherical shape without hints of surface changes. No change in ligand-bound particles was observed for the magnetic bioaffinity carrier.

The size of the beads before and after preparation of the bioaffinity carrier was then calculated from the SEM images (Figure 3B). Size of the individual beads before its modifications was 0.906 µm (±0.057); acquired bead sizes being close to the bead size stated by the manufacturer (0.88 µm). There was no significant change in size after the modification by the spacer and peptide.

Detection of anti-Aβ antibodies in human serum

The newly developed Aβ 1–42 peptide magnetic bioaffinity carrier was applied to detect natural N-terminal Aβ-Abs in the sample of control human serum (diluted in ratio of 1:100).
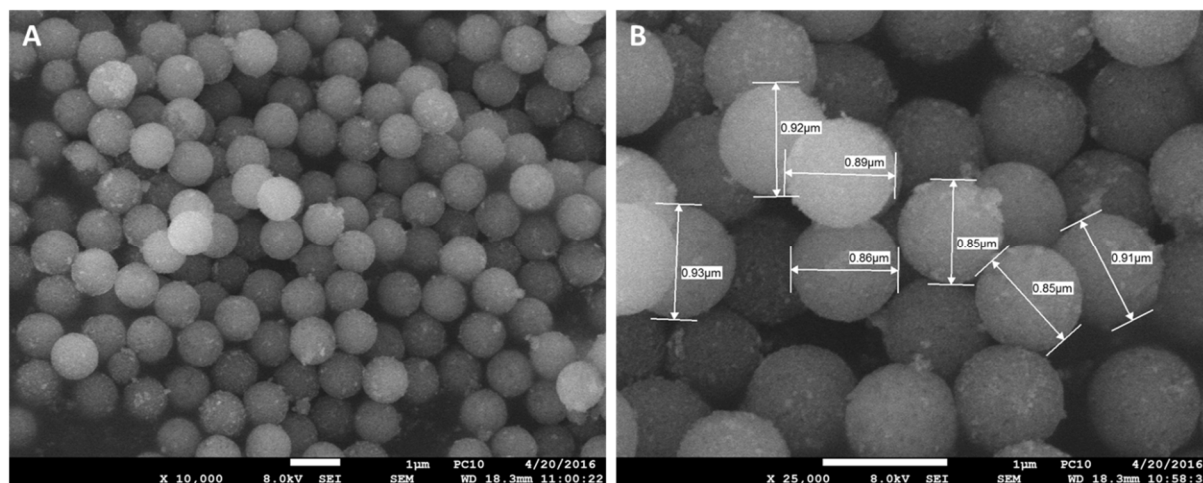

Fig. 3 Scanning electron microscopy of the bioaffinity carrier (A), bead size measurement (B)

To compare this developed system, we prepared the Aβ 1–42 peptide bioaffinity carrier without a bisamino-polyethylene glycol spacer. The spacer free Aβ 1–42 peptide bioaffinity carrier was prepared using the two-step carbodiimide method. Both bioaffinity carriers were blocked with 1% BSA to prevent adsorption of serum proteins to the carrier and simultaneously used for specific antibodies detection. Data, expressed as the mean optical density (OD) values ± SEM, responded to the solution after colorimetric anti-human IgG HRP conjugate development. The detection method and the evaluation have been taken from the previous report [1]. The interpretation of results on the Fig. 4 shows that the greater the OD value of the peptide carrier, the higher level of the anti-Aβ 1–42 Abs can be detected in the human serum.
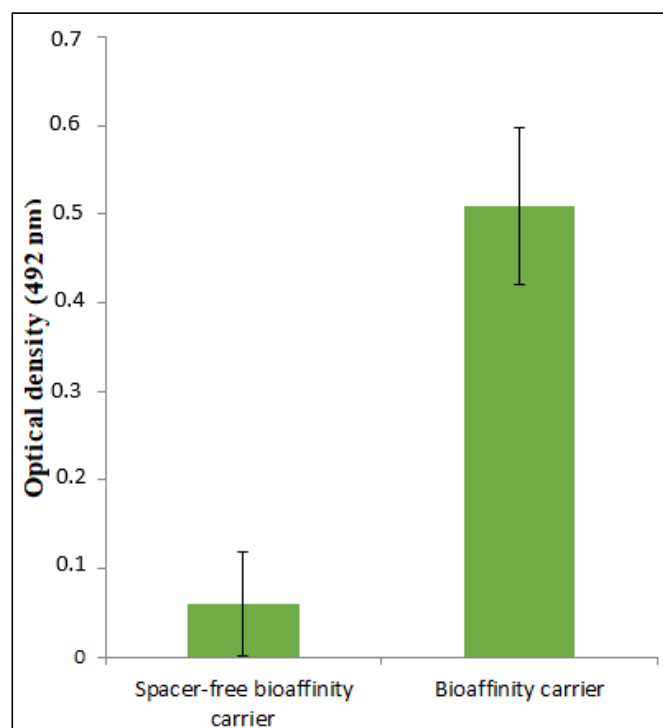
Fig. 4 Influence of the Aβ 1–42 peptide magnetic bioaffinity carrier preparation method to acquired values anti-Aβ Abs detected in control serum

OD values are acquired from three repetitions

Acquired values optical densities have shown that the spacer-free bioaffinity carrier had a significantly lower absorbance than the response of the spacer modified bioaffinity carrier. The peptide binding sites for spacer-free carriers compared to the spacer modified bioaffinity carrier were probably less accessible to anti-Aβ 1–42 Abs. Data suggest a significant positive effect of the bisamino-polyethylene glycol spacer use on the immobilized peptide availability to the specific antibody binding sites. Yet another possible explanation can also be the appropriate N terminal orientation of the immobilized Aβ 1–42 peptide related to the presence of natural anti-Aβ 1–42 Abs in the control serum.

In the next experiment, the Aβ 1–42 peptide magnetic bioaffinity carrier was used for analysis of different human serum samples. Bioaffinity carriers were blocked with 1% BSA. The aim of this pilot experiment was to observe different levels of specific antibodies known from the literature [16,18]. Serum samples included six randomly selected controls and fourteen patient sera from which six had belonged to patients with mixed cognitive impairment (MKP) and eight to the patients suffering from Alzheimer's disease (AD). Patient sera were provided by the National Institute of Mental Health in Klecany (see *Materials and methods*) and the designations of AD and MKP were based on confirmed clinical diagnoses. The sera tested were diluted at a ratio of 1:100.
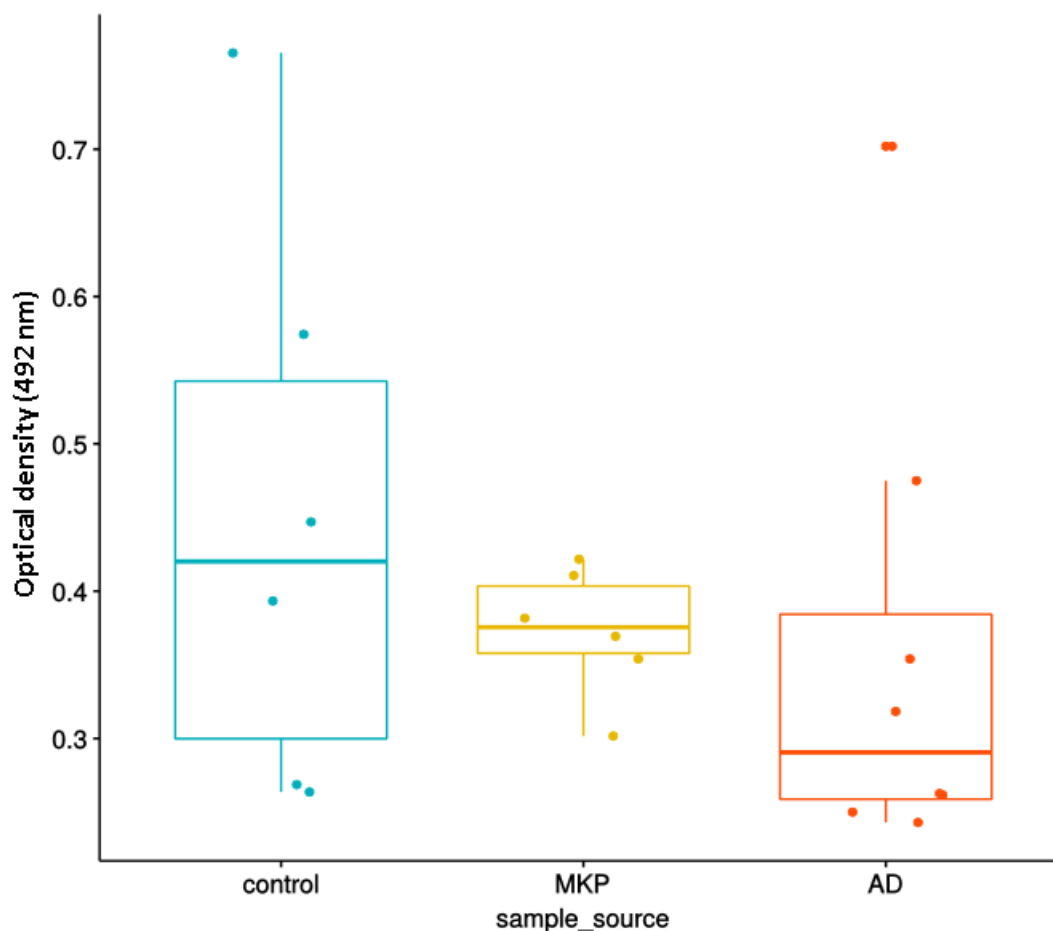
Fig. 5 Box plot of acquired values anti-Aβ Abs detected in control and patients sera using the Aβ 1–42 peptide magnetic bioaffinity carrier

MKP, mixed cognitive impairment, AD, Alzheimer disease, Values are acquired from three repetitions

Values of optical densities have been acquired from three analysis repetitions of each sample and are plotted in Fig. 5. The antibody levels measured in the control sera group showed a large dispersion. Similarly, large dispersion of data was evident from sera from patients with AD. A single group of MKP patient data gave homogeneous results. The developed magnetic bioaffinity carrier used for the anti-Aβ Abs detection was able of detecting anti-Aβ Abs with some differences between the groups of sera. The differences in the acquired data have been found not statistically significant (Kruskal–Wallis, p-value 0.2697). The low number of data and the sera examined did not allow us to evaluate the importance of the anti-Aβ Abs in serum.

Conclusions

A magnetic bioaffinity carrier with the amyloid β 1–42 peptide has been developed and tricine-SDS-PAGE used for verification of the immobilization efficiency. Also, SEM was used for verification of the particle size before and after ligand modification. The concentration of bisamino-polyethylene glycol spacer being necessary during peptide immobilization has been optimized and verified by beads zeta-potential measurements. Properties of the amyloid β 1–42 peptide bioaffinity carrier with spacer have proven to be useful as a solid phase for the detection of anti-amyloid antibodies in human serum. Detection of anti-amyloid beta antibodies in serum controls and patients with AD and MKP in the pilot experiment did not revealed statistically significant differences between the control samples and those obtained from patients with AD.

Shortcuts

Aβ	amyloid beta
Aβ-Abs	anti-A beta antibodies
AD	Alzheimer's disease
ELISA	enzyme-linked immunosorbent assay
HRP	horse radish peroxidase
IgG	immunoglobulin G
MKP	mixed cognitive impairment
OD	optical density

Acknowledgements

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