UNIVERSITÀ DEGLI STUDI FIRENZE

DOTTORATO DI RICERCA IN

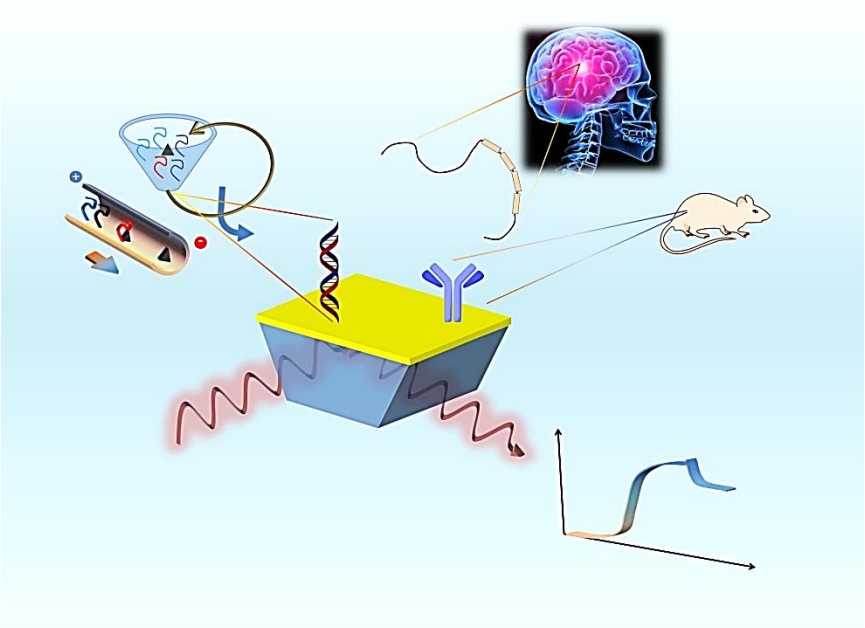
SCIENZE CHIMICHE

Curriculum: Chimica

CICLO XXIX

Coordinatore: Prof. Piero Baglioni

NOVEL APPROACHES BASED ON SURFACE PLASMON RESONANCE BIOSENSOR FOR MOLECULAR DIAGNOSIS OF ALZHEIMER'S DISEASE



Dottorando

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Sede amministrativa del dottorato – Università di Firenze

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Correlatori di tesi di dottorato sono la Dr.ssa Simona Scarano per Università di Firenze, e la Dr.ssa Corinne Ravelet, per l'Università di Grenoble- Alpes / *L'encadrement de la thèse a été effectué par Dr. Simona Scarano à l'Université de Florence, et Dr Corinne Ravelet, à l'Université Grenoble-Alpes.*

A mio nonno, esempio e punto di riferimento che ha contribuito con i suoi pregi e I suoi difetti a fare di me quello che sono. Non sarei mai arrivato fino a qui se non fossi stato testardo e perseverante quanto lo eri tu.

Abstract

Alzheimer's disease (AD) is a widespread pathogenic condition causing memory and behavior impairment mostly in elderlies because of the accumulation of amyloid beta peptide and tau protein in human brain. Current therapeutic approaches, based on the amyloid hypothesis, are unable to arrest the progression of the disease, hence early diagnosis is crucial for an effective intervention. Based on the updated criteria for AD probable diagnosis, and considering the limits associated with the actual analytical techniques, my work in this thesis was dedicated to develop novel strategies for AD diagnosis. The whole project focused on the analysis of tau protein by Surface Plasmon Resonance (SPR) biosensing. Such protein is well known for being relevant as neurodegenerative marker. In particular if the measurement of tau is associated with that of the amyloid beta peptide and that of the phosphorylated tau, the clinical specificity of this protein become significant to detect Alzheimer. Two aspects were studied; first of all an immunosensor was developed taking advantage of the well-established antigen-antibody interaction. After characterization of the analytical parameters of the direct assay (with primary antibody), a sandwich assay (using two monoclonal antibodies mapping on different analyte i.e. protein tau epitopes) was developed, allowing very low sensitivity to be obtained in artificial Cerebrospinal Fluid (aCSF). In particular to enhance the analytical signal Carbon Nano Tubes (CNTs) were used. Secondly, the research was focused on the selection of aptamers for tau. To this aim two SELEX (Systematic Evolution of Ligands by EXponential enrichment) methods were compared, both based on Capillary Electrophoresis (CE) for partitioning step of the process. Whether with CE-SELEX (first method), no

significant affinity improvement was measured, using the CE-Non-SELEX (second method) affinity of the DNA library for tau protein was consistently improved. After isolation of a limited population of aptamer candidates, five sequences were chosen to be analyzed for their affinity for the target. Fluorescence Anisotropy (FA) measurements and SPR highlight similar behavior for the selected sequences, despite the detection principles of these techniques are significantly different. In conclusion the work highlight versatility of SPR technology used both for quantitative analysis and for new selected aptamers characterization in terms of affinity for the analyte tau. The above mentioned versatility is of great interest in a field such AD, which is rapidly expanding. Lowering the total tau levels has been recently identified as a new goal for therapy. Therefore many drug candidates are likely going to be tested in the near future. SPR technology is already widely used in pharmaceutical industry to investigate novel molecules, since it gives access to a large panel of information. In this panorama aptamer technology may improve the overall quality of the analytical data, allowing better comparison among drug candidates. With respect of these receptors, the thesis opened the door to new studies for DNA aptamers to recognize tau, with considerable advantages in term of the receptor stability. Moreover the whole potential of DNA aptamers selected in this work still remain to be explored. New selection methodologies, combined with fast progression of bioinformatics tools might give rise to affinity improvement, which will lead to sensitivity improvement for tau detection in the next few years.

Outline

This PhD thesis is the result of a three-year project on the topic of early diagnosis of Alzheimer's disease supported by Università Italo-Francese (UIF), Vinci project (chapter III). Thanks to the support of this Institution, a collaborative effort between the Biosensor laboratory, Department of Chemistry "Ugo Schiff", of the University of Florence and the Department of Pharmacological Molecular chemistry (DPM) of the University of Grenoble, gave rise to an innovative approach for the detection of Alzheimer's disease core biomarkers present in biological fluids. The work was characterized by a carefully designed step-by-step process. Starting from the use of a conventional antibody-based technique, we progressed in two main directions which will be fully clarified in the text.

The whole project is built around tau protein (τ), which, as described in the introducing chapters (1 and 2), is one of currently accepted biomarker for Alzheimer together with Amyloid-β peptide (Aβ). In this introduction, in which a review published on *Analytica Chimica Acta (Scarano et al., 2016, 940, p. 21-37)* is attached, a critical report of the scientific literature is presented. After a short description of the biochemical role and structure of the core biomarkers for Alzheimer's disease, two aspects are analyzed. First of all novel biosensing strategies based on the well-known antibody-antigen interaction were reported. Both label-free and label-based techniques were considered and compared to existing techniques used in clinical routine. Secondly a survey on the emerging bio-mimetic receptors was carried out. Finally the theoretical principles of the techniques used in this thesis are presented.

The experimental part of the thesis is organized in two main blocks, any of which describes different aspects of the work.

Chapter 3 is focused on classical antigen-antibody interaction to develop a Surface Plasmon Resonance (SPR) sensor for tau protein quantification. This part of the thesis, published on *Biosensors and Bioelectronics,(In Press on Special Issue of the 26th International congress of Biosensors, doi: http://dx.doi.org/10.1016/j.bios.2016.08.078)* describes the use of two complementary monoclonal antibodies to design a sandwich assay. Multi Walled Carbon Nanotubes (MWCNTs) were explored as signal enhancers by labeling the secondary monoclonal antibody with these large nanostructures, thanks to a collaboration with Prof. Stefano Cicchi, (Department of Chemistry "Ugo Schiff, University of Florence) who kindly provided the MWCNTs.

In chapter 4 we investigated the possibility to obtain a new bio-mimetic receptor, based on single stranded DNA (ssDNA) aptamers, to compete with antibodies for the detection of tau protein in bioanalytical assays. As reported in the introduction, these receptors are usually selected *in vitro* by a process called SELEX (Systematic Evolution of Ligands by EXponential enrichment). The selection process starts using large DNA or RNA libraries and is always composed by three parts: separation, amplification by Polymerase Chain Reaction, and purification of amplified pool. Here using the same separation platform i.e. Capillary Electrophoresis (CE) two different selection procedures, named CE-SELEX and CE-Non-SELEX were tested and compared. SPR was also applied in the affinity determination of selected ssDNA for the target (tau).

Finally, in chapter 5 general conclusions and future perspectives are reported. Here the strong interdisciplinary character of the work was highlighted and appeared to be the key to obtain relevant scientific results.

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1 State of the art for bioanalytical approaches in Alzheimer's disease diagnosis

Alzheimer's disease first case was presented in Tübingen (Germany) for the first time at the beginning of the last century (1906) by Alois Alzheimer, a German physician who observed miliary bodies (amyloid plaques) and dense bundles of fibrils (neurofibrillary tangles) in the human brain of Auguste Deter [1]. Starting from that moment Alzheimer's disease research made incredible progresses both in the comprehension of the pathogenic mechanisms eventually leading to the disease, with the development of amyloid hypothesis [2], and in the field of analysis of the biochemical markers that have been discovered along the years.

In the analytical field main efforts ware devoted to detect the three core biomarkers commonly known as Alzheimer signature: Aβ (especially isoform of 42 amino acid residues), τ protein, and hyperphosphorylated τ [3]. They were included officially inn the algorithm for probable AD diagnosis in 2007 [4], after the criteria established 23-years earlier by the National Institute of Neurological Disorders and Stroke–Alzheimer Disease and Related Disorders (NINCDS–ADRDA) were extensively revised. Apart from imaging techniques, CerebroSpinal Fluid (CSF), for its direct contact with brain vessels, is up to now the preferred medium for analysis of all core biomarkers [5]. Immunochemical methods are in large majority both for commercial kits and for research. In parallel with conventional antigen-antibody assays, are reported new biomimetic receptors that emerged as promising for diagnosis. Nevertheless since considerably lower amount of works are reported for such receptors, they still hold great potential.

State of the art, including a perspective on the future improvement possibly desirable in the next years is reported here:

"Scarano, S., Lisi, S., Ravelet, C., Peyrin, E., Minunni, M., 2016. Detecting Alzheimer's disease biomarkers: From antibodies to new biomimetic receptors and their application to established and emerging bioanalytical platforms–A critical review. Anal. Chim. Acta 940, 21–37."

References

[1] R.A. Stelzmann, H.N. Schnitzlein, F. Reed Murtagh, An english translation of alzheimer's 1907 paper, "Uber eine eigenartige erkankung der hirnrinde," Clin. Anat. 8 (1995) 429–431.

[2] J.A. Hardy, G.A. Higgins, Alzheimer's disease: the amyloid cascade hypothesis, Science. 256 (1992) 184–185.

[3] L.M. Shaw, H. Vanderstichele, M. Knapik-czajka, C.M. Clark, P.S. Aisen, R.C. Petersen, K. Blennow, H. Soares, A. Simon, P. Lewczuk, R. Dean, E. Siemers, W. Potter, V.M.-Y. Lee, J.Q. Trojanowski, Q. John, Cerebrospinal fluid biomarker signature in Alzheimer's disease neuroimaging initiative subjects., Ann. Neurol. 65 (2009) 403–413.

[4] B. Dubois, H.H. Feldman, C. Jacova, S.T. DeKosky, P. Barberger-Gateau, J. Cummings, A. Delacourte, D. Galasko, S. Gauthier, G. Jicha, K. Meguro, J. O'Brien, F. Pasquier, P. Robert, M. Rossor, S. Salloway, Y. Stern, P.J. Visser, P. Scheltens, Research criteria for the diagnosis of Alzheimer's disease: revising the NINCDS-ADRDA criteria, Lancet Neurol. 6 (2007) 734–746.

[5] S. Schraen-Maschke, N. Sergeant, C.-M. Dhaenens, S. Bombois, V. Deramecourt, M.-L. Caillet-Boudin, F. Pasquier, C.-A. Maurage, B. Sablonnière, E. Vanmechelen, L. Buée, Tau as a biomarker of neurodegenerative diseases, Biomark. Med. 2 (2008) 363–384.

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Review

Detecting Alzheimer's disease biomarkers: From antibodies to new bio-mimetic receptors and their application to established and emerging bioanalytical platforms – A critical review

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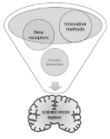
HIGHLIGHTS

- Alzheimer's biomarkers detection on conventional and innovative platforms are reviewed.
- Analytical performances in detecting amyloid-β and Tau protein species are reported.
- Critical aspects related to inter-laboratory variability of results are discussed.
- Emerging synthetic receptors for AD biomarkers detection are presented and compared.
- Future outlooks on alternative matrices and AD biomarkers are presented.

GRAPHICAL ABSTRACT



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ABSTRACT

The failure of therapeutic treatment of Alzheimer's disease (AD) patients can be related to the late onset of symptoms and, consequently, to a delayed pharmacological aid to counteract neurodegenerative progression. This is coupled to the fact that the diagnosis based on clinical criteria alone introduces high misdiagnosis rate. The availability of assessed biomarkers is therefore of crucial importance not only to counteract late diagnosis, but also to manage patients at high risk of AD development eligible for novel therapies. At the present time, amyloid-β peptides ($A\beta_{1\text{-}40}$ and $A\beta_{1\text{-}42}$ isoforms), alone or in combination with Tau protein (total and phosphorylated forms (p-tau)) constitute reliable AD biomarkers and result highly predictive of progression to AD dementia in patients with mild cognitive impairment (MCI), the earliest clinical presentation of AD. Improvement of existing diagnostic tools must take advantage of innovative bioanalytical approaches. In this review, starting from commercially available diagnostic platforms based on antibodies as recognition elements, we intended to provide a double point of view on the issue: 1) progresses achieved on innovative bioanalytical platforms (mainly sensors and biosensors) by using antibodies as consolidated receptors; 2) advance on promising bio-mimetic receptors alternative to antibodies in AD research, and their applications on conventional or innovative analytical platforms. In particular, we first focused on optical- (Propagating and Localized Surface Plasmon Resonance, named here SPR and LSPR) and electrochemical (voltammetric and impedimetric) transduction principles. Together with bioanalytical assays for AD biomarkers quantification, works aimed to investigate and understand their behavior, characteristics, and roles will also be considered in the discussion.

An increasing interest in new emerging biomimetic receptors for AD diagnosis, as a promising alternative to antibodies is noticed, thus the description of peptides, peptoids, nanobodies, aptamers, and

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1.1 Generalities of Alzheimer's disease

Alzheimer's disease (AD) was first described by Alois Alzheimer in 1907 as a severe neurodegenerative disorder characterized by progressive memory and cognitive impairment, with evolution to dementia and death observed on a 50-year-old woman [1], [2]. Brain histology related to this new clinical condition presented two main hallmarks, i.e. distinctive plaques and neurofibrillary tangles (NFTs) whose composition was clarified only in the eighties with the discovery of the amyloid beta peptide (Aβ) and the tau protein as main components of amyloid plaques (APs) and NFTs, respectively [3]. The World Alzheimer Report 2015 (http://www.worldalzreport2015.org) estimates that dementia syndromes affect over 46 million people worldwide, and approximately 60-70 % of them are diagnosed as AD, although the early and accurate diagnosis is still a challenge and a high percentage of patients are still misclassified or late diagnosed. [4]. Early intervention with improved accuracy of AD diagnosis may hence greatly increase the potential of therapeutic treatments as well as the assessment and validation of suitable biomarkers to support personalized health care. The latter area, exploiting companion diagnostics or theranostics, encompasses systems combining diagnosis, therapy, and monitoring, e.g. a test that qualifies a single patient for the most suitable treatment with a particular drug, at the right time and dose [5]. Despite huge efforts in this research field, up to now there is a lack of inexpensive, sensitive, and real-time detection strategies for the early detection and monitoring of AD biomarkers. This is due to the complex and partially unraveled questions regarding the production, accumulation and clearance patterns of assessed biomarkers under physiological and patho-physiological scenarios. In this framework, we feel that the design and development of new bioanalytical assays for AD diagnosis must be necessarily flanked by the definitive understanding of AD biomarkers

involvement in pathologic mechanisms, and their relationships. At this regard, functional and molecular brain imaging techniques are effectively improving and revising clinical diagnostic criteria of dementia-related neuropathologies in vivo. [6], [7], [8]. In particular, the observation of hippocampal atrophy and entorhinal cortex by advanced magnetic resonance imaging (MRI) techniques seems effective in distinguishing AD from healthy control subjects, whereas its utility remains unclear for differential diagnosis of other dementia. Molecular imaging techniques, such as 18F fluoro-deoxy glucose positron emission tomography (PET), using tracers for amyloid or tau biomarkers and invasive methods including cerebrospinal fluid (CSF) analysis of amyloid β and tau protein are also available to support the diagnosis of AD in vivo [7]. Therefore, significant advances can be foreseen by coupling information from AD biomarkers assays to different imaging-based techniques. Their combination is gradually anticipating to the presymptomatic stage of AD sensitive and specific diagnostic tools, which give information on the various pathological processes underpinning the different causes of dementia.

1.2 Overview on consolidated AD biomarkers

Nowadays, three core biomarkers are considered for routine diagnosis of AD in cerebrospinal fluid (CSF): amyloid beta peptides ($A\beta_{1\text{-}42}$ and $A\beta_{1\text{-}40}$), total tau protein (t-τ), and tau protein phosphorylated on threonine 181 (p-tau_{181}). Such markers are involved in the well-known amyloid cascade hypothesis, which has been the basis of research in the field for the last 20 years [9, 10] (sketched in Fig. 1-1). This milestone puts tau aggregation downstream to amyloid deposits; however, tau pathologies are

also described in absence of Aβ aggregates [11], and a profound revision of the whole mechanism is in progress [12].

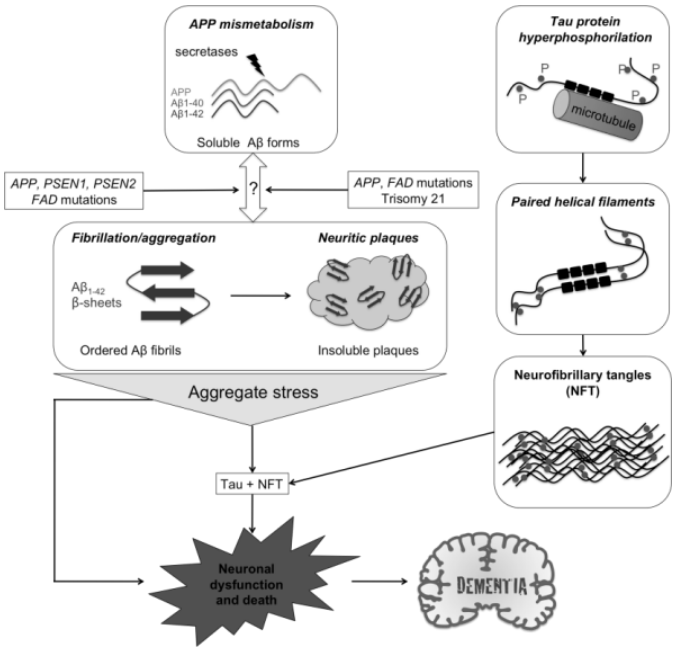

Fig. 1-1 Schematic diagram showing the involvement of Aβ and Tau protein in the amyloid cascade hypothesis, freely adapted from [10].

The Alzheimer's signature includes therefore all three biomarkers, in order to allow robust classification of AD patients compared to healthy subjects [13]. Moreover, new species such as amyloid-derived diffusible ligands (ADDLs) [14] or isoforms related to tau imbalance (such as four- and three-microtubule binding unit tau, 4R and 3R, respectively) [15], [16] have been recently considered as potential candidates for diagnosis.

Although great efforts have been realized in order to separate AD subjects from controls, a lack of reproducibility has been recently highlighted, leading to a collaborative work among several laboratories in order to reduce the observed variability [17]. Since CSF sampling is a quite invasive procedure compared to blood sampling, blood biomarkers would be of great interest. Despite efforts made to define reliable

molecular patterns to discriminate AD from other dementias or from healthy subjects, no convincing results have been reported [2]. Eventually attempts in combining information from peripheral biomarkers with processes related to aging (such oxidative stress) have also been conducted [18]. However, even if this multivariate approach is promising, no validated pattern of blood biomarkers is available [19].

1.2.1 Amyloid β peptides and derived species

Amyloid β peptides are a family of peptides with a molecular weight around 4 kDa produced by the cleavage of larger amyloid precursor protein (APP). Among the different forms produced during this process, peptides constituted of 40 ($A\beta_{1-40}$) and 42 ($A\beta_{1-42}$) amino acidic residues are prevalent. The clinical meaning of $A\beta_{1-40}$ and $A\beta_{1-42}$ for AD diagnosis has been confirmed by more than 2000 CSFs' patients analysis. Peptide concentration in age-matched healthy controls was found to decrease by 50% compared to AD subjects [20]. The ratio between the two amyloid species, *i.e.* $A\beta_{1-40}$ and $A\beta_{1-42}$, has also been proposed as AD hallmark, on the base of the so-called amyloid cascade hypothesis (Fig. 1-1). In fact, in this model, Hardy and Selkoe reported that AD pathogenesis is stated by an imbalance between $A\beta_{1-42}$ production and its clearance [21], giving good diagnostic information to distinguishing AD subjects from healthy population (better for early onset Alzheimer's disease). In fact physiological peptide concentration usually exceeds 110 pM, while a consistent reduction is observed in cerebrospinal fluid of AD patients (table 1-1). Amyloid β monomers aggregate to the final state of mature fibrils, *via* the formation of the so-called amyloid-derived diffusible ligands (ADDLs). ADDLs are small aggregates displaying high toxicity and diffusivity into neuronal cells compared to amyloid monomers and neuritic plaques. The analysis of ADDLs, while challenging, seems also

very promising for the early AD detection [22] and would provide a unifying mechanism for AD, accounting not only for memory loss but also the major features of neuropathology (synapse loss, tau phosphorylation, oxidative damage, and specific cell death).

table 1-1: Levels of Alzheimer's disease biomarkers and relative cut-off values, obtained by ELISA. Conversion is realized considering 4300 Da for $A\beta_{1-42}$ and 45900 Da for tau protein. n.d. means not significant values for sporadic Alzheimer's disease.

Biomarker	Healthy subject (ng L^{-1})	AD subject (ng L^{-1})
$A\beta_{1-42}$	794±20 (184.3 pM)	<500 (<116.3 pM)
Total tau protein (21-50 years)	136±89 (3.0 pM)	n.d.
Total tau protein (51-70 years)	243±127 (5.3 pM)	>450 (>9.8 pM)
Total tau protein (>71 years)	341±171 (7.4 pM)	>600 (>13.1 pM)
Phosphorilated tau protein (181)	23±2 (0.5 pM)	>60 (>1.3 pM)

1.2.2 Tau proteins and derived isoforms

Tau protein (50–65 kDa) belongs to microtubule associated protein family (MAP) and is expressed in human neurons, oligodendrocytes, and astrocytes within the central nervous system (CNS) and peripheral nervous system (PNS) [23], [24]. In axons, where it is primarily found, tau protein is involved in microtubules polymerization and stabilization through its interaction with tubulin (Fig. 1-1). The tau structure can be divided in four regions, i.e. the N-terminal region, the proline-rich domain, the microtubule-binding domain, and the C-terminal region [25]. The human tau gene (*MAPT*) contains 16 exons, with alternative splicing of exons 2, 3, and 10, which yields six isoforms, ranging in length from 352 to 441 amino acids. Inclusion of exon 2 results in 1N isoforms, the inclusion of both exons 2 and 3 produces 2N isoforms, whereas isoforms lacking both exons are named 0N [15]. The microtubule-binding domain is characterized by the presence of three (3R isoforms) or four (4R isoforms) repetitive units before the C-terminal region. Compared to amyloid peptides, tau proteins are well soluble in water and stable in the absence of

aggregation inducers, due to its hydrophilic primary structure, which is also responsible for its natively unfolded structure [26].

The role of tau regions in aggregation has been studied under several conditions. Von Bergen and colleagues discovered the minimal sequence able to aggregate in paired helical filaments (PHF), which eventually lead to tangles in AD [27]. Since interaction with microtubules is regulated from tau phosphorylation states, phospho-tau species have gained significant attention for explaining AD pathological mechanism [28]. Comparison among amino acid phosphorylation sites (85 potential serine (S), threonine (T), and tyrosine (Y)) of tau protein in normal and AD brain has been reported. In healthy subjects, a low level of phosphorylation (11.8% i.e. 10 with tau phosphorylated over 85 subjects) of analyzed soluble tau was found [28], [29], while in insoluble aggregated tau, extracted from tauopathy brain, higher phosphorylation levels, *i.e.* about 50% (approximately 45 different serine, threonine, and tyrosine phosphorylation sites), were found [27], [30], [31], [32], [33].

The higher phosphorylation levels in AD patients have been considered as AD biomarker for the development of bioassays. In particular, attention has been focused on two phosphorylation sites, respectively serine 181 (p-tau181) and threonine 231 (p-tau231) since their measurement proved to be informative for classifying AD from relevant differential diagnoses: p-tau_{181} enhances the classification between AD and dementia with Lewy bodies, and p-tau_{231} is able to differentiate between AD and frontotemporal dementia (FTD) [34].

Along with hyperphosphorylation, other tau post-translational modifications have been related to tauopathies, such as glycosylation, glycation, prolyl-isomerization, cleavage or truncation, nitration, polyamination, ubiquitination, sumoylation, oxidation and aggregation [35].

1.3 Immuno-based platforms on the market: ELISA and xMAP assays

At the present time, the most widely used immunoassay platforms for CSF AD biomarkers detection (Aβ peptides 1-40 and 1-42, total tau (6 isoforms), and p-tau$_{181}$) are provided by Fujirebio-Innogenetics (Innogenetics Inc., Alpharetta, GA) and consist in a singleplex enzyme-linked immunosorbent assay (ELISA) kit and a micro-bead based Multi-Analyte Profile (xMAP) (also recently reviewed by Kang and colleagues [36]). The bioanalytical strategy of the two immunoassays benefits from the development of commercial monoclonal antibodies (mAbs) for each target molecule, working in sandwich format. No cross-reactivity between monoclonal antibodies has been noted [37], [38], and both immunoassays work in cerebrospinal fluid (CSF). If ELISA-based kit performs one biomarker at a time in parallel reactions on immunoplate, xMAP technology allows multiplexed analysis. table 1-1 shows physiological levels of all core biomarkers, quantified by ELISA. xMAP technology (Luminex®) indeed performs discrete assays on the surface of color coded beads, then read by multiple lasers or LEDs, reporting the reactions occurred on each individual microsphere [39]. On the basis of xMAP technology, Olsonn and colleagues developed a cytometry-based assay for the multi-analyte determination of core AD biomarkers coupling cytometry to the use of labeled spherical microbeads [40]. The spheres may be loaded with up to a hundred combinations of two selected dyes and then separated by flow cytometry (sketched in Fig. 1-2). Details of the xMAP platform and its functioning are reported in the relative caption.

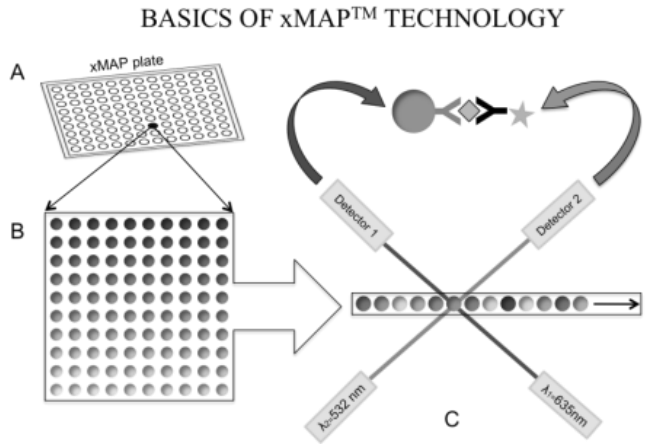

Fig. 1-2: Sketched representation of xMAP™ technology. Each plate well (A) contains a 10x10 microspheres set obtained by varying the ratio of two fluorophores (B), in order to classify each microsphere on the basis of a unique fluorescent signature. This allows for multiple analyte profiling in a single sample. Classification is allowed by flow cytometry system coupled to two lasers (C). Detector 1 (635 nm) is used to identify the interrogated bead. Each bead is functionalized with a primary antibody, enabling the capture of the analyte from complex media. Detector 2 (532 nm) excites phycoerythrin (PE), used as label of the secondary antibody, to quantify the binding event

Absolute results obtained through this approach are similar to those obtained by means of ELISA. However the direct comparison of results obtained by ELISA and xMAP requires appropriate models to convert the biomarkers levels between the two platforms, as reported by several authors [41]. Declared ranges for calibrators of ELISA-based kits (lyophilized peptide solutions) are 7.8-1000 ng L^{-1} for $A\beta_{1-40}$ (Limit of Detection (DL) not provided), 62.5-4000 ng L^{-1} for $A\beta_{1-42}$ (DL= 65 ng L^{-1}), 15.6-1000 ng L^{-1} for p-tau181 (DL= 13 ng L^{-1}), and 50-2000 ng L^{-1} for total tau (DL= 34 ng L^{-1}, all six isoforms from 352 to 441 aa). xMAP-based kit for the same CSF AD biomarkers, INNO-BIA AlzBio3, is furnished with a ready-to-use calibrator in solution offering wider calibrator ranges in a semi-automated assay platform with minimal intra-sample variance and limits of detection slightly lower than ELISA-based kits. A detailed paper reporting all the operative steps and related expedients to minimize the variability of results has been published by

Kang and colleagues [42]. The same article summarizes the clinical performances of AD CSF biomarkers measured by xMAP-Luminex platform.

Recently, Fujirebio-Innogenetics put on the market a new assay kit for $A\beta_{1-40}$ and $A\beta_{1-42}$ quantification in plasma instead of CSF. Repeatability for both assays showed a coefficient of variation (CV%) below 10%, with a limit of detection (LOD) in the pM range for all biomarkers with a slightly extended dynamic range and higher accuracy for xMAP technology, which results in an appropriate assay platform to incorporate diagnostic biomarkers into routine clinical practice.

Even though conventional methods show good analytical performances, the lack of reference CSF matrix for calibrators and of standard reference materials for each biomarker is the main source of high inter-laboratory variability, which raises the CV% up to 30%. This and other causes of the observed inter-laboratory variability are now being considered by Alzheimer's biomarker standardization initiative (ABSI) members taking into account pre-analytical and analytical factors in large inter-laboratory studies. Among various sources, also batch to batch variability related with antibody production is considered in order to obtain harmonization of the results [17].

In this framework, the availability of effective monoclonal antibodies targeting AD biomarkers [43] and new emerging synthetic and/or bio-mimetic receptors have stimulated the development of alternative bio-analytical approaches for AD biomarkers detection. Next paragraphs review these emerging approaches and the related analytical strategies.

1.4 Innovative bioanalytical platforms based on immunodetection

1.4.1 Propagating Surface Plasmon Resonance (SPR)

Several biosensors, based on various transduction principles, are reported in literature for AD biomarkers detection, whose performances have also been critically reviewed [44], [45]. Label free optical techniques, such as Surface Plasmon Resonance (SPR, conventional and imaging) [46], [47], [48] or Localized SPR (LSPR) [49], as well as electrochemical techniques, offer the possibility to detect biomolecular interactions with high sensitivity, and in real time. In table 1-2, an overview of these approaches based on label free biosensing is reported.

table 1-2: Immuno-based label free methods for Alzheimer's disease (AD) core biomarkers detection. (1): Technique; SPR: Surface Plasmon resonance; LSPR: Localized SPR; CNT-FET: carbon nanotubes-field effect transistor; SWV: square wave voltammetry; EIS: electrochemical impedance spectroscopy; ADDLs: amyloid-derived diffusible ligands; CSF: cerebrospinal fluid; mAb: monoclonal antibody.

Transduction principle	(1)	Target	LOD	Medium	Detection strategy	Antibody	Ref
Optical	SPR	$A\beta_{1-40}$	0.3 fM	buffer	sandwich	Fragmented mAb	[53]
		$A\beta_{1-40}$ $A\beta_{1-42}$	20.0 pM	CSF	sandwich	mAb	[56]
		tau (captured) $A\beta_{1-42}$ (flowed)	3.0 nM	buffer	sandwich	mAb	[58]
		$A\beta_{1-42}$	0.2 µM	buffer	direct	mAb	[61]
		ADDLs	280 nM	buffer	sandwich	mAb	[62]
		ADDLs	n.d.	buffer	direct	mAb	[63]
	LSPR	ADDLs	10.0 pM	human brain cells and CSF	sandwich	mAb	[22]
		Tau	0.2 pM	buffer	direct	mAb, protein G oriented	[67]
Electrochemical	SWV	$A\beta_{1-40}$ $A\beta_{1-42}$	20.0nM	rat CSF	direct	mAb	[74]
	EIS	$A\beta_{1-42}$	0.57 nM	buffer	direct	mAb, protein G oriented	[75]
	CNT-FET	$A\beta_{1-42}$	2 nM	serum	direct	mAb	[76]

SPR is one of the most relevant and well known analytical platforms in biomolecular interaction assessment, and during recent years, several

works in the field of AD have appeared [50], [51], [52], even if only a few of them are intended for biomarkers quantification. Lee and colleagues quantified $A\beta_{1-40}$ by SPR immunosensing in a sandwich format exploiting gold nanoparticles (GNPs) for signal enhancement [53] (Fig. 1-3).

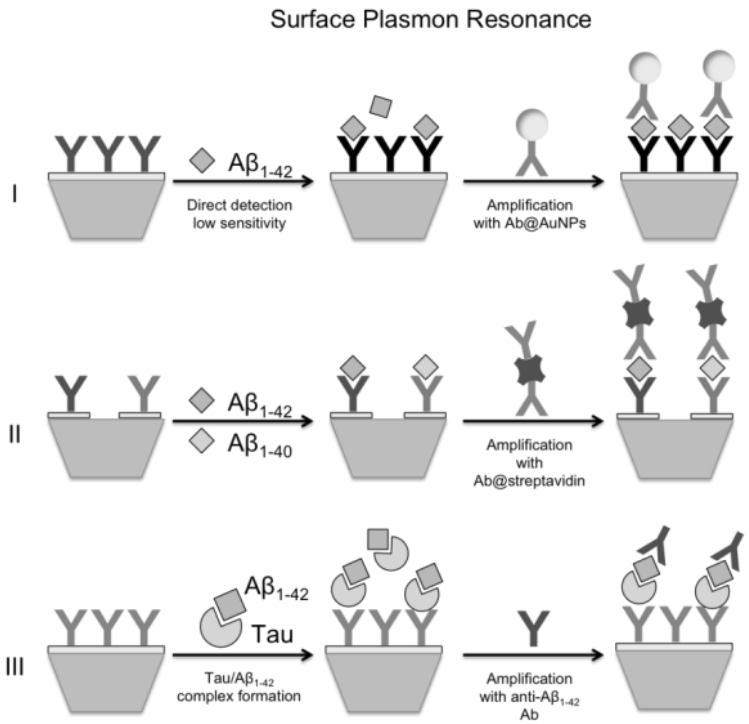

Fig. 1-3: SPR-based detection of amyloid $A\beta_{1-40/1-42}$ biomarkers by immunosensing strategies involving the use of a secondary antibody to enhance the SPR signal. The enhancement can be obtained by means of molecular architectures in which the secondary Ab is directly coupled to gold nanoparticles (I) [53], multiple site recognition proteins, i.e. streptavidin (II) [56], or by label-free detection exploiting the mass enhancement of tau/$A\beta_{1-42}$ binding [58].

The low molecular weight of Aβ (about 4 kDa) limits both the SPR angle change and the detection limit, requiring the set-up of molecular strategies able to amplify the assay response.

With GNP-enhanced SPR, the observed detection limit in buffer was declared at 0.2 pM (1 pg L^{-1}), by using a fragmented mAb immobilized as receptor in an oriented fashion via gold/thiol linkage. Non-specific binding

or possible interferences were not considered, which is often crucial when dealing with fragmented Abs that suffer from non-specific interactions [54]. In another work, ethylene glycol was used to address this issue by passivating the surface of an SPR biosensor realized for the simultaneous detection of Aβ species [55]. Through biotinylated mAbs anchored on streptavidin, used both as capturing agents and signal amplifiers, Aβ species were detected in the buffer and CSF, with an estimated LOD of 3.5 pM for all the species. The biosensor was fully regenerable with reproducibility lower than 10% (CV%). To the best of our knowledge, this is the only work in which SPR was used to detect analytes in real matrices [56] (Fig. 1-3).

Guo and colleagues proved tau-$A\beta_{1-42}$ complex formation by SPR, with a K_d in the low nM range [57]. On this basis, Homola's group exploited the tau-$A\beta_{1-42}$ complex formation through a sandwich assay in which anti-tau antibody was immobilized on the surface in order to capture tau-$A\beta_{1-42}$ complex from the solution [58] (Fig. 1-3). The direct signals obtained were then amplified by a secondary anti-$A\beta_{1-42}$ antibody binding the tau-$A\beta_{1-42}$ complex. No information about regeneration was given.

One of the major advantages of SPR is to exploit physical properties of metal structures or surfaces to enhance the analytical signal. If the use of GNPs is quite common [59], a different approach relies on the constitution of a bimetallic Ag/Au film. Indeed the combination of the narrower resonance curve of Ag (compared to Au) and very little optical leakage of waveguide layers are able to improve significantly signal to noise ratio [60]. The developed technology was used for $A\beta_{1-42}$ detection in buffer at concentrations comparable to clinical level of the peptide in CSF (25-500 pM, i.e. 100-2000 ng L^{-1}) [61]. However, such strategy compared with the sandwich approach previously illustrated, could be more prone to interferences coming from real samples. Thus in this case, deeper

investigation of possible non-specific signal should be considered. Apart from quantitative aspects, SPR technology gives the possibility to investigate peculiar behaviors of the biomolecules involved in the affinity recognition, and this feature has been exploited also in the AD biomarkers field. In particular, different monoclonal antibodies against Aβ oligomers may highlight the existence of antibody-dependent aggregation patterns [62], [63]. For example, clones 82E1 and 12F4 for Aβ, when exploited on SPR as bioreceptors, displayed the ability to differentiate association patterns in Aβ oligomers growth [62], *i.e.* while clone 82E1 infers a single sigmoidal signal, a double sigmoidal trend was elicited by 12F4 clone. Conversely, mAb for ADDL (clone A11) together with mAb for amyloid fibrils (clone OC) were immobilized by Yi and colleagues to evaluate aggregation over time in presence of Aβ aggregation modulators such as copper [63]. The use of oligomers-specific antibodies could also be proposed to reduce sample pretreatment. Finally, in a work carried out by our group [51] on SPR, we exploited Aβ monomers as fibrils elongation triggers. This allowed to study aggregation trends in the presence or not of clioquinol, a proposed inhibitor of Aβ fibrils elongation, also in combination with piezoelectric transduction. Real time and label free monitoring allowed by SPR gives the exquisite chance of understanding and evaluating the inhibitory action of clioquinol as well as of any other putative active substance of interest for therapeutic purposes.

1.4.2 Localized Surface Plasmon Resonance

Surface Plasmon oscillation can occur eventually in metallic nanostructures, and LSPR is displayed by the characteristic wavelength of maximum absorbance (λ_{max}) within the visible-near infrared range depending on size, shape, and material (typically gold and silver, or their alloys) [64]. By modifying nanostructures surface with biomolecules

specific for a target analyte (nucleic acids sequence, peptide, antibody, carbohydrate etc.) and interrogating them with light, LSPR response correlates with analyte concentration. It can be recorded as λ_{max} shift of the extinction spectrum and it is elicited by local change of the refractive index, which is induced by the biomolecule film formed at nanomaterial surface after biorecognition. Furthermore, LSPR-based sensing presents reduced background noise by localizing the "sensing depth" close to the surface (10-20 nm), improving the detection limits as compared to propagating SPR (SPR and SPRimaging). This feature is of paramount importance since turns into a low or negligible matrix effect, one of the major requirements in bioanalysis on biological samples. LSPR measurements can be performed in solution or on planar arrays of nanostructures and represents a new emerging and highly performing optical label free-based sensing technique.

Van Duyne's group first exploited LSPR in the field of AD research, in order to assess the interaction between anti-ADDLs antibodies and amyloid oligomers [65] (Fig. 1-4)

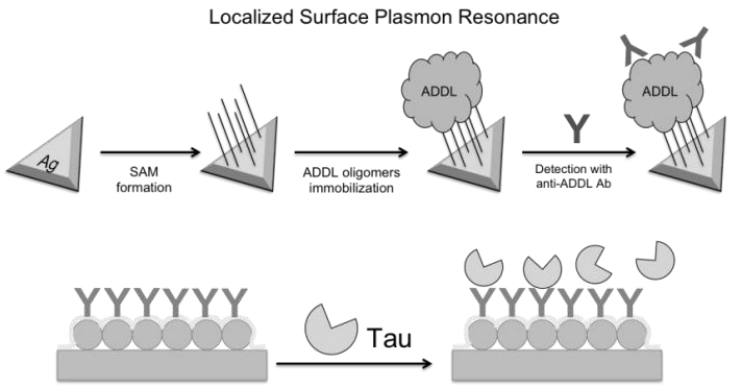

Fig. 1-4 Reported optical techniques relied on LSPR of nanostructured metals (silver and gold) to transduce biomolecular interactions between antibodies and AD biomarkers. On the top, silver nanoprisms used for detection of amyloid-derived diffusible ligands (ADDLs) [65]. On the bottom, silica nanoparticles coated with thin layer of gold exploited for tau detection [67].

ADDLs were immobilized on mono-disperse, surface-confined silver nanotriangles obtained by Nanosphere lithography (NSL). Polystyrene nanospheres, forming a hexagonal close-packed monolayer of spheres, served as a deposition mask. The sensor was hence exposed to various concentrations of anti-ADDLs antibodies. Nanoparticles anchorage was carried out on glass substrate avoiding the use of the classic chromium adhesion layer, which displayed to dramatically affect the selectivity of the biosensor. This effect is likely due to the higher binding affinity of Cr for COOH than SH groups in alkanethiols [66], leaving the thiol available to form disulfide bonds with cysteines of anti-ADDL molecules. Primary anti-ADDLs antibody was immobilized on AgNPs and then incubated with various oligomers concentrations. The direct signal was then amplified by a secondary antibody reaching LOD at a low pM level both in buffer, brain extract homogenates, and CSF. Moreover, models developed to quantify the target, revealed different classes of ADDLs with different binding affinity for the receptors depending on the concentration level (<10 pM or > 10 pM ADDLs). Such feature further demonstrates the

versatility of SPR biosensing as compared to conventional immuno-based assays [22]. More recently, tau protein was revealed by LSPR by Vestergaard and colleagues by immuno-based direct detection [67] (Fig. 1-4). Here nanostructured resonating surface was achieved by growing a thin gold layer on a substrate of silica nanoparticles. The LSPR change was elicited by the binding between the monoclonal anti-tau antibody (clone not specified, immobilized via protein G orientation) and tau protein (60 KDa) in buffer solution. Despite the reported LOD was 0.167 pM (10 ng L^{-1}) tau, lower than the cut-off value of 3.5 pM (195 ng L^{-1}) (for AD) for tau protein in CSF, the result has not been tested on real samples. Moreover, the specificity was tested on bovine serum albumin (BSA), instead of human serum albumin that is the most abundant protein of CSF (able to properly simulate the possible unspecific matrix effect on the biosensor).

As a whole, LSPR biosensing is under fast evolution and promises exciting results through the fine tunability of the nanomaterial used, the simplicity of its signal detection, coupled to high sensitivity and selectivity. Moreover, LSPR biosensing is foreseen to be easily miniaturized for point-of-care test platforms [68], [69], so we feel that there will be a dramatic increase of works devoted to LSPR-based diagnostic methods in next years.

1.4.3 Electrochemical (bio)sensors

Label free electrochemical strategies to detect peptides and proteins can take advantage from intrinsic electro-active amino acids, mainly tyrosine and tryptophan, as exhaustively reviewed by Tamiya and colleagues [50]. The tyrosine residue of Aβ peptide (10^{th} position) has been thus exploited for the first time by Vestergaard and colleagues in more than one work [71], [72], [73]. The first work dealt with the electrochemical monitoring

of aggregation patterns of amyloid beta peptides ($A\beta_{1-40}$ and $A\beta_{1-42}$) using three different voltammetric techniques at a glassy carbon electrode (GCE) [71]. The approach allowed the authors not only to evidence the progressive elongation of Aβ fibrils (confirmed by thioflavin T and AFM) by increasing the incubation time, but also to characterize that the two isoforms are featured by well distinguishable aggregation rates. The longer the amyloid beta incubation time, the lower the oxidation signal of tyrosine. This suggests that the observed trend could be elicited by the conformational change of the peptides during the aggregation process. Afterwards, the same approach was exploited to investigate the phosphorylation degree of Aβ [72], since they found that the process affects the tyrosine electro-oxidation and, finally, to follow tyrosine kinase activity (responsible for Aβ phosphorylation in the presence of ATP) and its modulation in the presence of an inhibitor [73]. The reported approaches are sketched in Fig. 1-5.

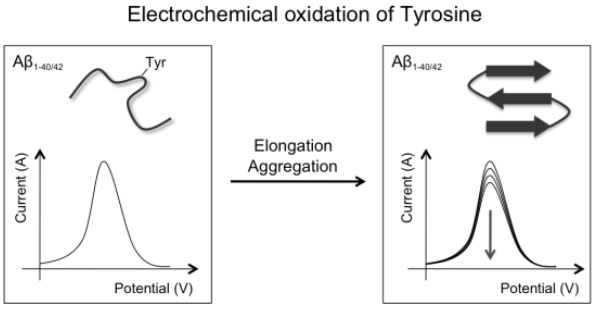

Fig. 1-5 Representation of label free electrochemical detection based on tyrosine oxidation. The $A\beta_{1-42}$ elongation/aggregation induces a progressive decrease of Tyr signal, allowing also for the differential monitoring of aggregation patterns of Aβ species [71], [72], [73]

Prabhulkar and colleagues developed a multiplexed, implantable immunosensor to detect Aβ isoforms using triple barrel carbon fiber microelectrodes as sensing platform [74]. Two carbon fiber microelectrodes were separately modified with two mAbs specific for the

C-terminal of the Aβ species (mHJ2 for $A\beta_{1-40}$ and mHJ7.4 for $A\beta_{1-42}$). The third one, mHJ5.1 clone, recognizes the central domain of both Aβ isoforms (amino acids 17–28) without detectable cross-reactivity toward other APP fragments. The behavior of tyrosine oxidation peak was monitored by square wave voltammetry (SWV) allowing simultaneous and label free detection of $A\beta_{1-40}$ and $A\beta_{1-42}$ species in rat CSF. Authors report that the biosensor showed non-significant interference with other (not specified) proteins containing Tyr residues, with an inter-electrode precision of 14.2% (CV%) over dilutions of analytes. The limit of detection for both Aβ isoforms resulted 20 nM even if calibration plots reported by the authors show dose-response correlation quite far from linearity, mainly for $A\beta_{1-40}$. Despite quantitative aspects, the novelty of the approach among the other electrochemistry-based examples is undoubtedly the concept of an implantable biosensor able to detect the two Aβ isoforms on single triple barrel carbon microelectrodes, with perspectives for *in vivo* analyses.

Electrochemical Impedance Spectroscopy (EIS) has been reported for the Aβ isoforms detection, by coupling immunosensing to disposable printed electrodes [75]. On impedimetric sensors, detection is based on the principle that any substance tethered on the electrode elicits a change of the measured impedance. Therefore, any change in the impedance spectra can be related to the change of interface properties. In a step-wise fashion, authors explored the detection limit enhancement by three different modifications of microelectrodes, before anti-amyloid antibody immobilization. In brief, these involve 1) direct amino coupling of the mAb on sensor surface; 2) growth of gold nanoparticles followed by amino coupling of the mAb on sensor surface; 3) growth of gold nanoparticles followed by protein G immobilization to obtain the most favored orientation of mAb on the surface. The results obtained are quite

expected and displayed a progressive improvement of the detection limit over the three methods, with the best LOD at 0.57 nM, by applying the method 3). At that stage of development, the authors focused the work on mAb immobilization strategy to improve the sensitivity of the assay of monomer Aβ isoforms (in standard conditions). They concluded by anticipating that the near future work will expand the approach also to the quantitative detection of oligomeric amyloid beta species.

A different strategy was employed by Oh and colleagues [76]. A carbon nanotubes film-based biosensor was developed with metal semiconductor field effect transistor structure (CNT-MESFET). In such device, classical p-n junction is replaced by Schottky barrier provided by the contact between a metal (i.e. gold) and a semiconductor. Here the electrical conductance change is used as analytical signal, thus it is related to target concentration. Anti-$A\beta_{1-42}$ antibody was oriented by using the Z domain of protein A displayed on the *E. Coli* outer membrane linked to the surface [77]. Using this approach, a very high sensitivity was obtained (see table 1-2) for amyloid detection in human serum, *i.e.* LOD of 0.25 pM (1 pg mL^{-1}). However, no information about other analytical figures of merit was reported.

Tau misfolding during the early stage of its auto aggregation process was also studied by EIS and cyclic voltammetry [78]. The tau-tau binding, established between tau immobilized on gold electrode and tau monomers in solution, was investigated and compared to the behavior of p-tau_{181} and BSA, displaying specificity. A linear correlation between charge transfer resistance to the redox probe ($[Fe(CN)_6]^{3-/4-}$) and tau monomer concentration was found from 0.2 to 1.0 µM. Also in this case, the study provides very interesting information on tau aggregation in standard conditions, and could be further developed to work in real samples.

1.4.4 Strategies to improve the analytical performances

Among labels employed in immuno-based detection strategies, four main classes can be identified in works dealing with bioassays for AD biomarkers detection. In particular, they consist of 1) nucleic acids conjugated to antibodies or immobilized on gold nanoparticles (GNPs) for bio-barcode assays; 2) GNPs in spectroscopic techniques such as Surface Enhanced Raman Spectroscopy (SERS) and Two-Photons Rayleigh Scattering (TPRS); 3) fluorescent dyes in fluorescence or FRET measurements; 4) catalytic enzymes, i.e. alkaline phosphatase, in electrochemical methods. Labels are generally used either as signal reporters and/or to improve the sensitivity of the method. table 1-3 and Fig. 1-6 summarize the principles of signal enhancement for the label-based methods discussed here.

table 1-3: Label-based techniques and labels used for AD diagnosis. (a) iPCR: immuno-PCR; RLS: Resonance Light Scattering; TPRS: Two Photons Rayleigh Scattering; SERS: Surface Enhanced Raman Spectroscopy; FRET: Forster Resonance Electron Transfer; (b) GNPs: gold nanoparticles; ALP: ALkaline Phosphatase.

Technique (a)	Label (b)	Role of the label	References
immuno-PCR	DNA	Antibody-DNA conjugates. DNA is amplified by PCR. Final DNA concentration is related to the starting analyte amount.	[79], [82], [85], [88], [89]
Bio-barcode	DNA-GNPs	Bio-barcode DNA and antibody are both immobilized on GNPs. After PCR amplification, the final bio-barcode concentration is related to the starting analyte amount.	[80], [90]
RLS/Dot-blot immunoassay	GNPs	GNPs aggregation induces increased RLS intensity, which is directly related to analyte concentration	[92]
TPRS		GNPs aggregation elicits changes in scattering properties	[93]
SERS		GNPs aggregation elicits enhancement of SERS spectrum	[94]
FRET	Fluorescent label	Analyte aggregation induces proximity of labelled antibodies	[95]
Fluorescence intensity		Fluorescent dye labelled to secondary antibody elicit signal	[96], [97]
Electrochemical	ALP	Catalysis of electroactive substrates to form detectable products	[98], [99]

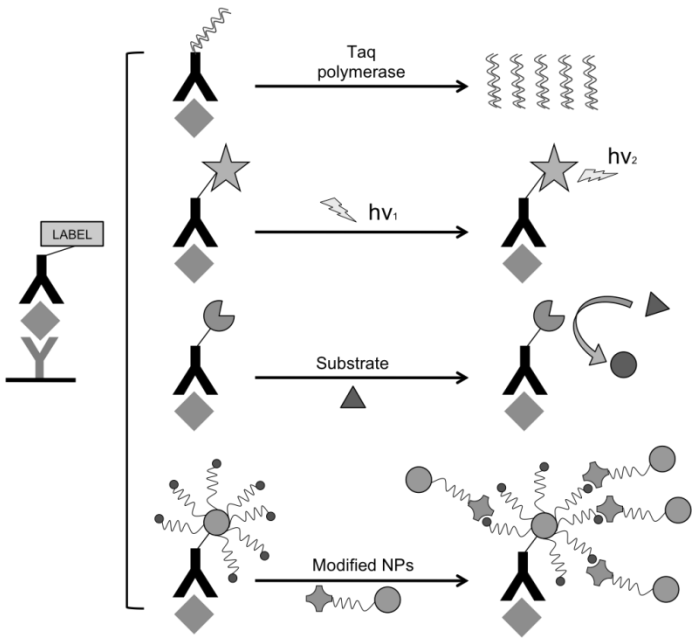

Fig. 1-6 Sketched representation (not to scale) of classes of labels used in bioanalytical methods for AD biomarkers detection discussed in the review. From top to bottom: DNA sequences in immuno-PCR, fluorescent probes in fluorescence-based methods, catalytic enzymes in electrochemical detection, and use of metallic nanoparticles in optical methods. All the strategies are based on the sandwich format.

Nucleic acids as enhancers

Properties of nucleic acids as labels are generally exploited on two different bioanalytical platforms: immuno-PCR (iPCR) [79] and bio-barcode analyses [80]. While the first one usually requires the bioconjugation between an antibody and a DNA strand, the second one takes advantage of the immobilization of a bio-barcode DNA sequence on GNPs. In a typical iPCR protocol, a double-stranded DNA sequence is bound to an antibody addressing the target analyte similarly to classic immuno-based assays [81]. Afterward, if the analyte target binds to the Ab, the DNA reporter sequence undergoes amplification by polymerase chain reaction (PCR) and finally is quantitatively detected in real time (real-time PCR) or by means of end point PCR and gel electrophoresis detection, after recovery of the strands. Among AD biomarkers, Aβ1-40

and tau protein (total tau, 3R/4R isoforms, and phosphorylated tau) have been detected by iPCR while, to our best knowledge, Aβ1-42 detection has not been yet attempted.

Hashimoto and colleagues detected Aβ1-40 in microdissected neurons by iPCR using simultaneous addition of the analyte and antibody-DNA conjugate to pre-coated ELISA plate [82]. Quantification was achieved after overnight incubation with VIC/TAMRA probe (ThermoFisher Scientific). LOD in buffer was 0.03 fmol per well and 100% recovery was found when the pre-treated matrix was fortified with known amount of the analyte. Since imbalance of tau isoforms in CSF has been proposed as promising information in AD differential diagnosis [15], [83], iPCR and ELISA tests for 3R and 4R tau were developed to this aim [84], [85]. Artificial CSF, 1:1 diluted with buffer, was used as simulated matrix, and anti-tau/DNA conjugate was used as secondary antibody, further amplified by real-time PCR. LOD of 0.2 pM was obtained in both analytes, with two orders of magnitude improvement versus ELISA developed by the same authors. Inter-assay repeatability was found to be 10-25%, with 90% recovery for both analytes. After optimization, the method was applied to human CSF samples, revealing analytical performances comparable to standard conditions. Phosphorylated tau species, i.e. p-tau 212, 214, 231, 235, were analyzed after optimization of the iPCR protocol on synthetic peptides. While reported in the scientific literature [86], [87], such phosphorylation sites have still unclear role in AD diagnosis. Nonetheless, here again iPCR demonstrated a good sensitivity, comparable or higher than CSF level of the analytes (LOD 0.04 pM for all analytes), and allowed measurements in this matrix. The detected antibody-DNA conjugate in this case was against digoxigenin, used as label for secondary anti-tau antibody (phosphorylation specific) [88].

Total tau detection was recently achieved by a different nano-iPCR approach [89]. Anti-tau capturing antibody was exposed to samples (tau calibrators in buffer solutions or undiluted CSF with unknown analyte amount) and incubated overnight. Then both anti-tau secondary antibody and DNA label sequences were immobilized on GNPs, thus no antibody-DNA conjugate was employed. Finally, functionalized GNPs were added and then amplified by real-time PCR. The method demonstrated a wide range of linearity (0.1-500 pM in matrix, R^2=0.99). In this asset, the strategy proposed by Stegurová and colleagues merges iPCR-based analysis with the basic concept of bio-barecode. Bio-barcode was introduced by Mirkin's group in 2003 for detecting prostate specific antigen (PSA) [80] and defines a designed DNA strand used as specific code for the target of interest. Compared to iPCR, bio-barcode analysis: i) needs no conjugation between the antibody and the DNA strand, ii) is realized in a homogeneous medium, and iii) increases DNA/antibody ratio. Through bio-barcode analysis, ADDLs were detected for the first time at fM level in 30 CSF samples (LOD 0.1 fM) [90] in a sandwich like assay on magnetic microparticles (MMPs). The primary antibody was immobilized on MMPs, capturing ADDLs. Then a secondary antibody, conjugated to GNPs, together with a DNA strand complementary to biobarcode DNA, was added. Watson-Crick interactions between immobilized strand and bio-barcode were then removed to recover the latter from the solution. Finally, the bio-barcode strand was immobilized onto nitrocellulose membrane and recognized by GNPs functionalized with complementary DNA probe via DNA-DNA interactions. The detection was realized by scanometric analysis, in which the intensity of scattered light is registered and enhanced by the presence of GNPs and Ag reduction [91]. The above DNA-based approaches are also summarized in table 1-3.

Gold nanoparticles

The optical properties of GNPs are widely exploited as labels, and besides classical spectrophotometric measurements based on LSPR, Resonance Light Scattering (RSL) results a very useful approach when aggregation of nanoparticles is under investigation. In fact, recording RLS at or near the absorption wavelength of the colloidal nanomaterial, e.g. gold nanoparticles, the aggregation trend elicits a strong enhancement of RLS, proportional to the analyte inducing the phenomenon. For $A\beta_{1-42}$ detection, RLS-based method coupled to a sandwich strategy was developed by dot-blot immunoassay [92]. The analyte was recognized by two monoclonal antibodies, the secondary carrying a biotin tag to bind streptavidin-coated GNPs. After 30 minutes of incubation, RSL signal was detected. Signal enhancement depends on the size of GNPs aggregates, which correlates with the analyte concentration. $A\beta_{1-42}$ (spiked in the buffer and undiluted CSF) was tested from 20 nM to 2 μM, obtaining a linear dose-response trend. While subnanomolar concentrations of $A\beta_{1-42}$ are usually reported in literature, the authors detected 5 nM in CSF. Nonetheless, such concentration is beyond calibration limits, thus a greater variability is possibly present.

Tau protein was detected by Neely and colleagues [93], who functionalized GNPs with monoclonal anti-tau antibodies. Two-Photon Rayleigh Scattering (TPRS) intensity was used to detect tau in buffer from 7.5 nM down to 0.02 pM (LOD). Signal intensity change is due to the increased multipolar contribution elicited by GNPs aggregation in the presence of tau. BSA and heme (3000 ng mL^{-1}) produced negligible signal, demonstrating the selectivity of the method under tested conditions. However, the repeatability of the assay and its application in CSF samples were not examined.

Finally, Surface Enhanced Raman Spectroscopy (SERS) was used to detect tau in buffer by monitoring of 5,5-dithiobis(2-dinitrobenzoic acid) (DTNB) Raman spectrum [94]. A sandwich assay was established between a polyclonal antibody, immobilized in combination with DTNB on GNPs, and a monoclonal antibody immobilized on magnetic particles. These latter ones were used to obtain maximum recovery yield during the washing steps of the procedure and are potentially useful for tau extraction from CSF, although no real samples were evaluated in the paper. In the presence of the analyte GNPs undergo aggregation, and SERS spectrum was recorded. The peak at 1332 cm^{-1} allowed for tau quantification from 500 nM down to 25 fM (R^2=0.994) with no interference coming from BSA and IgG (host species not specified) at 500 nM. The above GNPs-based approaches are also summarized in table 1-3.

Fluorescent labels

Fluorescent molecules were used as labels to sort ADDLs by size through flow cytometry coupled to Forster Resonance Electron Transfer (FRET) [95]. Two monoclonal antibodies (clones 4G8 and 6E10) were labeled with Alexafluor 488 (donor) or Alexafluor 594 (acceptor), respectively. Depending on the aggregation state of the analyte, the antibodies are sufficiently close (<10 nm) to enable the electron transfer and distinguish ADDLs from amyloid monomers, with a LOD of 10 pM. The assay, applied to 1:1 diluted CSF, displayed a CV% < 5% in all the tested samples. Ammar and colleagues developed an immunosensor by immobilizing anti $A\beta_{1-42}$ antibodies onto silicon surface [96]. Analyte in buffer solution (0.5-1.7 µM) was detected by a sandwich immunoassay, in which the secondary antibody was labeled with cyanine 5 (Cy5). The dose-response was linear within the investigated range (R^2=0.988), but the

obtained LOD resulted above the physiological levels of $A\beta_{1-42}$ in human CSF.

Higher sensitivity was realized by exploiting the Si/SiO_2 property of enhancing the fluorescence intensity by modulating SiO_2 layers thickness to have a constructive interference [97]. $A\beta_{1-42}$ detection was realized in artificial CSF by sandwich assay in which secondary biotinylated mAbs bind a cyanine 3-labeled streptavidin, to give fluorescent signal. The method was linear (R^2=0.993) between 0.9 nM and 20 pM, with an estimated LOD of 18.25 pM. Moreover, the microarray format allowed multiplexing determination by using only 0.4 nL of sample volume. The above fluorescent label-based approaches are also summarized in table 1-3.

Alkaline phosphatase

Alkaline phosphatase (ALP) is largely exploited in bioassays as a label, in view of its outstanding merits. Its use by means of its catalytic activity has been reported also in the field of AD biomarkers detection. Two recent works are based on electrochemical detection of amyloid species, in which a competition takes place between biotinylated $A\beta_{1-42}$ and the native form of the peptide. In the first one, Screen Printed Electrodes (SPE) were used by Rama and colleagues [98] to immobilize biotinylated $A\beta_{1-42}$ *via* streptavidin binding on SPE surface, after its nanostructuring with gold. Then anti-amyloid antibodies and unlabeled antigens were incubated over the sensor. Finally, anti-rabbit IgG labeled with alkaline phosphatase, was added, together with a mixture of 3-indoxyl phosphate with silver ions (3-IP/Ag^+) used as the ALP substrate. The detected signal raised from the reduction of silver ions a. In artificial CSF, the method gave a linear response in the range 0.125-125 pM (0.5-500 ng L^{-1}, R^2=0.991), with a LOD of 0.025 pM in buffer solutions (real samples were not tested).

The second example utilized the red-ox cycle of p-aminophenol (p-AP) as the substrate for ALP-streptavidin conjugate [99]. In presence of tris(2-carboxyethyl)phosphine (TCEP), p-AP red-ox cycle was extended with a significant increase of the amperometric current, allowing for the simultaneous detection of total Aβ and $A\beta_{1-42}$ species. Sensor response decreased when amyloid concentration increased, with an estimated detection limit of 5 pM for both species at mAb-modified electrode, tested in spiked artificial CSF. Both examples highlight the successful potential of electrochemical-based immunosensing by means of ALP-based enzymatic cycling leading to signal enhancement. The above ALP-based approaches are summarized in table 1-3.

Concluding remarks

In summary, immuno-based bioanalytical platforms such as ELISA and XMAP are still the gold reference for conventional AD hallmarks detection at clinical level, integrated to advanced functional and molecular brain imaging techniques. Amyloid-β monomers, *i.e.* $A\beta_{1-40}$ and $A\beta_{1-42}$, are still currently the major players in this field, on the basis of the classical interpretation of the ACH which assigns the pathogenic trigger to these APP sub-products. However, diagnostic meaning of $A\beta_{1-40}/A\beta_{1-42}$ ratio and ADDLs oligomers detection are emerging as complementary information. Tau protein, and some of the possible phosphorylated forms (mainly p-tau_{181}), flanks Aβ in AD biomarkers analyses, even if the related literature is still limited with respect to amyloid-based assays. Emerging immuno-based platforms are still restricted to research level, but their contribution is of utmost importance in the discovery of new biomarkers, creative and sensitive analytical strategies (label-free or label-based), and innovative analytical platforms (e.g. optical and electrochemical (bio)sensors). Depending on the target analyte, especially for amyloid

monomers and oligomers, immunoassays generally require sandwich-based detection strategies and signal enhancers to push detection limits toward clinically significant values. As a whole, antibodies are still the bioreceptors of choice in this field, applied to established and innovative bioanalytical platforms. However, the following paragraphs will discuss the growing role of synthetic (bio)receptors as an alternative to antibodies in AD biomarkers detection.

1.5 Biomimetic receptors as alternative to immuno-detection

In the last decades, great efforts have been directed toward the design and the selection of innovative synthetic receptors that may realize the dream of effective alternatives to classical antibodies. At present, several classes of synthetic receptors have been widely tested at research level and are now also available on the market. Biomimetic receptors share with their natural counterpart the unique feature of recognizing their target analytes in a specific and selective manner, being at the same time smaller in size and easier to be chemically modified. Moreover, their *in-vitro* production permits in general low costs and high stability of the products, which can be elicited toward wider panels of targetable analytes [100]. The most exploited biomimetic receptors for protein analysis are undoubtedly aptamers [101], [102], peptides or peptoids binding proteins [103], and molecularly imprinted polymers (MIP) [104], [105]. Surprisingly, in the field of biomimetic receptors for AD diagnosis, the presence of aptamers and MIPs is still limited. On the contrary, peptides and peptoids (unnatural synthetic N-substituted oligoglycines binding proteins [106], [107], [108]) have found major application as biomimetic receptors.

1.5.1 Peptides and analogues

As shown in table 1-4, their use is prevalently found in electrochemical techniques based on impedance (Electrochemical Impedance Spectroscopy, EIS), and voltammetry (Square Wave Voltammetry (SWV) and Differential Pulse Voltammetry (DPV)).

table 1-4 Biomimetic receptors used for AD biomarkers detection. (a): EIS, Electrochemical Impedance Spectroscopy; SWV, Square Wave Voltammetry; DPV, Differential Pulse Voltammetry.; SPRi, Surface Plasmon Resonance Imaging. (b) ADDLs: Amyloid-Derived Diffusible Ligands. (c) CHO: Chinese hamster ovary cells; CSF: cerebrospinal fluid.

Transducer	Technique (a)	Analyte (b)	Biomimetic receptor	Matrix (c)	LOD	Reference
Electrochemical	EIS	ADDLs	PrP^c fragment	CHO cells	100 pM	[113]
	EIS	ADDLs	PrP^c fragment	serum	3 pM	[114]
	EIS	ADDLs	Nanobodies (A4, A5)	CSF	n.d.	[125]
	SWV	ADDLs	I10 peptide	serum	480 pM	[123]
	SWV	ADDLs	I10 peptide	buffer	48 pM	[115]
	DPV	$A\beta_{1-42}$	Gelsolin	rat CSF and serum	50 pM	[126]
Optical	SPRi	$A\beta_{1-42}$	ADP3 peptoid	serum	2.2 µM	[118]

Among synthetic peptides, Prion Protein fragment (PrP) [109], [110] and I10 peptide, [111] emerged over the last years as extremely promising synthetic receptors for ADDLs and Aβ bioassays.

The cellular prion protein (PrP^C) has been identified in the genome-wide screen as a high-affinity receptor for Aβ oligomers [110], and many studies have also indicated that the core region of PrP^C responsible for the AβO/PrP^C interaction is PrP(95–110, THSQWNKPSKPKTNMK), which is located within the unstructured N-terminal region of PrP^C. It has also been shown that sub-stoichiometric amounts of PrP^C (as small as 1/20) relative to Aβ strongly inhibit amyloid fibrils formation [112]. This effect is specific to the unstructured N-terminal domain of PrP^C and electron microscopy indicates that PrP^C is able to trap Aβ in an oligomeric form.

Rushworth and colleagues immobilized the PrP peptide via biotin/neutravidin interaction [113]. A polymer, polytyramine/poly-3-(4-hydroxyphenyl propionic acid) (POPA), is used on screen-printed gold electrodes to minimize non-specific interactions during the analysis. The synthetic oligomers were incubated on the sensor surface for 20 minutes (1 pM to 1 μM) prior to EIS measurements; then, by increasing ADDLs concentration, a decrease in the charge transfer resistance was observed (Pearson R value = -0.991). In order to evaluate the response in real conditions, the ADDLs produced in Chinese Hamster Ovary (CHO) cells (used as a model for human oligomers) were analyzed, revealing the ability of the sensor to work in complex media.

Liu and co-workers developed a sandwich-based assay in which PrPC was used both for capturing ADDLs and for achieving their detection by means of electrochemical reaction [114]. Cysteine-containing PrP(95–110) was immobilized on a gold electrode to capture Aβ oligomers; then, alkaline phosphatase (ALP)-conjugated PrP(95–110) was used for the recognition of the captured analytes and the generation of electroactive species, leading to a limit of detection of 3 pM (calculated *versus* the molecular weight of the Aβ monomer). Sato and colleagues [111] observed that GxxxG (G=glycine) motifs play a key role as inhibitors able to block fibrils formation. These authors started from a general eight amino acid peptide, RGTFEGKF-NH_2 (I1), characterized by alternated hydrophilic and hydrophobic amino acids. In β-strand secondary structure, the hydrophilic amino acids line one face of the peptide, RxTxExKx, whereas hydrophobic amino acids line the opposite face, xGxFxGxF. The alternating small and large amino acids on the hydrophobic face displayed a stacking activity, and among twelve peptides derived from the first, a number of them confirmed a binding ability in the nanomolar range. In particular, the linear peptide I10 showed the ability to specifically interact

with ADDLs by hydrophobic interactions. The dynamic properties of I10 peptide have been exploited in a novel peptide-based sensing strategy by making use of the analyte-binding induced change of surface Electron Transfer (ET), as well as the fine-tuned Square Wave Voltammetry (SWV) frequency [115]. In particular, the peptide probe carrying an N-terminal modification with 11-mercaptoundecanoic acid (MUA), and a C-terminal modification with Ferrocene, is self-assembled on gold electrodes. After saturation of the surface with 9-mercapto-1-nonanol (MNH), the electrode was incubated (20 h at room temperature, 10 mM PBS, pH 7.4) with concentrations of $A\beta_{1-42}$ soluble oligomer and then rinsed with water. Since SWV currents relative to target-free and target-bound states result very different as the frequency of the square-wave pulse is altered, authors finely tuned this parameter. At 5 Hz they found that target binding can induce slowdown of surface ET, with consequent amplification of the response. Conversely, under the same conditions, the flexible, target-free state of I10 peptide corresponds to a suppressed signal. This allowed for the $A\beta_{1-42}$ soluble oligomer calibration following a signal-on strategy down to 240 pM of the protein in buffer solution. Recovery was assayed in simulated serum spiked with $A\beta_{1-42}$ in undiluted fetal bovine serum with relative errors (%) lower than 5%. In the wake of these first examples, the research for new and improved peptides with similar characteristics have been designed and tested as inhibitors [116]. It is easily predictable that these peptides will be soon exploited in new bioanalytical assays for AD markers detection.

Similarly to peptides, peptoids are unnatural synthetic N-substituted oligoglycines that have been selected from the libraries and applied as antigen surrogates to identify and isolate target antibodies in body fluids [117]. This N-substitution prevents the intra-backbone (BB) hydrogen bonding that is seen in proteins and polypeptides, providing an

opportunity to explore polymer properties and folding in the absence of BB hydrogen bonding. Very recently, a work aimed to $A\beta_{1-42}$ detection in serum samples by SPR imaging biosensing has been published exploiting the peptoid ADP3 [118]. This peptoid was first selected by Reddy and colleagues by a comparative screening of a peptoid library against AD and normal sera, searching for candidate IgG biomarkers and their relative capture agents based on an immunoassay method [108]. They found that ADP3 retains the ability to distinguish AD from healthy sera through its interaction with some biomolecular signature present in AD sera, i.e. $A\beta_{1-42}$ or unknown IgGs present in AD sera.

1.5.2 Cucurbit[n]urils

Another successful example are cucurbit[n]urils (n=5,6,7,8,10) [119], macrocylic compounds composed by glycoluril monomers linked by methylene bridges. Their cavities are highly symmetrical, with two identical openings of variable size depending on n, and have been extensively studied for their host-guest chemistry [120]. Their potential role as drug deliverers has strongly attracted researchers in supramolecular chemistry first [121], but recent advancements in modification and functionalization opened new routes for their application in several fields [122], including sensing. A recent, elegant example combines the I10 protein binding peptide cited above [111] as capturing receptor with cucurbit[8]uril as signal reporter [123]. Such approach was applied to ADDLs and Tumor Necrosis Factor (TNF) indirect detection. Depending on analyte concentration, after its capture by the peptide, the remaining free molecules are able to interact with cucurbituril and its electrochemical reporter. Thus an inverse relation is established between analyte concentration and the signal. This approach allowed for detecting ADDLs in buffer at a concentration of 48 pM.

1.5.3 Single-domain antibodies

The use of single-domain antibodies (also called nanobodies) [124] has also to be mentioned in this framework. Their minimal size, great stability, reversible refolding and outstanding solubility in aqueous solution, is combined with the ability to specifically recognize unique epitopes with subnanomolar affinity. In the race toward new effective receptors for AD diagnosis, A4 nanobody [124] specific for Aβ has been immobilized over a printed circuit and detected by EIS [125]. A second clone, D5, specific for α-synuclein (Parkinson's disease biomarker), was used in parallel experiments and both nanoclones displayed coherent results in diluted CSF on AD, PD patients, or healthy controls.

1.5.4 Gelsolin

Finally, gelsolin was recently reported in two works of Yu and co-workers [126], [127] based on electrochemical transduction. This secretory protein is known to selectively bind soluble Aβ monomers (both $A\beta_{1–40}$ and $A\beta_{1–42}$) in a concentration-dependent manner, but not their oligomeric and fibril forms [128]. Gelsolin is therefore a promising alternative receptor for the detection of total soluble $A\beta_{1–40/1-42}$ in CSF, even if it does not allow for the differentiation between the two forms. Both the cited works use screen-printed carbon electrodes (SPCEs) as electrode surface, which undergo modification with multi-walled carbon nanotubes and gold nanoparticles (GNPs) to facilitate the electron transfer on the film. Gelsolin is then immobilized by amino-coupling and the remaining film surface is finally blocked through bovine serum albumin (BSA) incubation. The direct assay is performed by addition of $A\beta_{1–40/1-42}$ on the sensor surface. To realize the sandwich assay, GNPs in solution are

functionalized with gelsolin and thionine [126], or Horseradish peroxidase (HRP) [127] and added to $A\beta_{1-40/1-42}$ molecules already bound to the electrode. The secondary recognition is allowed since gelsolin displays multiple site recognition ability on $A\beta_{1-40/1-42}$ species. By monitoring thionine reduction signal, a linear relationship was found between 0.1 and 50 nM of Aβ monomers, with a LOD of 50 pM. The inclusion of HRP enzymatic reaction in the detection strategy further improved the sensitivity down to 28 pM. Both methods allowed the differentiation between healthy and AD rat' CSFs, and high selectivity against potential sources of interference, *i.e.* Aβ oligomers, fibrils and protofibrils, was also demonstrated.

1.5.5 State of art on aptamers and MIPs for AD diagnosis

Aptamers are usually selected *in vitro* by Systematic Evolution of Ligands by Exponential enrichment (SELEX), fully reviewed elsewhere [129]. Compared to antibodies, they show no immunogenicity, smaller size, and can be labeled with multiple fluorophores to ensure multi modal recognition [130]. Since the 1990's, with two seminal works in this field, references [101], [102], a huge number of aptamers for therapeutic and/or diagnostic purposes have been selected and tested, two among all the thrombin [131], [132], [133] and TAT protein aptamers [134], [135]. Despite aptamers have stimulated wide interest in clinical diagnostics and therapeutic applications, up to now only few attempts have been directed toward their selection for AD biomarkers detection. An aptamer for $A\beta_{1-40}$ was developed by Ylera and colleagues, who immobilized the target on an affinity chromatography separation column [136]. After eight rounds of selection, several sequences were isolated with apparent dissociation constants (K_d) spanning from 29 to 48 nM. The lowest K_d was obtained for β55 oligonucleotide, which interacted only with amyloid fibrils. Such

feature was due to the specific interaction of the aptamer with β-sheets assumed by the target during aggregation. Such hypothesis was also supported by Rahimi and colleagues [137], who used covalently stabilized $A\beta_{1-42}$ trimers as target for the aptamer selection. After 12 rounds of selection based on filter binding partitioning technique, 33 clones were selected. Surprisingly, no interaction with trimers was found for the two best candidates (KM33, KM41). Otherwise, the aptamers showed affinity toward several kinds of amyloid fibrils, either formed by Aβ peptides or by other amyloidogenic molecules such as lysozyme, prion peptide etc. Despite the scarce specificity showed as receptors, aptamers could find use in replacing amyloidogenic marker (thioflavine) to detect early β-sheets structures. This suggestion was exploited for *ex vivo* application of β55 aptamer on natural amyloid plaques [130]. From this point of view, aptamers showed better features compared to antibodies because of the ease of labeling (by inserting fluorescent nucleotides) and delivery into living cells. Nonetheless, the absence of analytical applications and the observed cross-reactivity, suggest the need for further efforts in the aptamer selection for AD biomarkers detection. In this framework, also aptamers against whole tau protein have not yet been developed and proposed. A pioneering selection was recently carried out by Flu-Mag SELEX [138], against a peptide corresponding to amino acids 226-240 of the tau protein, in which the target analyte is bound to magnetic beads allowing for their easy separation. Thirteen rounds of selection were performed obtaining 47 clones with the best binding affinity of K_d=76 nM. To note, preliminary experiments have been reported by joint work by Minunni and Peyrin groups for the capillary electrophoresis-SELEX based selection of DNA aptamers against whole protein tau [139], and a very recent work has also been reported [140].

Finally, MIPs development for large biomolecule binding, e.g. proteins, is an emerging field that only in the last years has found some innovative strategy to obtain reliable results applicable to bioassays [141]. One of the most promising approaches is undoubtedly the so called 'epitope imprinting', which differs from past strategies by substituting the classical whole molecule imprinting method with a smart and effective imprinting of short (≥ 9 aa) and surface epitopes [142] of the protein. To do this, synthetic peptides are exploited to mimic the epitope, and tailored mixtures of monomers and cross linkers (depending on the amino acids composition) are used through different methods to synthesize MIPs, even at nanoscale [143].

Epitope imprinting has been therefore attempted for the imprinting of the C-termini of $A\beta_{1-40}$ and $A\beta_{1-42}$ corresponding to the sequences $A\beta_{33-40}$ and $A\beta_{33-42}$. MIPs were synthesized using two hexapeptides as templates [144], and their binding properties were evaluated comparing MIP affinity with non-imprinted polymers (NIPs) after incubation for 24 h of polymer particles with the analyte under denaturing conditions. The most promising polymers were immobilized on solid phase extraction cartridges used to enrich serum samples spiked both with 625 nM (2.5 mg L^{-1}) of the templates and full length $A\beta_{1-40}$ (5 μg L^{-1}) or $A\beta_{1-42}$ (1 μg L^{-1}). Eluted fractions were analyzed by HPLC showing linear dependence (R^2=0.999) between 0.125-12.5 μM (0.5-50 mg L^{-1}). Recoveries at spiked levels were also analyzed by SDS-PAGE of eluted fractions. Interestingly, MIPs presented similar performances both for templates and for full-length species. This attempt, the first at our best knowledge, shows that MIPs are promising new biosynthetic receptors with encouraging perspective, both in fundamental studies of peptide aggregation and AD diagnostics.

1.5.6 Concluding remarks

In summary, new routes are opening toward the application of biomimetic receptors in the field of AD biomarkers detection, mainly peptides and peptoids. The advantages offered by their unique characteristics (mainly ease of production, modification, and improved stability compared to antibodies) can be effectively exploited to transduce electrochemical or optical signals. Great efforts are still needed to expand their applicability, mainly aimed to explore and control their specificity toward target analytes in real matrices. Sensing platforms, from electrochemical to optical ones, find room to host synthetic bioreceptors with high versatility and potentialities, but new sensitive and assessed detection strategies are still required. In this panorama, the selection of aptamers and the synthesis of molecularly imprinted polymers (MIPs) targeting AD biomarkers could further enrich the possibilities given by biosynthetic receptors in rivaling antibodies. However, much work is still needed in this direction, and the overall feeling is that results strictly depend on the elucidation of the physiopathological mechanisms of AD, under continuous revision, and the identification of effective biomarkers.

1.6 Conclusions and future outlooks

This review provides a critical viewpoint on bioanalytical approaches to CSF AD biomarkers detection, moving from conventional assays based on antibodies as bioreceptors (ELISA and xMAP) toward innovative approaches both in terms of bioanalytical platforms and synthetic receptors. Conventional methods are still the best choice for routine diagnosis, while more likely emerging techniques are going to play a role in the study of marker levels variability and aggregation patterns. Among currently investigated AD biomarkers, we focused the discussion on the

most validated core biomarkers, i.e. $A\beta_{1\text{-}40/1\text{-}42}$, total-τ, and p-$tau_{181}$. At present, the majority of the works is related to the investigation of amyloid species, while only few focused on the tau protein detection. A similar trend is found considering the capturing probes, *i.e.* antibody-based assays are predominant compared to the exploration of new receptors for AD detection.

Signal amplification based on labeled or unlabeled strategies showed to be effective to achieve adequate sensitivity, but related protocols are usually more complex with possible analyte loss over steps. Moreover, conjugation of antibodies to labels is a delicate step which may result in reproducibility loss and increase of analysis costs. Concerning the choice of selective and sensitive bioreceptors as an alternative to antibodies, a number of encouraging examples are emerging in the literature. Most of these synthetic receptors are peptides or related species, while aptamers and MIPs still found very few applications to biomarker quantification. As a whole, their employment as synthetic receptors is still in its germinal stage, but promising results have already been obtained.

Some aspects related to AD biomarkers detection, besides those covered here, deserve to be mentioned in conclusion. In particular, the identification of alternative sampling matrices and new hallmarks has undoubtedly a relevant role in the near future of AD diagnosis [145]. CSF is still considered the elective source for biomarker detection, as it is in direct contact with the extracellular space of the brain and can reflect biochemical changes that occur inside the brain. However, alternative matrices are currently under consideration for conventional biomarkers detection, *i.e.* serum/plasma, urine, saliva [146], [147], [148]. At the same time, alternative matrices are of great interest also in the identification of new AD biomarkers [149]. To our best knowledge, despite the obvious advantages non-invasive and peripheral sampling, to date controversial

and limited results have been reported in literature [150], and a clinical applicability is far from reality. Brain derived proteins exist in much smaller concentrations in peripheral blood or urine than in CSF, due to the function of the blood–brain barrier and the large total volume of blood and urine in which they are diluted; further complications include significant binding of many proteins of interest and rapid clearance from the blood, which may make many conventional assays insufficiently sensitive [151]. This may change with the development of more sensitive metabolomic and proteomic approaches being used for biomarker discovery.

The route toward early and effective AD diagnosis is still a challenge, but the perspective of achieving reliable, sensitive and hopefully predictive bioassays is mandatory. Several technical aspects such as sampling, handling and storage of CSF samples are related to inter-laboratory variability, which results significant even if tested on validated commercial platforms. Moreover, the complexity of AD pathogenesis, far from being unraveled, results in the production of several related biomarker species (i.e. monomers, oligomers, fibrils, etc.). This reflects and could eventually elicit the currently investigated inter-laboratory variability, thus going deeper into the research in this field is necessary. Behind these considerations related to *ex vivo* analysis, the role of *in vivo* imaging approaches, which achieved important results in the field, should be finally considered, believing that joint efforts interesting progresses in AD anticipated diagnostic may come in the next future.

References

[1] L. Jarvik and H. Greenson, About a peculiar disease of the cerebral cortex. By Alois Alzheimer, 1907, Alzheimer Dis. Assoc. Disord. 1 (1987) 3–8.

[2] H. Hippius, and G. Neundörfer. The discovery of Alzheimer's disease, Dialogues Clin. Neurosci. 5 (2003) 101–108.

[3] K. Blennow, H. Hampel, M. Weiner, H. Zetterberg, Cerebrospinal fluid and plasma biomarkers in Alzheimer disease, Nat. Rev. Neurol. 6 (2010) 131–144.

[4] C. Humpel, Identifying and validating biomarkers for Alzheimer's disease, Trends Biotechnol. 29 (2011) 26–32.

[5] A.P.F. Turner, Biosensors: sense and sensibility, Chem. Soc. Rev. 42 (2013) 3184-3196.

[6] J.T. O'Brien, Role of imaging techniques in the diagnosis of dementia. Br. J. Radiol. 2 (2007) S71-77.

[7] L. Narayanan and A.D. Murray, What can imaging tell us about cognitive impairment and dementia? World J. Radiol. 8 (2016) 240-254.

[8] R.M. Ahmed, R.W. Paterson, J.D. Warren, H. Zetterberg, J.T. O'Brien, N.C. Fox, G.M. Halliday, J.M. Schott, J. Biomarkers in dementia: clinical utility and new directions, Neurol. Neurosurg. Psychiatry 85 (2014) 1426-1434.

[9] J.A. Hardy, G.A. Higgins, Alzheimer's disease: the amyloid cascade hypothesis, Science, 256 (1992) 184–185.

[10] C. Reitz, Alzheimer's Disease and the Amyloid Cascade Hypothesis: A Critical Review, Int J. Alzheimers Dis. 2012 (2012), Article ID 369808, 1-11.

[11] F. Hernández, J. Avila, Tauopathies, Cell. Mol. Life Sci. 64 (2007) 2219–2233.

[12] K. Herrup, The case for rejecting the amyloid cascade hypothesis, Nature Neurosci. (2015) 794–799.

[13] L.M. Shaw, H. Vanderstichele, M. Knapik-czajka, C.M. Clark, P.S. Aisen, R.C. Petersen, K. Blennow, H. Soares, A. Simon, P. Lewczuk, R. Dean, E. Siemers, W. Potter, V.M. Lee, J.Q. Trojanowski, Alzheimer's Disease Neuroimaging Initiative, Cerebrospinal fluid biomarker signature in Alzheimer's disease neuroimaging initiative subjects, Ann. Neurol. 65 (2009) 403–413.

[14] C. Haass, D.J. Selkoe, Soluble protein oligomers in neurodegeneration: lessons from the Alzheimer's amyloid β-peptide, Nat. Rev. Mol. Cell Bio. 8 (2007) 101–112.

[15] L. Buée, T. Bussière, V. Buée-Scherrer, A. Delacourte, P.R. Hof, Tau protein isoforms, phosphorylation and role in neurodegenerative disorders, Brain Res. Rev. 33 (2000) 95–130.

[16] N. Sergeant, A. Delacourte, L. Buée, Tau protein as a differential biomarker of tauopathies, Biochim. Biophys. Acta 1739 (2005) 179–197.

[17] H. Vanderstichele, M. Bibl, S. Engelborghs, N. Le Bastard, P. Lewczuk, J.L. Molinuevo, L. Parnetti, A. Perret-Liaudet, L.M. Shaw, C. Teunissen, D. Wouters, K. Blennow, Standardization of preanalytical aspects of cerebrospinal fluid biomarker testing for Alzheimer's disease diagnosis: a consensus paper from the Alzheimer's Biomarkers Standardization Initiative, Alzheimers Dement. 8 (2012) 65–73.

[18] F. Mangialasche, M.C. Polidori, R. Monastero, S. Ercolani, C. Camarda, R. Cecchetti, P. Mecocci, Biomarkers of oxidative and nitrosative damage in Alzheimer's disease and mild cognitive impairment, Ageing Res. Rev. 8 (2009) 285–305.

[19] H. Hampel, D. Prvulovic, S. Teipel, F. Jessen, C. Luckhaus, L. Frölich, M.W. Riepe, R. Dodel, T. Leyhe, L. Bertram, W. Hoffmann, F. Faltraco, German Task Force on Alzheimer's Disease (GTF-AD), The future of Alzheimer's disease: the next 10 years, Prog. Neurobiol. 95 (2011) 718–728.

[20] H. Hampel, K. Bürger, S.J. Teipel, A.L.W. Bokde, H. Zetterberg, K. Blennow, Core candidate neurochemical and imaging biomarkers of Alzheimer's disease, Alzheimers Dement. 4 (2008) 38–48.

[21] J. Hardy, D. J. Selkoe, The amyloid hypothesis of Alzheimer's disease: progress and problems on the road to therapeutics, Science 297 (2002) 353–356.

[22] A.J. Haes, L. Chang, W. L. Klein, R.P. Van Duyne, Detection of a biomarker for Alzheimer's disease from synthetic and clinical samples using a nanoscale optical biosensor, J. Am. Chem. Soc. (2005) 2264–2271.

[23] Y. Gu, F. Oyama, and Y. Ihara, Tau is widely expressed in rat tissues, J. Neurochem. 67 (1996) 1235–1244 .

[24] J.Q. Trojanowski, T. Schuck, M.L. Schmidt, V.M. Lee, Distribution of tau proteins in the normal human central and peripheral nervous system, J. Histochem. Cytochem. 37 (1989) 209–215.

[25] E.M. Mandelkow, O. Schweers, G. Drewes, J. Biernat, N. Gustke, B. Trinczek, and E. Mandelkow, Structure, Microtubule Interactions, and Phosphorylation of Tau Protein, Ann. N. Y. Acad. Sci. 777 (1996) 96-106.

[26] S. Jeganathan, M. Von Bergen, E.M. Mandelkow, E. Mandelkow, The natively unfolded character of Tau and its aggregation to Alzheimer-like paired helical filaments, Biochemistry 47 (2008) 10526–10539.

[27] M. Von Bergen, P. Friedhoff, J. Biernat, J. Heberle, E.M. Mandelkow, E. Mandelkow, Assembly of protein into Alzheimer paired helical filaments depends on a local sequence motif (306 VQIVYK 311) forming β structure, Proc. Natl. Acad. Sci. 97 (2000) 5129–5134.

[28] M. Kolarova, F. García-Sierra, A. Bartos, J. Ricny, D. Ripova, Structure and pathology of tau protein in Alzheimer disease, Int. J. Alzheimers. Dis. 2012 (2012) 1-13.

[29] D.P. Hanger, H.L. Byers, S. Wray, K.Y. Leung, M.J. Saxton, A. Seereeram, C.H. Reynolds, M.A. Ward, and B.H. Anderton, Novel phosphorylation sites in tau from Alzheimer brain support a role for casein kinase 1 in disease pathogenesis, J. Biol. Chem. 282 (2007) 23645-23654.

[30] D.P. Hanger, J.P. Brion, J.M. Gallo, N.J. Cairns, P.J. Luthert, and B.H. Anderton, Tau in Alzheimer's disease and Down's syndrome is insoluble and abnormally phosphorylated, Biochem. J. 275 (1991) 99–104.

[31] M. Morishima-Kawashima, M. Hasegawa, K. Takio, M. Suzuki, H. Yoshida, K. Titani, and Y. Ihara Proline-directed and non-proline-directed phosphorylation of PHF-tau, J. Biol. Chem. 270 (1995) 823–829.

[32] D.P. Hanger, J.C. Betts, T.L. Loviny, W.P. Blackstock, and B.H. Anderton, New phosphorylation sites identified in hyperphosphorylated tau (paired helical filament-tau) from Alzheimer's disease brain using nanoelectrospray mass spectrometry, J. Neurochem. 71 (1998) 2465–2476.

[33] I.A. Tavares, D. Touma, S. Lynham, C. Troakes, M. Schober, M. Causevic, R. Garg, W. Noble, R. Killick, I. Bodi, D.P. Hanger, and J.D. Morris, Prostate-derived sterile 20-like kinases (PSKs/TAOKs) phosphorylate tau protein and are activated in tangle-bearing neurons in Alzheimer disease, J. Biol. Chem. 288 (2013) 15418–15429.

[34] K. Buerger, R. Zinkowski, S.J. Teipel, T.H. Arai, H.K. Blennow, K.N. Andreasen, K. Hofmann-Kiefer, D. Kerkman, C. McCulloch, R. Kohnken, F. Padberg, T. Pirttila, M.B. Schapiro, S.I. Rapoport, H.J. Moller, P. Davies, H. Hampel, Differential diagnosis of Alzheimer disease with cerebrospinal fluid levels of tau protein phosphorylated at threonine 231, Arch. Neurol. 59 (2002) 1267–1272.

[35] L. Martin, X. Latypova, F. Terro, Post-translational modifications of tau protein: implications for Alzheimer's disease, Neurochem. Int. 58 (2011) 458-471.

[36] J.H. Kang, M. Korecka, J.B. Toledo, J.Q. Trojanowski, L.M. Shaw, Clinical utility and analytical challenges in measurement of cerebrospinal fluid amyloid-β(1-42) and tau proteins as Alzheimer disease biomarkers, Clin. Chem. 59 (2013) 903–916.

[37] E. Vanmechelen, H. Vanderstichele, P. Davidsson, E. Van Kerschaver, B. Van Der Perre, M. Sjogren, N. Andreasen, K. Blennow, Quantification of tau phosphorylated at threonine 181 in human cerebrospinal fluid : a sandwich ELISA with a synthetic phosphopeptide for standardization, Neurosci. Lett. 285 (2000) 49–52.

[38] H. Vanderstichele, E. Van Kerschaver, C. Hesse, P. Davidsson, M.-A. Buyse, N. Andreasen, L. Minthon, A. Wallin, K. Blennow, E. Vanmechelen, Standardization of measurement of β-amyloid (1-42) in cerebrospinal fluid and plasma, Amyloid 7 (2000) 245–258.

[39] R.F. Gordon, R.L. McDade, Multiplexed quantification of human IgG, IgA, and IgM with the FlowMetrix system, Clin. Chem, 43 (1997) 1799–1801.

[40] A. Olsson, H. Vanderstichelen, N. Andreasen, G. De Meyer, A. Wallin, B. Holmberg, L. Rosengren, E. Vanmechelen, K. Blennow, Simultaneous measurement of β-amyloid(1-42), total tau, and phosphorylated tau (Thr181) in cerebrospinal fluid by the xMAP technology, Clin. Chem. 51 (2005) 336–345.

[41] L.-S. Wang, Y.Y. Leung, S.-K. Chang, S. Leight, M. Knapik-Czajka, Y. Baek, L.M. Shaw, V.M.-Y. Lee, J.Q. Trojanowski, and C.M. Clark, Comparison of xMAP and ELISA assays for detecting CSF biomarkers of Alzheimer's Disease, J. Alzheimers Dis. 31 (2012) 439–445.

[42] J.H. Kang, H. Vanderstichele, J.Q. Trojanowski, L.M. Shaw, Simultaneous analysis of cerebrospinal fluid biomarkers using microsphere-based xMAP multiplex technology for early detection of Alzheimer's disease, Methods 56 (2012) 484-493.

[43] J.K.H. Liu, The history of monoclonal antibody development - Progress, remaining challenges and future innovations, Ann. Med. Surg. 3 (2014) 113–116.

[44] A. Kaushik, R.D. Jayant, S. Tiwari, A. Vashist, M. Nair, Nano-biosensors to detect beta-amyloid for Alzheimer's disease management, Biosens. Bioelectron. 80 (2016) 273–287.

[45] H.V.S. Ganesh, A.M. Chow, K. Kerman, Recent advances in biosensors for neurodegenerative disease detection, Trends Anal. Chem. Epub 23 February 2016.

[46] J. Homola, Surface Plasmon Resonance Sensors for Detection of Chemical and Biological Species, Chem. Rev. 108 (2008) 462–493.

[47] S. Scarano, M. Mascini, A.P.F. Turner, M. Minunni, Surface plasmon resonance imaging for affinity-based biosensors, Biosens. Bioelectron. 25 (2010) 957–966.

[48] S. Mariani, M. Minunni, Surface plasmon resonance applications in clinical analysis, Anal. Bioanal. Chem. 406 (2014) 2303–2323.

[49] K.A. Willets, R.P.V. Duyne, Localized surface plasmon resonance spectroscopy and sensing, Annu. Rev. Phys. Chem. 58 (2007) 267–297.

[50] L. Liu, N. Xia, J. Wang, Potential applications of SPR in early diagnosis and progression of Alzheimer's disease, RSC Adv. 2 (2012) 2200-2204.

[51] X.R. Cheng, V.W. Sze Hung, S. Scarano, M. Mascini, M. Minunni, K. Kerman, Label-free methods for probing the interaction of clioquinol with amyloid-β, Anal. Methods 4 (2012) 2228–2232.

[52] A. Barrantes, J. Sotres, M. Hernando-Pérez, M.J. Benítez, P.J. de Pablo, A.M. Baró, J. Avila, J.S. Jimenez, Tau aggregation followed by atomic force microscopy and surface plasmon resonance, and single molecule tau-tau interaction probed by atomic force spectroscopy, J. Alzheimers. Dis. 18 (2009) 141–151.

[53] J.H. Lee, D.Y. Kang, T. Lee, S.U. Kim, B.K. Oh, J.W. Choi, Signal enhancement of surface plasmon resonance based immunosensor using gold nanoparticle-antibody complex for β-Amyloid (1-40) detection, J. Nanosci Nanotechnol. 9 (2009) 7155–7160.

[54] A. Makaraviciute, A. Ramanaviciene, Site-directed antibody immobilization techniques for immunosensors, Biosens. Bioelectron. 50 (2013) 460–471.

[55] G.B. Sigal, C. Bamdad, A. Barberis, J. Strominger, G.M. Whitesides, A self-assembled monolayer for the binding and study of Histidine-tagged proteins by surface plasmon resonance, Anal. Chem. 68 (1996) 490–497.

[56] N. Xia, L. Liu, M.G. Harrington, J. Wang, F. Zhou, Regenerable and simultaneous surface plasmon resonance detection of Aβ(1−40) and Aβ(1−42) peptides in cerebrospinal fluids with signal amplification by streptavidin conjugated to an N-terminus-specific antibody, Anal. Chem. 82 (2010) 10151–10157.

[57] J. Guo, T. Arai, J. Miklossy, P.L. Mcgeer, Aβ and tau form soluble complexes that may promote self aggregation of both into the insoluble forms observed in Alzheimer's disease, Proc. Natl. Acad. Sci. 103 (2006) 1953–1958.

[58] Z. Kristofikova, D. Ripova, K. Hegnerová, J. Sirova, J. Homola, Protein tau-mediated effects on rat hippocampal choline transporters CHT1 and tau-amyloid β interactions, Neurochem. Res. 38 (2013) 1949–1959.

[59] G. Spoto, M. Minunni, Surface Plasmon Resonance Imaging: What Next?, J. Phys. Chem. Lett. 3 (2012) 2682–2691.

[60] K.S. Lee, J.M. Son, D.Y. Jeong, T.S. Lee, W.M. Kim, Resolution enhancement in surface plasmon resonance sensor based on waveguide coupled mode by combining a bimetallic approach, Sensors 10 (2010) 11390–11399.

[61] Y.K. Lee, K.-S. Lee, W.M. Kim, Y.-S. Sohn, Detection of Amyloid-β42 using a waveguide-coupled bimetallic surface plasmon resonance sensor chip in the intensity measurement mode, PLoS ONE 9 (2014) e98992.

[62] P. Palladino, A.M. Aura, G. Spoto, Surface plasmon resonance for the label-free detection of Alzheimer's β-amyloid peptide aggregation, Anal. Bioanal. Chem. 408 (2016) 849–854.

[63] X. Yi, C. Feng, S. Hu, H. Li, J. Wang, Surface plasmon resonance biosensors for simultaneous monitoring of amyloid-beta oligomers and fibrils and screening of select modulators, Analyst 141 (2016) 331–336.

[64] D. Erickson, S. Mandal, A.H.J. Yang, B. Cordovez, Nanobiosensors: optofluidic, electrical and mechanical approaches to biomolecular detection at the nanoscale, Microfluid. Nanofluidics 4 (2007) 33–52.

[65] A.J. Haes, W.P. Hall, L. Chang, W.L. Klein, R.P.V. Duyne, A localized surface plasmon resonance biosensor: first steps toward an assay for Alzheimer's disease, Nano Lett. 4 (2004) 1029–1034.

[66] D.R. Jung and A.W. Czanderna, Metal overlayers on organic functional groups of self-assembled monolayers, VI. X-ray photoelectron spectroscopy of Cr/COOH on 16-mercaptohexadecanoic acid, J. Vac. Sci. Technol. A 13 (1995) 1337-1344.

[67] M.D. Vestergaard, K. Kerman, D.-K. Kim, H.M. Hiep, E. Tamiya, Detection of Alzheimer's tau protein using localised surface plasmon resonance-based immunochip, Talanta. 74 (2008) 1038–1042.

[68] F.B. Myers, and L.P. Lee, Innovations in optical microfluidic technologies for point-of-care diagnostics, Lab. Chip, 8 (2008) 2015-2031.

[69] D. Erickson, S. Mandal, A.H.J. Yang, B. Cordovez, Nanobiosensors: optofluidic, electrical and mechanical approaches to biomolecular detection at the nanoscale, Microfluid. Nanofluid., 4 (2008) 33–52.

[70] M. Vestergaard, K. Kerman, E. Tamiya, An Overview of Label-free Electrochemical Protein Sensors, Sensors 7 (2007) 3442–3458.

[71] M. Vestergaard, K. Kerman, M. Saito, N. Nagatani, Y. Takamura, A rapid label-free electrochemical detection and kinetic study of Alzheimer's amyloid β aggregation, J. Am. Chem. Soc. 37 (2005) 11892–11893.

[72] K. Kerman, M.D. Vestergaard, M. Chikae, S. Yamamura, E. Tamiya, Label-free electrochemical detection of the phosphorylated and non-phosphorylated forms of peptides based on tyrosine oxidation, Electrochem. Commun. 9 (2007) 976–980.

[73] K. Kerman, M. Vestergaard, E. Tamiya, Label-free electrical sensing of small-molecule inhibition on tyrosine phosphorylation, Anal. Chem. 79 (2007) 6881–6885.

[74] S. Prabhulkar, R. Piatyszek, J.R. Cirrito, Z.-Z. Wu, C.-Z. Li, Microbiosensor for Alzheimer's disease diagnostics: detection of amyloid β biomarkers, J. Neurochem. 122 (2012) 374–381.

[75] T. Lien, Y. Takamura, E. Tamiya, M.C. Vestergaard, Modified screen printed electrode for development of a highly sensitive label-free impedimetric immunosensor to detect amyloid β peptides, Anal. Chim. Acta 892 (2015) 69–76.

[76] J. Oh, G. Yoo, Y.W. Chang, H.J. Kim, J. Jose, E. Kim, J.C. Pyun, K.H. Yoo, A carbon nanotube metal semiconductor field effect transistor-based biosensor for detection of amyloid-beta in human serum, Biosens. Bioelectron. 50 (2013) 345–350.

[77] J. Jose, M. Park, J.-C. Pyun, E. coli outer membrane with autodisplayed Z-domain as a molecular recognition layer of SPR biosensor, Biosens. Bioelectron. 25 (2010) 1225–1228.

[78] J.O. Esteves-Villanueva, H. Trzeciakiewicz, S. Martic, A protein-based electrochemical biosensor for detection of tau protein, a neurodegenerative disease biomarker, Analyst 139 (2014) 2823–2831.

[79] T. Sano, C. Smith, C. Cantor, Immuno-PCR: very sensitive antigen detection by means of specific antibody-DNA conjugates, Science 258 (1992) 120–122.

[80] J.-M. Nam, C.S. Thaxton, C.A. Mirkin, Nanoparticle-based bio-barcodes for the ultrasensitive detection of proteins, Science 301 (2003) 1884–1886.

[81] C.M. Niemeyer, M. Adler, R. Wacker, Detecting antigens by quantitative immuno-PCR, Nat. Protoc. 2 (2007) 1918–1930.

[82] M. Hashimoto, M. Aoki, B. Winblad, L.O. Tjernberg, A novel approach for Aβ1–40 quantification using immuno-PCR, J. Neurosci. Meth. 205 (2012) 364–367.

[83] M.G. Spillantini, M. Goedert, Tau protein pathology in neurodegenerative diseases, Trends Neurosci. 2236 (1998) 428–433.

[84] C. Luk, Y. Compta, N. Magdalinou, M.J. Martí, G. Hondhamuni, H. Zetterberg, K. Blennow, R. Constantinescu, Y. Pijnenburg, B. Mollenhauer, C. Trenkwalder, J. Van Swieten, W.Z. Chiu, B. Borroni, A. Cámara, P. Cheshire, D.R. Williams, A.J. Lees, R. de Silva, Development and assessment of sensitive immuno-PCR assays for the quantification of cerebrospinal fluid three- and four-repeat tau isoforms in tauopathies, J. Neurochem. 123 (2012) 396–405.

[85] C. Luk, G. Giovannoni, D.R. Williams, A.J. Lees, R. de Silva, Development of a sensitive ELISA for quantification of three- and four-repeat tau isoforms in tauopathies, J. Neurosci. Meth. 180 (2009) 34–42.

[86] H. Hampel, K. Buerger, R. Zinkowski, S.J. Teipel, A. Goernitz, N. Andreasen, M. Sjoegren, J. DeBernardis, D. Kerkman, K. Ishiguro, H. Ohno, E. Vanmechelen, H. Vanderstichele, C. McCulloch, H.J. Moller, P. Davies, K. Blennow, Measurement of phosphorylated Tau epitopes in the differential diagnosis of Alzheimer disease: a comparative cerebrospinal fluid study, Arch. Gen. Psychiatry 61 (2004) 95–102.

[87] J. Avila, J.J. Lucas, M. Perez, F. Hernandez, Role of tau protein in both physiological and pathological conditions, Physiol. Rev. 84 (2004) 361–384.

[88] D. Singer, H. Soininen, I. Alafuzoff, R. Hoffmann, Immuno-PCR-based quantification of multiple phosphorylated tau-epitopes linked to Alzheimer's disease, Anal. Bioanal. Chem. 395 (2009) 2263–2267.

[89] L. Stegurová, E. Dráberová, A. Bartos, P.P. Dráber, D. Rípová, P. Dráber, Gold nanoparticle-based immuno-PCR for detection of tau protein in cerebrospinal fluid, J. Immunol. Methods 406 (2014) 137–142.

[90] D.G. Georganopoulou, L. Chang, J. Nam, C.S. Thaxton, E.J. Mufson, W.L. Klein, C.A. Mirkin, Nanoparticle-based detection in cerebral spinal fluid of a soluble pathogenic biomarker for Alzheimer's disease, Proc. Natl. Acad. Sci. 102 (2005) 2273–2276.

[91] T.A. Taton, C.A. Mirkin, R.L. Lestinger, Scanometric DNA Array Detection with Nanoparticle Probes, Science 289 (2000) 1757–1760.

[92] C. Wang, D. Liu, Z. Wang, Gold nanoparticle based dot-blot immunoassay for sensitively detecting Alzheimer's disease related β-amyloid peptide, Chem. Comm. 48 (2012) 8392–8394.

[93] A. Neely, C. Perry, B. Varisli, A.K. Singh, T. Arbneshi, D. Senapati, J.R. Kalluri, P.C. Ray, Ultrasensitive and highly selective detection of Alzheimer's disease biomarker using two-photon Rayleigh scattering properties of gold nanoparticle, ACS Nano 3 (2009) 2834–2840.

[94] A. Zengin, U. Tamer, T. Caykara, A SERS-based sandwich assay for ultrasensitive and selective detection of Alzheimer's Tau protein, Biomacromolecules 14 (2013) 3001–3009.

[95] A.N. Santos, S. Torkler, D. Nowak, C. Schlittig, M. Goerdes, T. Lauber, L. Trischmann, M. Schaupp, M. Penz, F.W. Tiller, G. Bohm, Detection of amyloid-β oligomers in human cerebrospinal fluid by flow cytometry and fluorescence resonance energy transfer, J. Alzheimers Dis. 11 (2007) 117–125.

[96] M. Ammar, C. Smadja, L.G.T. Phuong, M. Azzouz, J. Vigneron, A. Etcheberry, M. Taverna, E. Dufour-Gergam, A new controlled concept of immune-sensing platform for specific detection of Alzheimer's biomarkers, Biosens. Bioelectron. 40 (2013) 329–335.

[97] P. Gagni, L. Sola, M. Cretich, M. Chiari, Development of a high-sensitivity immunoassay for amyloid-β 1–42 using a silicon microarray platform, Biosens. Bioelectron. 47 (2013) 490–495.

[98] E.C. Rama, M.B. González-García, A. Costa-García, Competitive electrochemical immunosensor for amyloid-β 1-42 detection based on gold nanostructurated screen-printed carbon Electrodes, Sensor. Actuat. B-Chem. 201 (2014) 567–571.

[99] L. Liu, Q.G. He, F. Zhao, N. Xia, H.J. Liu, S.J. Li, R.L. Liu, H. Zhang, Competitive electrochemical immunoassay for detection of β-amyloid (1–42) and total β-amyloid peptides using p-aminophenol redox cycling, Biosens. Bioelectron. 51 (2014) 208–212.

[100] F.L. Dickert, Biomimetic receptors and sensors, Sensors 14 (2014) 22525–22531.

[101] A.D. Ellington, J.W. Szostak, In vitro selection of RNA molecules that bind specific ligands, Nature 346 (1990) 818–822.

[102] C. Tuerk, L. Gold, Systematic evolution of ligands by exponential enrichment: RNA ligands to bacteriophage T4 DNA polymerase, Science 249 (1990) 505–510.

[103] P. Colas, B. Cohen, T. Jessen, I. Grishina, J. McCoy, R. Brent, Genetic selection of peptide aptamers that recognize and inhibit cyclin-dependent kinase 2, Nature 380 (1996) 548–550.

[104] K. Haupt, K. Mosbach, Molecularly imprinted polymers and their use in biomimetic sensors, Chem. Rev. 100 (2000) 2495–2504.

[105] C. Alexander, H.S. Andersson, L.I. Andersson, R.J. Ansell, N. Kirsch, I.A. Nicholls, J. O'Mahony, M.J Whitcombe, Molecular imprinting science and technology: a survey of the literature for the years up to and including 2003, J. Mol. Recognit. 19 (2006) 106–180.

[106] C.M. Gao, A.Y. Yam, X. Wang, E. Magdangal, C. Salisbury, D. Peretz, R.N. Zuckermann, M.D. Connolly, O. Hansson, L. Minthon, H. Zetterberg, K. Blennow, J.P. Fedynyshyn, S. Allauzen, β40 oligomers identified as a potential biomarker for the diagnosis of Alzheimer's disease, PLoS ONE 5 (2010) e15725.

[107] A.Y. Yam, X. Wang, C.M. Gao, M.D. Connolly, R.N. Zuckermann, T. Bleu, J. Hall, J.P. Fedynyshyn, S. Allauzen, D. Peretz, C.M. Salisbury, A Universal method for detection of amyloidogenic misfolded proteins, Biochemistry 50 (2011) 4322–4329.

[108] M.M. Reddy, R. Wilson, J. Wilson, S. Connell, A. Gocke, L. Hynan, D. German, T. Kodadek, Identification of candidate IgG biomarkers for Alzheimer's disease via combinatorial library screening, Cell 144 (2011) 132–142.

[109] M.P. Lambert, K. Barlow, B. Chromy, C. Edwards, R. Freed, M. Liosatos, T.E. Morgan, I.B. Rozovsky, B.K.L. Trommer, K.L. Viola, P. Wals, C. Zhang, C.E. Finch, G.A. Krafft, W.L. Klein, Diffusible, nonfibrillar ligands derived from Aβ 1-42 are potent central nervous system neurotoxins, Proc. Natl. Acad. Sci. 95 (1998) 6448–6453.

[110] J. Laurén, D.A. Gimbel, H.B. Nygaard, J.W. Gilbert, S.M. Strittmatter, Cellular prion protein mediates impairment of synaptic plasticity by amyloid-β oligomers, Nature 457 (2009) 1128–1132.

[111] T. Sato, P. Kienlen-Campard, M. Ahmed, W. Liu, H. Li, J.I. Elliott, S. Aimoto, S.N. Constantinescu, J.N. Octave, S.O. Smith, Inhibitors of amyloid toxicity based on β-sheet packing of Aβ40 and Aβ42, Biochemistry 45 (2006) 5503–5516.

[112] N.D. Younan, C.J. Sarell, P. Davies, D.R. Brown, J.H. Viles, The cellular prion protein traps Alzheimer's Aβ in an oligomeric form and disassembles amyloid fibers, FASEB J. 27 (2013) 1847–1858.

[113] J.V. Rushworth, A. Ahmed, H.H. Griffiths, N.M. Pollock, N.M. Hooper, P. Millner, A label-free electrical impedimetric biosensor for the specific detection of Alzheimer's amyloid-β oligomers, Biosens. Bioelectron. 56 (2014) 83–90.

[114] L. Liu, N. Xia, M. Jiang, N. Huang, S. Guo, S. Li, S.L. Zhang, Electrochemical detection of amyloid-β oligomer with the signal amplification of alkaline phosphatase plus electrochemical–chemical–chemical redox cycling, J. Electroanal. Chem. 754 (2015) 40–45.

[115] H. Li, Y. Cao, X. Wu, Z. Ye, G. Li, Peptide-based electrochemical biosensor for amyloid β1-42 soluble oligomer assay, Talanta 93 (2012) 358–363.

[116] C. Barucker, H.J. Bittner, P.K.-Y. Chang, S. Cameron, M.A. Hancock, F. Liebsch, S. Hossain, A. Harmeier, H. Shaw, F.M. Charron, M. Gensler, P. Dembny, W. Zhuang, D. Schmitz, J.P. Rabe, Y. Rao, R. Lurz, P.W. Hildebrand, R.A McKinney, G. Multhaup, Aβ42-oligomer Interacting Peptide (AIP) neutralizes toxic amyloid-β42 species and protects synaptic structure and function, Sci. Rep. 5 (2015) 15410.

[117] R.J. Simon, R.S. Kania, R.N. Zuckermann, V.D. Huebner, D.A. Jewell, S. Banville, S. Ng, L. Wang, S. Rosenberg, C.K. Marlowe, Peptoids: A modular approach to drug discovery, Proc. Natl. Acad. Sci. USA 89 (1992) 9367–9371.

[118] Z. Zhao, L. Zhu, X. Bu, H. Ma, S. Yang, Y. Yang, Z. Hu, Label-free detection of Alzheimer's disease through the ADP3 peptoid recognizing the serum amyloid-beta42 peptide, Chem. Commun. 51 (2015) 718–721.

[119] J.W. Lee, S. Samal, N. Selvapalam, H.J. Kim, K. Kim, Cucurbituril homologues and derivatives: new opportunities in supramolecular chemistry, Acc. Chem. Res. 36 (2003) 621–630.

[120] W.L. Mock, Cucurbituril, in: Comprehensive Supramolecular Chemistry, Vögtle, F., Ed.; Pergamon: Oxford. 2 (1996) 477-493.

[121] S. Walker, R. Oun, F.J. McInnes, N.J. Wheate, The potential of Cucurbit[n]urils in drug delivery, Isr. J. Chem. 51 (2011) 616–624.

[122] K. Kim, N. Selvapalam, Y.H. Ko, K.M. Park, D. Kim, J. Kim, Functionalized cucurbiturils and their applications, Chem. Soc. Rev. 36 (2007) 267–279.

[123] H. Li, H. Xie, Y. Cao, X. Ding, Y. Yin, G. Li, A general way to assay protein by coupling peptide with signal reporter via supermolecule formation, Anal. Chem. 85 (2013) 1047–1052.

[124] G. Hassanzadeh-Ghassabeh, N. Devoogdt, P. De Pauw, C. Vincke, S. Muyldermans, Nanobodies and their potential applications, Nanomedicine 8 (2013) 1013–1026.

[125] A. Zameer, S. Kasturirangan, S. Emadi, S.V. Nimmagadda, M.R. Sierks, Anti-oligomeric Aβ single-chain variable domain antibody blocks Aβ-induced toxicity against human neuroblastoma cells, J. Mol. Biol. 384 (2008) 917–928.

[125] M.R. Sierks, G. Chatterjee, C. Mcgraw, S. Kasturirangan, P. Schulz, S. Prasad, CSF levels of oligomeric alpha-synuclein and β-amyloid as biomarkers for neurodegenerative disease, Integr. Biol. 3 (2011) 1188–1196.

[126] Y. Yu, L. Zhang, C. Li, X. Sun, D. Tang, G. Shi, A method for evaluating the level of soluble β-amyloid (1-40/1-42) in Alzheimer's disease based on the binding of gelsolin to β-amyloid peptides, Angew. Chem. Int. Ed. 53 (2014) 12832–12835.

[127] Y. Yu, X. Sun, D. Tang, C. Li, L. Zhang, D. Nie, X.X. Yin, G.Y. Shi, Gelsolin bound β-amyloid peptides (1–40/1–42): Electrochemical evaluation of levels of soluble peptide associated with Alzheimer's disease, Biosens. Bioelectron. 68 (2015) 115–121.

[128] V.P. Chauhan, I. Ray, A. Chauhan, H.M. Wisniewski, Binding of gelsolin, a secretory protein, to amyloid β-protein, Biochem. Bioph. Res. Co. 258 (1999) 241–246.

[129] R. Stoltenburg, C. Reinemann, B. Strehlitz, SELEX- a (r)evolutionary method to generate high-affinity nucleic acid ligands, Biomol. Eng. 24 (2007) 381–403.

[130] C.T. Farrar, C.M. William, E. Hudry, T. Hashimoto, B.T. Hyman, RNA aptamer probes as optical imaging agents for the detection of amyloid plaques, PLoS One 9 (2014) e89901.

[131] L.C. Bock, L.C. Griffin, J.A. Latham, E.H. Vermaas, J.J. Toole, Selection of single-stranded DNA molecules that bind and inhibit human thrombin, Nature 355 (1992) 564–566.

[132] R.F. Macaya, P. Schultze, F.W. Smith, J.A. Roe, J. Feigon, Thrombin binding DNA aptamer forms a unimolecular quadruplex structure in solution, Proc. Natl. Acad. Sci. 90 (1993) 3745–3749.

[133] S. Tombelli, M. Minunni, M. Mascini, Aptamers-based assays for diagnostics, environmental and food analysis, Biomol. Eng. 24 (2007) 191–200.

[134] S. Tombelli, M. Minunni, E. Luzi, M. Mascini, Aptamer-based biosensors for the detection of HIV-1 Tat protein, Bioelectrochemistry 67 (2005) 135–141.

[135] R. Yamamoto, S. Toyoda, P. Viljanen, K. Machida, S. Nishikawa, K. Murakami, K. Taira, P.K. Kumar, In vitro selection of RNA aptamers that can bind specifically to Tat protein of HIV-1, Nucleic Acids Symp. Ser. 34 (1995) 145–146.

[136] F. Ylera, R. Lurz, V.A. Erdmann, J.P. Fürste, Selection of RNA aptamers to the Alzheimer's disease amyloid peptide, Biochem. Biophys. Res. Commun. 290 (2002) 1583–1588.

[137] F. Rahimi, K. Murakami, J.L. Summers, C.-H.B. Chen, G. Bitan, RNA aptamers generated against oligomeric Aβ40 recognize common amyloid aptatopes with low specificity but high sensitivity, PLoS ONE 4 (2009) e7694.

[138] R. Stoltenburg, C. Reinemann, B. Strehlitz, FluMag-SELEX as an advantageous method for DNA aptamer selection, Anal. Bioanal. Chem. 383 (2005) 83–91.

[139] S. Lisi, E. Fiore, Y. Boehmann, S. Scarano, M. Minunni, E. Peyrin, C. Ravelet, CE-Non-SELEX for isolation of aptamers directed against protein tau, Aptamers in Bordeaux, International conference on Aptamer Biology, Chemistry and Technologies, (2016) June 24-25, Bordeaux, France.

[140] J.H. Kim, E. Kim, W.H. Choi, J. Lee, J.H. Lee, H. Lee, D.E. Kim, Y.H. Suh, M.J. Lee, Inhibitory RNA Aptamers of Tau Oligomerization and Their Neuroprotective Roles against Proteotoxic Stress, Mol. Pharm. 13 (2016) 2039-2048.

[141] M.J. Whitcombe, I. Chianella, L. Larcombe, S.A. Piletsky, J. Noble, R. Porter, A. Horgan, The rational development of molecularly imprinted polymer-based sensors for protein detection, Chem. Soc. Rev. 40 (2011) 1547–1571.

[142] A. Bossi, F. Bonini, A. Turner, S. Piletsky, Molecularly imprinted polymers for the recognition of proteins: The state of the art, Biosens. Bioelectron. 22 (2007) 1131–1137.

[143] A. Poma, A.P. Turner, S.A. Piletsky, Advances in the manufacture of MIP nanoparticles, Trends Biotechnol. 28 (2010) 629–637.

[144] J.L. Urraca, C.S.A. Aureliano, E. Schillinger, H. Esselmann, J. Wiltfang, B. Sellergren, Polymeric complements to the Alzheimer's disease biomarker β-Amyloid isoforms Aβ1–40 and Aβ1–42 for blood serum analysis under denaturing conditions, J. Am. Chem. Soc. 133 (2011) 9220–9223.

[145] R. Craig-Schapiro, A.M. Fagan, D.M. Holtzman, Biomarkers of Alzheimer's disease, Neurobiol. Dis. 35 (2009) 128–140.

[146] M. Shi, Y.-T. Sui, E.R. Peskind, G. Li, H. Hwang, I. Devic, C. Ginghina, J.S. Edgar, C. Pan, D.R. Goodlett, A.R. Furay, L.F. Gonzalez-Cuyar, and Jing Zhang,

Salivary tau species are potential biomarkers of Alzheimer Disease, J. Alzheimers Dis. 27 (2011) 299–305.

[147] P. Mehta, T. Pirttila, B. Patrick, M. Barshatzky, S. Mehta, Amyloid β protein 1–40 and 1–42 levels in matched cerebrospinal fluid and plasma from patients with Alzheimer disease, Neurosci. Lett. 304 (2001) 102–106.

[148] F. Song, A. Poljak, G.A. Smythe, P. Sachdev, Plasma biomarkers for mild cognitive impairment and Alzheimer's disease, Brain Res. Rev. 61 (2009) 69–80.

[149] L. Moreno, R. Osta, and A.C. Calvo, New perspectives in the search for reliable biomarkers in Alzheimer disease, The Eur. J. Psychiat., 29 (2015) 51-65.

[150] P.R. Wattamwar and P.S. Mathuranath, An overview of biomarkers in Alzheimer's disease, Ann. of India Acad. Neurol. 2 (2010) 116-S123.

[151] K.A. Bates, G. Verdile, Q.-X. Li, D. Ames, P. Hudson, C.L. Masters and R.N. Martins, Clearance mechanisms of Alzheimer's amyloid-b peptide: implications for therapeutic design and diagnostic tests, Mol. Psychiatry 14 (2009) 469–486.

2 Basic Principles of biosensing and selection technique

Beyond the issues presented in the review, few words are worthy to be said on the theoretical principles of the techniques which are the basis for this work. In the next two paragraphs basics of the biosensing technique, *i.e.* SPR and on aptamer selection based on capillary electrophoresis as partitioning technique are reported.

2.1 Principle of SPR biosensors

IUPAC (International Union of Pure and Applied Chemistry) [1] define a biosensor as:

> *"A device that uses specific biochemical reactions mediated by isolated enzymes, immunosystems, tissues, organelles or whole cells to detect chemical compounds usually by electrical, thermal or optical signals."*

Such devices can be distinguished in Affinity Based biosensors (ABB) or catalytic biosensors which differs from the nature of the signal measured after the interaction take place. Despite the most successful example of commercial biosensors is represented by catalytic biosensor for glucose monitoring [2], growing applications of ABB are reported in scientific literature. Several transducers and various receptors of different nature exist to convert the biological interaction to a readable analytical signal (Fig. 2-1).

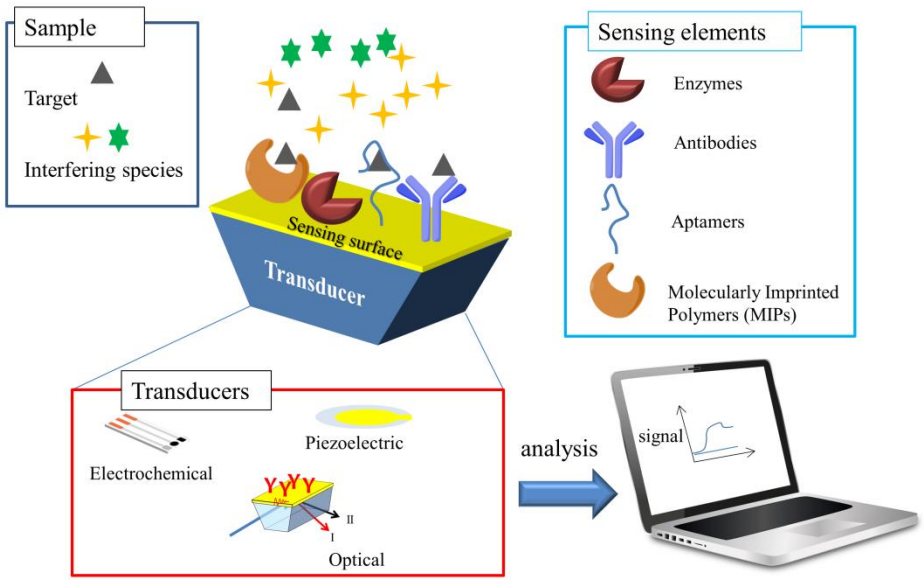

Fig. 2-1: Biosensor general scheme.

Among existing transducers, Surface Plasmon Resonance (SPR) sensors are widely applied in clinical diagnosis and many other research field (e.g. in drug discovery for reducing *in vivo* tests) [3–5]. The basic principle that allows the transduction is related with the generation of Surface Plasmon-Polaritons (SPPs), which are electromagnetic waves that propagates at the interface between a semi-infinite metal and a semi-infinite dielectric. SPPs have a magnetic vector parallel to the plane defined by the interface and are characterized by a propagation constant (β_{SP}) dependent on permittivity of both materials (equation 1).

$$\beta_{SP} = k\sqrt{\frac{\varepsilon_d\,\varepsilon_m}{\varepsilon_d+\varepsilon_m}} \qquad \text{eq. (1)}$$

Such equation describes SPPs only if real part of permittivity for metal (ε_m) is negative and that of dielectric (ε_d) is positive ($\varepsilon_m < -\varepsilon_d$). When visible or near-infrared light waves are used, silver and gold are among

those metals able to fulfill the criteria to generate SPPs, with a propagation depth of 100 – 500 nm, for a dielectric with a refractive index (η) of 1.32. Intensity of the magnetic field decays exponentially into both media, however the majority of the field is concentrated on the dielectric. Because of that when SPPs are excited and investigated with a light wave, refractive index (related to permittivity) variations in the dielectric medium are detected with extreme sensitivity [4,6,7]. However the direct interaction of the wave vector with metal films does not allow SPPs to be excited. Hence in the SPR instrument described in the experimental part of the thesis, as in many other apparatus, a prism (made of BK7 glass, η_p~1.52) is used as coupler in order to allow the component of the wave vector parallel to the interface, to match that of the SPPs. The light wave (λ) must pass through the prism in Krescthmann configuration of attenuated total reflection (ATR), generating an evanescent wave penetrating the metal film. In matching conditions, depending from the incident angle (θ) of light, the propagation constant of the evanescent wave can be adjusted to match that of the SPPs and part of the energy of the wave vector is transferred to the SPPs (equation 2).

$$\frac{2\pi}{\lambda}\eta_p \sin(\theta) = Re\{\beta_{SP}\} \quad \text{eq. 2}$$

Using thin metal films (~50 nm thickness) the evanescent wave reaches the opposite boundary of the film itself, hence it serves as a probe to investigate the events involving the superior dielectric layer (within the limit of the magnetic field) [8]. Under coupling conditions, change in the propagation constant (*i.e.:* due to analyte-receptor interaction) affects the properties of the incident light wave (i.e.: change of SPR angle, change of intensity, and change of the phase), that can be detected label-free and in

real time by a photomultiplier detector (in our case) to produce the analytical signal.

Absence of labeling processes is one of the main feature of the SPR, especially when the technique is compared with ELISA (Enzyme Linked ImmunoSorbent Assay). Moreover time of analysis, multiplexing capability [9] and multiple usage of a single biochip [10] can be also mentioned as three of the main benefits available for SPR sensors. Technologic developments and coupling between SPR biochip and now widely available smartphones opened the route for Point-of-Care (POC) testing by surface plasmon resonance based sensors, although further improvements need to be done (i.e. temperature control) [11,12].

Similarly to ELISAs, several assay format can be designed for SPR. Sandwiches are widely used for proteins or (more generally) those molecules presenting at least two epitopes that are recognized by a primary and a secondary antibody [3]. Despite the success of this approach, the low levels of some proteins in biological fluids, as is the case for Alzheimer's biomarkers, require further efforts to enhance sensitivity. Several works reported the use of gold or silver nanoparticles (GNPs or AgNPs) as label for the secondary antibody, to greatly increase the sensitivity of SPR thanks to mass due to NP size and to the coupling occurring between the plasmons on the sensor surface and those of the NPs [13–15]. Other approaches allowing significant gain in sensitivity consist in nanostructuring the sensor surface using metal nanoparticles [15,16], or with single- or multiple graphene layers [17].

Apart from the very high sensitivity in quantitative analysis, SPR also holds the ability to monitor biomolecular interaction in real time, elucidating the kinetic and thermodynamic parameters that drive the formation of affinity complex. Absence of a label for monitoring kinetic interactions, greatly facilitated many tasks such as a comparison among

different species and drug-target interactions. Nevertheless determination of kinetic association and dissociation constants require carefully designed experiments to avoid misleading interpretation of SPR data. Several are, indeed, the events that might negatively affect the correct determination of kinetic (and thermodynamic) parameters associated with a biochemical interaction. Some of them, such as mass transport effect, might be easily interpreted and corrected simply by using the proper mathematical model to fit the SPR sensorgram, whether it is more difficult to correctly interpret biphasic interaction. A complete survey of the amount of information derivable from SPR data is reported by Rich and Myszaka [18].

In this work, SPR was used both for its excellent sensitivity properties and for the above mentioned capability in monitoring biomolecular interactions in real time, giving access to kinetic information about the binding that are usually more difficult to be obtained with labeled approaches.

2.2 SELEX and Non-SELEX: advantages and drawbacks compared to other existing separation techniques

In this section basic principles of Capillary Electrophoresis SELEX and Non-SELEX (CE-SELEX and CE-Non- SELEX respectively) are reported. Since 1990 when aptamers were firstly selected [19,20], the selection process has been extensively modified. Many excellent reviews summarized the advances made in aptamer selection technologies [21,22] which is still evolving nowadays, with the growing application made by bioinformatics tools [23].

Despite its fast evolution, main steps of selection process can be easily represented and are summarized in Fig. 2-2

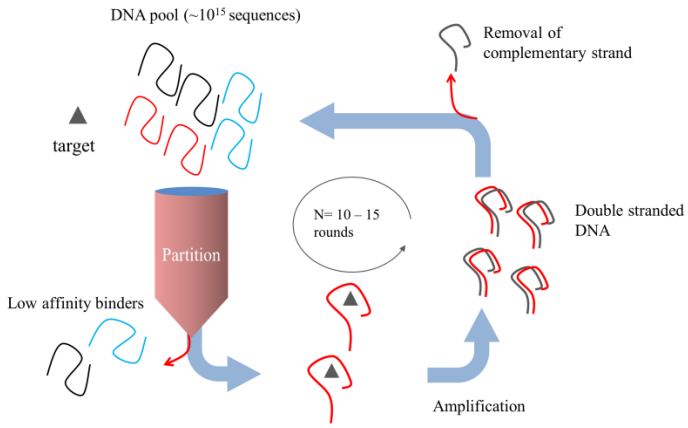

Fig. 2-2: General representation of Systematic Evolution of Ligands by EXponential enrichment (SELEX).

After the chemical synthesis (*step 1*) of a DNA or RNA library, usually containing ~10^{15} molecules, this large, heterogeneous pool of sequences is put in contact with the molecule for which one wants to select the aptamer (*step 2*). A separation technique is then used to select only the single strands DNA (ssDNA) or RNA sequences able to bind the target, enriching the library with most affine molecules. After the enrichment, Polymerase Chain Reaction (PCR) (*step 3*) is exploited for their properties

to greatly amplify the selected sequences by generating double-strand DNA molecules (dsDNA) which are finally purified (*step 4*) to re-start the cycle. For conventional selection methodologies indeed up to 15 cycles are needed to obtain potential aptamer candidates, making the process laborious and time-consuming. With respect of separation methods they are well described by Gopinath [24] and reported in table 2-1.

table 2-1: Separation methodologies used for aptamer selection

Separation technique	Small molecules/Proteins	Advantage	Drawback	Reference
Nitrocellulose membrane	- /+	· easy to use	· high number of selection cycles · poor binding for small molecules and peptide	[19]
Affinity based surface	+/+	· used for protein and small molecules	· need of target specificity or tags for immobilization on the support	[25]
Surface Plasmon Resonance	+/++	· determination of K_d during SELEX	· possible surface degradation along rounds	[26]
Gel shift	- /++	· no need of expensive instrument	· slow · low separation efficiency	[27,28]
Affinity Chromatography	++/+	· very common	· high target amount · difficile elution of best binders	[29,30]
Magnetic Beads	- /++	· no need to expensive instrument		[31,32]
Flow Cytometry	For cell-Selex	· allows direct affinity evaluation	· expensive, require high-specialized operator	[33]
Capillary Electrophoresis	- /++	· homogeneous · high separation efficiency	· need of electrophoretic mobility difference	[34,35]

The efficiency of the partitioning step is crucial, since the maximum affinity improvement per round is closely related to that [32]. As reported by Irvine et al., in any partitioning process some parasite sequences interacts with the target molecule and migrates with the sequences

presenting lower K_d, although the affinity of these parasite sequences is not as good as that of the best binder in the pool [36].

Any method has advantages and drawbacks, but as a general rule they all require immobilization of the target on a support. One of the few methodology that does not require target immobilization is based on the properties of nitrocellulose membranes, which were used to separate RNA molecules from protein [37] prior to be applied to selection [19]. However, because of the low efficiency of separation technique, several rounds were needed to complete the selection. Reducing the number of selection rounds is not only necessary in order to rapidly obtain aptamers, but also to i) avoid loss of rare (but highly affine) sequences [38], ii) reduce errors introduced by PCR along rounds [39,40], and iii) limit parasite species enrichment.

Capillary Electrophoresis-SELEX was introduced by Bowser's group [34,35] to overcome some of the limitations of selection procedures. In particular, thanks to separation efficiency of CE, the selection rounds were significantly reduced from usual 8-15 to only 2. Secondly, using CE the target-DNA (or RNA) complexes are formed in free solution, thus they are fully accessible during the separation. Finally, because the process takes place in homogeneous solution, the negative selection rounds that serve to eliminate the sequences directed to the immobilization support are no more required in this approach.

A lower amount of sequences is generally introduced in the capillaries for electrophoretic separation (10^{12} versus 10^{15}), though a very high diversity is usually recovered from the analysis of selection results. Thus the number of independent aptamers available might be greatly increased compared to conventional selection techniques. Perhaps one of the major inconvenient associated with CE-SELEX is related to the principle that allows the separation between DNA and the target. In fact, the

electrophoretic mobility of the species depends on the charge/mass ratio, thus aptamers against small molecules (i.e.: MW<500 Da) are more difficult to select.

Due to its ability to monitor affinity of the affinity complex, CE responds to all characteristics one needs during aptamer selection: *i)* monitor separation efficiency between target and the library; *ii)* determine kinetic and thermodynamic parameters of the interaction; *iii)* select aptamers with pre-defined binding ability [41].

To evaluate affinity of target-DNA complex, the species are combined to form an equilibrium mixture (EM). Then a plug of the mixture is injected in the capillary and undergoes separation in non-equilibrium conditions. Ideally the following electropherogram should be obtained (Fig. 2-3).

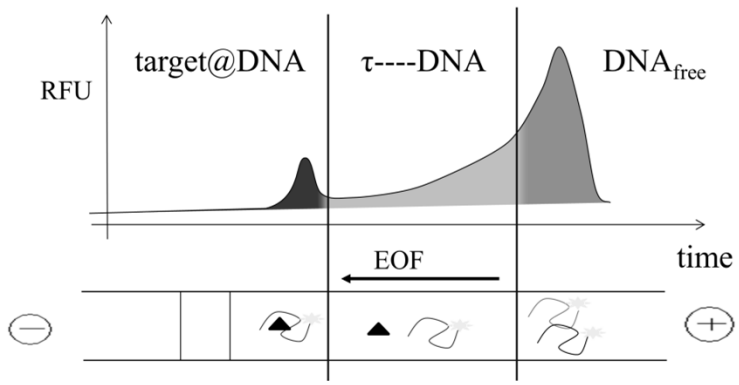

Fig. 2-3: Electropherogram of the Equilibrium Mixture (EM). Upper panel represents peak migration times of stable target-DNA complex (target@DNA), dissociating complex, and free DNA respectively (from left to right). Lower panel shows position of the species in the capillary tube during separation in normal polarity.

DNA molecules which form stable complex (low K_d) with the target usually migrate faster than DNA sequences with lower affinity, and free DNA. Since libraries are usually fluorescently labeled, all these species might be identified with high sensitivity by Laser Induced Fluorescence

(LIF) detector [41]. During the selection protocol, the various zones of the electropherogram are separated by collecting fractions at different time. The user defines the amplitude of collection window (containing sequences that are later amplified by PCR) which generally starts from the migration time of the affinity complex, and finish when the peak of free DNA starts to exit from the capillary. Modification of collection window amplitude affects significantly the stringency of the selection. In fact using short-time collection windows (end of collection immediately after the affinity complex peak) only stable DNA-target complex are selected, whether with long-time windows more diversity is maintained within the library. Negative selection is carried out simultaneously, by eliminating the fraction representing free DNA alone. Evaluation of affinity improvement is realized by estimating the areas under the curve (AUC) of each species detected by LIF.

The concept of CE-Non-SELEX was introduced later in two works [42,43]. The process was characterized by absence of amplification steps between the partitioning steps. As major advantage, CE-Non-SELEX allows to select aptamers considerably faster than any other selection methodology. For example, aptamers for h-Ras (a protein involved in cell proliferation) were obtained after only three rounds of selection in only few working hours according to the authors. Moreover the absence (or reduction) of PCR amplification steps, allows rare, very affine sequences, to be selected by maintaining the initial high diversity of the library [22]. On the other end, for the same reason, data issued of CE-Non-SELEX might be difficult to be interpreted without using some data analysis technique. In chapter 3 of the thesis the results of CE-SELEX and CE-Non-SELEX are compared to the selection of aptamers for tau protein, in view of aptamer applications to diagnosis of Alzheimer's disease.

References

[1] A.D. McNaught, A. Wilkinson, Compendium of chemical terminology- the "Gold book," Oxford, UK, 1997.

[2] A.P.F. Turner, Biosensors: sense and sensibility, Chem. Soc. Rev. 42 (2013) 3184–3196.

[3] S. Mariani, M. Minunni, Surface plasmon resonance applications in clinical analysis., Anal. Bioanal. Chem. 406 (2014) 2303–2323.

[4] J. Homola, Surface plasmon resonance sensors for detection of chemical and biological species., Chem. Rev. 108 (2008) 462–493.

[5] W. Huber, SPR-Based Direct Binding assay in drug discovery, in: M. Cooper, L. Mayr (Eds.), Label-Free Technol. Drug Discov., John Wiley and son Ltd, 2011: pp. 270–282.

[6] J. Homola, Present and future of surface plasmon resonance biosensors, Anal. Bioanal. Chem. 377 (2003) 528–539.

[7] J. Homola, Surface Plasmon Resonance Based Sensors, Springer, Berlin, 2006.

[8] H. Raether, Surface plasmons on smooth and rough surfaces and on gratings, Springer tracts in modern physics (vol 111), Springer, 1988.

[9] S. Scarano, M. Mascini, A.P.F. Turner, M. Minunni, Surface plasmon resonance imaging for affinity-based biosensors., Biosens. Bioelectron. 25 (2010) 957–66.

[10] A. Kausaite-Minkstimiene, A. Ramanaviciene, J. Kirlyte, A. Ramanavicius, Comparative study of random and oriented antibody immobilization techniques on the binding capacity of immunosensor, Anal. Chem. 82 (2010) 6401–6408.

[11] P. Preechaburana, M.C. Gonzalez, A. Suska, D. Filippini, Surface plasmon resonance chemical sensing on cell phones, Angew. Chem. Int. Ed. 51 (2012) 11585–11588.

[12] Y. Liu, Q. Liu, S. Chen, F. Cheng, H. Wang, W. Peng, Surface Plasmon Resonance Biosensor Based on Smart Phone Platforms., Sci. Rep. 5 (2015) 12864.

[13] L.M. Zanoli, R. D'Agata, G. Spoto, Functionalized gold nanoparticles for ultrasensitive DNA detection., Anal. Bioanal. Chem. 402 (2012) 1759–1771.

[14] G. Spoto, M. Minunni, Surface Plasmon Resonance Imaging : What Next ?, J. Phys. Chem. Lett. (2012) 2682–2691.

[15] M. Stewart, Nanostructured Plasmonic Sensors, Chem. Rev. 108 (2008) 494–521.

[16] S. Mariani, M.L. Ermini, S. Scarano, F. Bellissima, M. Bonini, D. Berti, M. Minunni, Improving surface plasmon resonance imaging of DNA by creating new gold and silver based surface nanostructures, Microchim. Acta. 180 (2013) 1093–1099.

[17] Y. Liu, X. Dong, P. Chen, Biological and chemical sensors based on graphene materials, Chem. Soc. Rev. 41 (2012) 2283.

[18] R.L. Rich, D.G. Myszka, Survey of the year 2007 commercial optical biosensor literature, J. Mol. Recognit. 21 (2008) 355–400.

[19] A.D. Ellington, J.W. Szostak, In vitro selection of RNA molecules that bind specific ligands, Nature. 346 (1990) 818–822.

[20] C. Tuerk, L. Gold, Systematic evolution of ligands by exponential enrichment: RNA ligands to bacteriophage T4 DNA polymerase, Science. 249 (1990) 505–510.

[21] R. Stoltenburg, C. Reinemann, B. Strehlitz, SELEX--a (r)evolutionary method to generate high-affinity nucleic acid ligands., Biomol. Eng. 24 (2007) 381–403.

[22] M. Darmostuk, S. Rimpelová, H. Gbelcová, T. Ruml, Current approaches in SELEX : An update to aptamer selection technology, Biotechnol. Adv. 33 (2015) 1141–1161.

[23] M. Blind, M. Blank, Aptamer Selection Technology and Recent Advances, Mol. Ther. Acids. 4 (2015) e223.

[24] S.C.B. Gopinath, Methods developed for SELEX, Anal. Bioanal. Chem. 387 (2007) 171–182.

[25] A. Rhodes, A. Deakin, J. Spaull, B. Coomber, A. Aitken, P. Life, S. Rees, The generation and characterization of antagonist RNA aptamers to human oncostatin M., J. Biol. Chem. 275 (2000) 28555–28561.

[26] M. Khati, M. Schüman, J. Ibrahim, Q. Sattentau, S. Gordon, W. James, Neutralization of Infectivity of Diverse R5 Clinical Isolates of Human Immunodeficiency Virus Type 1 by gp120-Binding 2 'F-RNA Aptamers, J. Virol. 77 (2003) 12692–12698.

[27] J. Charlton, G.P. Kirschenheuter, D. Smith, Highly potent irreversible inhibitors of neutrophil elastase generated by selection from a randomized DNA-valine phosphonate library, Biochemistry. 36 (1997) 3018–3026.

[28] D. Smith, G.P. Kirschenheuter, J. Charlton, D.M. Guidot, J.E. Repine, In vitro selection of RNA-based irreversibile inhibitors of human neutrophil elastase, Chem. Biol. 2 (1995) 741–750.

[29] S. Tombelli, M. Minunni, M. Mascini, Analytical applications of aptamers, Biosens. Bioelectron. 20 (2005) 2424–2434.

[30] D. Nieuwlandt, M. Wecker, L. Gold, In Vitro Selection of RNA Ligands to Substance P, Biochemistry. 34 (1995) 5651–5659.

[31] R. Stoltenburg, C. Reinemann, B. Strehlitz, FluMag-SELEX as an advantageous method for DNA aptamer selection, Anal. Bioanal. Chem. 383 (2005) 83–91.

[32] M.R. Gotrik, T.A. Feagin, A.T. Csordas, M.A. Nakamoto, H.T. Soh, Advancements in Aptamer Discovery Technologies, Acc. Chem. Res. 49 (2016) 1903–1910.

[33] X. Yang, X. Li, T.W. Prow, L.M. Reece, S.E. Bassett, B.A. Luxon, N.K. Herzog, J. Aronson, R.E. Shope, J.F. Leary, D.G. Gorenstein, Immunofluorescence assay and flow-cytometry selection of bead-bound aptamers, Nucleic Acids Res. 31 (2003) e54–e54.

[34] S.D. Mendonsa, M.T. Bowser, In vitro selection of high-affinity DNA ligands for human IgE using capillary electrophoresis., Anal. Chem. 76 (2004) 5387–5392.

[35] S.D. Mendonsa, M.T. Bowser, In Vitro Selection of Aptamers with Affinity for Neuropeptide Y Using Capillary Electrophoresis, J. Am. Chem. Soc. 127 (2005) 9382–9383.

[36] D. Irvine, C. Tuerk, L. Gold, Selexion. Systematic evolution of ligands by exponential enrichment with integrated optimization by non-linear analysis, J. Mol. Biol. 222 (1991) 739–761.

[37] T.I. Přistoupil, M. Kramlova, Microchromatographic separation of ribonucleic acids from proteins on nitrocellulose membranes, J. Chromatogr. A. 32 (1968) 769–770.

[38] M. Djordjevic, SELEX experiments: New prospects, applications and data analysis in inferring regulatory pathways, Biomol. Eng. 24 (2007) 179–189.

[39] M.U. Musheev, S.N. Krylov, Selection of aptamers by systematic evolution of ligands by exponential enrichment: addressing the polymerase chain reaction issue., Anal. Chim. Acta. 564 (2006) 91–96.

[40] F. Tolle, J. Wilke, J. Wengel, G. Mayer, By-product formation in repetitive PCR amplification of DNA libraries during SELEX., PLoS One. 9 (2014) e114693.

[41] M. Berezovski, A. Drabovich, S.M. Krylova, M. Musheev, V. Okhonin, A. Petrov, S.N. Krylov, Nonequilibrium capillary electrophoresis of equilibrium mixtures: a universal tool for development of aptamers., J. Am. Chem. Soc. 127 (2005) 3165–3171.

[42] M. Berezovski, M. Musheev, A. Drabovich, S.N. Krylov, Non-SELEX selection of aptamers., J. Am. Chem. Soc. 128 (2006) 1410–1411.

[43] M. V Berezovski, M.U. Musheev, A.P. Drabovich, J. V Jitkova, S.N. Krylov, Non-SELEX: selection of aptamers without intermediate amplification of candidate oligonucleotides., Nat. Protoc. 1 (2006) 1359–1369.

3 Immunosensing for protein tau: carbon nanotubes for signal amplification

As described in the introducing chapter (chapter 1), detection of tau protein is usually carried by using time-consuming (*i.e.:* ELISA) or with highly expensive instrumental apparatus. These techniques, despite their consolidated applicability need to take advantage from labelling molecules (a chromophore or a fluorophore) for the detection of the target.

Starting from the well-established affinity and selectivity properties of commercially available antibodies against tau protein, here we explored the use of Surface Plasmon Resonance (SPR) to develop a label-free, regenerable biosensor to detect tau.

First step in this sense was devoted to the investigation of the analytical performances of SPR immunosensor developed with a monoclonal antibody, mapping the central region of the analyte, immobilized onto the gold surface by simple amino-coupling approach. Here we demonstrated that, by this method, tau was easily detected with good repeatability (below 10% average CV) both in buffer and in simulated matrix solution, developed to reproduce the saline, and protein composition of CerebroSpinal Fluid (CSF). In the latter, the analytical signal of protein tau was maintained for a longer period compared to buffer, suggesting a role of dissolving medium composition tau preservation.

From this first approach the use of an amplification strategy seemed mandatory to significantly improve the sensitivity toward pM levels (cut-off for Alzheimer's probable diagnosis). To this aim a sandwich assay was developed, using of a secondary antibody directed to the N-terminal part of tau. The signal was increased, but further amplification was needed. Therefore, although label-free feature was lost, Multi-walled Carbon

Nanotubes (MWCNTs) were employed to fulfill the sensitivity requirement for tau.

Experimental Limit of Detection at pM level was obtained, and total amount of secondary antibody necessary for the analysis was also decreased. Regeneration of the sensor became more complicated, if compared with previous experiments. Successful removal of carbon nanotubes from the surface was achieved by considering the length dispersion associated with these nanostructures. As demonstrated here, and in previous works, oxidized nanotubes lengths span from 0.2 to 2.0 µm. Because of that, to restore the regeneration process antibody-MWCNTs conjugate injection in SPR was preceded by a mild centrifugation step aimed to keep low- size nanotubes only. Despite this could negatively affect signal enhancement this was not observed in this case, with a global increase in sensitivity of about two orders of magnitude compared to the initial direct assay, set up with the primary antibody only. Further studies might allow to improve the LOD in the near future. Better size-selection protocol for MWCNTs can be conceived, to improve sensitivity as well as repeatability. Since MWCNT length is easily monitored by Transmission Electron Microscopy (TEM), accurate measurement of this parameter can be considered to evaluate sample dispersion. Total antibody amount fixed at the nanotube surface could also be controlled either by thermogravimetric analysis or Atomic Force Microscopy (AFM). Nevertheless the intrinsic complexity of the nanostructures might lead to difficile data interpretation and need therefore to be studied in depth prior to be applied.

The results of this work are reported in *Biosensor and Bioelectronics*, special issue of the 26th international congress of Biosensors, in the form of Short communication *(doi:* 10.1016/j.bios.2016.08.078)

"Toward sensitive immuno-based detection of Tau protein by Surface Plasmon Resonance coupled to carbon nanostructures as signal amplifiers"

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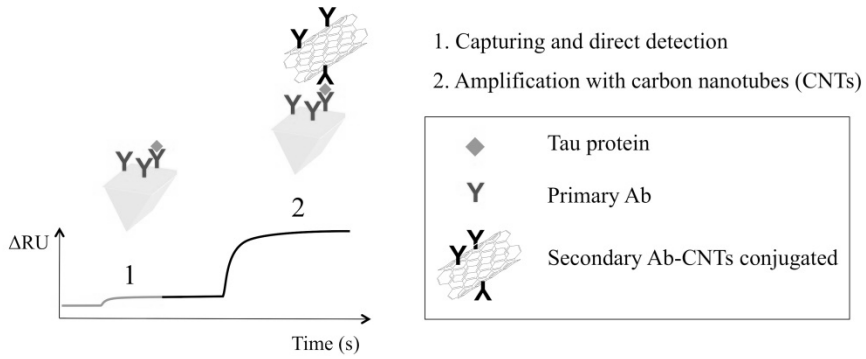
Graphical Abstract



Abstract

Interest on Tau protein is fast increasing in Alzheimer's disease (AD) diagnosis. There is the urgent need of highly sensitive and specific diagnostic platforms for its quantification, also in combination with the other AD hallmarks. Up to now, SPR has been poorly exploited for tau detection by immunosensing, due to sensitivity limits at nanomolar level, whereas the clinical requirement is in the picomolar range. Molecular architectures built in a layer-by-layer fashion, biomolecules and nanostructures (metallic or not) may amplify the SPR signal and improve the limit of detection to the desired sensitivity. Mostly gold nanostructures

are widely employed to this aim, but great interest is also emerging in Multi Walled Carbon Nanotubes (MWCNTs). Here MWCNTs are modified and then decorated with the secondary antibody for tau protein. Eventually we took advantage from MWCNTs-antibody conjugate to obtain a sandwich-based bioassay with the capability to increase the SPR signal of about 10^2 folds compared to direct detection and conventional unconjugated sandwich. With respect to these results, we hope to give a strong impulse for further investigation on studying possible roles of carbon nanotubes in optical-based biosensing.

Keywords: Carbon nanotubes; tau protein; Alzheimer's disease; Surface Plasmon Resonance; signal amplification.

3.1 Introduction

Tau protein is one of the validated biomarkers for Alzheimer's disease (AD) and tauopathies diagnosis in general [1–3]. Sensing and biosensing approaches to the detection of AD biomarkers were recently reviewed [4,5], highlighting how majority of works in AD research is directed toward other biomarkers, i.e. amyloid beta monomers and derived species. Despite SPR is described in routine diagnostics for clinical analyses [6,7], SPR-based tau biosensing was attempted only by few works [8–10], and none of them approaches the detection of tau in real matrix, *i.e.* CerebroSpinal Fluid (CSF). In this framework, we developed an SPR immunosensor to detect tau protein at nanomolar concentration, both in buffer and artificial CSF (aCSF) by direct and sandwich-based detection. Since the use of metallic nanostructures emerged in the last years for SPR signal amplification [11,12], we recently exploited gold nanoparticles-DNA conjugates to this aim, in DNA sandwich-like detection strategies

[13,14]. Herein, we combine the enhancing properties given by Multi-Walled Carbon Nano Tubes (MWCNTs) [15,16] to a classic immuno-based sandwich format for tau detection. Such nanostructures have been reported in biosensing [17] however their use in SPR is still scarcely described [15]. Being inspired by these approaches, and considering that significant clinical levels of tau protein are in the range of pM [18], we developed a sandwich strategy in which the secondary monoclonal antibody (mAb_2) is coupled to MWCNTs (MWCNTs-mAb_2) via amino-coupling reaction. These nanostructured amplifiers were successfully exploited for signal amplification, obtaining a gain of more than two orders of magnitude respect to the unlabeled strategy.

3.2 Materials and methods

3.2.1 Materials and methods

List of chemicals, SPR instrumentation, biochip modification, MWCNTs characterization by TEM and elemental analysis, together with the oxidation and functionalization protocols are all reported in Supplementary material (Section 3.5.1).

3.2.2 Analysis

Three assays were designed (Fig. 3-1) and compared: a) a direct assay on the primary mAb (39E10 clone, hereafter mAb_1), b) a sandwich assay by adding the secondary mAb (tau 12 clone, hereafter mAb_2) after the direct assay and c), a sandwich assay in which mAb_2 is covalently coupled to MWCNTs (MWCNTs-mAb_2).

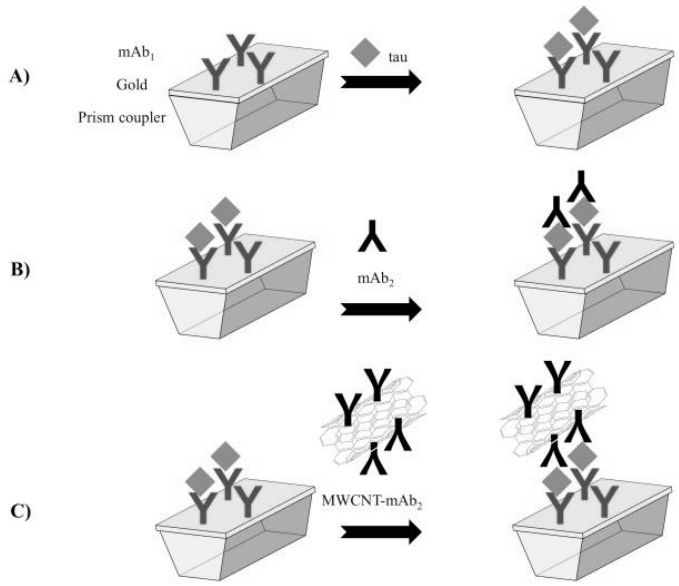


Fig. 3-1: Design of SPR assays for tau quantification. A) direct approach. Antibody immobilized by amino-coupling interacted with tau. Injection of the analyte is followed by the injection of secondary mAb2 (Tau12, panel B) to amplify the signal. C) Tau12 coupled to MWCNTs in a sandwich-like format to obtain signal amplification.

Tau protein (diluted in 1X HBS-EP or aCSF) was injected at different concentrations, ranging from 125 pM-250 nM. Interaction with mAb_1 lasted 4 minutes for measurements performed in 1X HBS-EP buffer and 5 minutes in aCSF. The sandwich approach was carried out using mAb_2 at 10 mg L^{-1} for 6 minutes of contact time, when used unconjugated, while injection lasted 6 or 15 minutes in presence of MWCNT-mAb_2 conjugates. HSA at 100 mg L^{-1} (the most abundant protein in CSF) [19], mAb_2 (Tau12) in absence of tau, and MWCNTs-mAb_2 (on non-functionalized cell) were tested as negative controls to check possible non specific contributions. All measurements were realized at 5 µL min^{-1} flow rate, using 1X HBS-EP as running buffer.

3.3 Results and discussion

3.3.1 Direct assay

A pre-concentration test was realized to optimize pH conditions during immobilization of mAb_1, finding the best accumulation effect at pH 4.0 (2440.7 RU, see Fig. S3-1).

After immobilization, the biosensor was calibrated in 1X HBS-EP buffer and aCSF on tau concentration spanning from 7 nM to 250 nM, showing different behaviors in the two media (Fig. S3-2 and Fig. S3-3). Limit of detection (LOD) in both media resulted similar (7.8 nM and 15.0 nM respectively), but tau showed positive properties when dissolved in simulated matrix than buffer. In particular, an extended linearity (125.0 nM versus 31.2 nM) and an improved repeatability, as demonstrated by a lower coefficient of variation in aCSF (5.8%) than in buffer (7.8%), were obtained. LOD observed in our experiments resulted similar to that reported by Homola's group, who used a 2-D Self Assembled Monolayer (SAM) to build a sandwich strategy to detect tau-amyloid complex formation [9]. In our case, nM range was reached by direct approach, probably thank to the 3-D character of dextran surface which allows to obtain a higher mAb surface density [6,20]. More than 30 measurements were realized over the same chip, after regeneration of the surface injecting short pulses of NaOH (Fig. S3-4), which did not damage the sensor as demonstrated by the absolute RU variation of the baseline between two consecutive cycles (±2.2 RU) (data not shown). As negative control, aCSF containing physiological HSA levels was tested, displaying negligible signal in the absence of tau (3.2 ± 2.2 RU). Investigations on tau protein preservation (62.5 nM) in the two tested media revealed about 89% (45.5±3.1 RU) recovery for tau protein kept overnight at 4 °C in aCSF, while only about 42% (16.6±5.1 RU) in 1X HBS-EP buffer under

the same conditions (Fig. S3-5). In analytical perspective, these results support the need for stable Certified Standard Materials (CRMs) to validate methods for AD clinical diagnosis. Such materials, because of their validated composition, could help in reducing the interlaboratory variability associated with AD biomarker measurements [21,22].

3.3.2 Sandwich assay

The sandwich approach, performed by injecting mAb_2 after tau binding on mAb_1 allowed to enhance the analytical signal through mass enhancement in a time dependent manner, as demonstrated by increasing tau-mAb_2 incubation time from 2 to 6 minutes (Fig. S3-6, upper panel). The maximum enhancement of the analytical signal was obtained by using 10 mg L^{-1} of mAb_2, displaying a concentration dependent response which indicates the specific binding between tau and mAb_2 (Fig. S3-6, lower panel). The absence of non-specific interaction of mAb_2 with mAb_1 immobilized on the chip surface was assessed. As negative control, HSA at physiological concentration (e.g. 100 mg L^{-1}) was tested giving negligible signal (Fig. 3-2A).

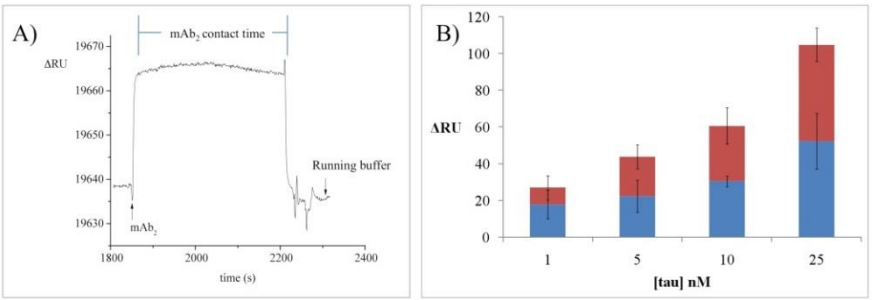


Fig. 3-2: Sandwich assay for tau protein. A) mAb_2 injection in absence of tau protein. Arrows indicate beginning of injection and end of signal monitoring. B) signal obtained for four tau levels. Blue bars represent the results for direct assay, while red bars take into account amplification with the secondary antibody (mAb_2).

Under these conditions, tau protein was calibrated in aCSF between 1 and 25 nM (Fig. 3-2B), obtaining a significant enhancement of the SPR signal respect to direct detection, with a slope increment of about six folds (from 0.579 to 3.179) and a linear correlation (R^2) of 0.995. The estimated LOD for tau protein was consequently lowered to 2 nM (vs 15 nM by direct detection). Despite the sandwich assay improved the sensitivity, this result is still far from the pM range needed for detecting tau protein in clinical samples [2]. Therefore, we investigated the use of nanostructures to further amplify the signal.

3.3.3 Coupling mAb2 to MWCNTs as signal enhancers for SPR analysis

MWCNTs were exploited as mass enhancers after their conjugation to the secondary antibody to elicit SPR signal amplification. In fact, mAb_2 carrying these nanostructures (Fig. S3-7) at the sensing interface should provide a consistent refractive index change, as already reported for the detection of erythropoietin by SPR [15]. To use these nanostructures overcoming hydrophobic nature associated with their pristine structure is mandatory. Dispersibility of pristine nanotubes is in fact very low in aqueous solutions [23], which are the ideal media for protein analysis. The oxidation of the original material was hence performed both to obtain

stable suspensions in aqueous solutions and to provide carboxylic groups on MWCNTs surface to covalently link mAb_2. Activation of the functional groups was verified by IR spectroscopy and elemental analysis demonstrating the increase of nitrogen amount on MWCNTs surface (Fig. S3-8). To effectively bind mAb_2 MWCNTs, a fine optimization of the whole protocol was necessary, i.e. sonication time, coupling medium composition, and mAb_2 concentration, as discussed in section 3.5.2 of supplementary material. These parameters resulted crucial to minimize inter-particles aggregation and the desired functionalization of MWCNTs with mAb_2. After a preliminary 1:10 dilution, the conjugates were initially tested (in 1X HBS-EP buffer) on 7 nM tau previously bound on mAb_1, obtaining an enhancement of the SPR signal of about two orders of magnitude respect to the signal recorded by direct assay (from 5 RU to 567 RU) (Fig. 3-3A). However, despite this huge amplification, the regeneration of sensor surface under these conditions resulted impossible, probably because of the presence of MWCNTs-mAb_2 in the micrometers range of size, more prone to aggregate at the sensor surface than shorter structures.

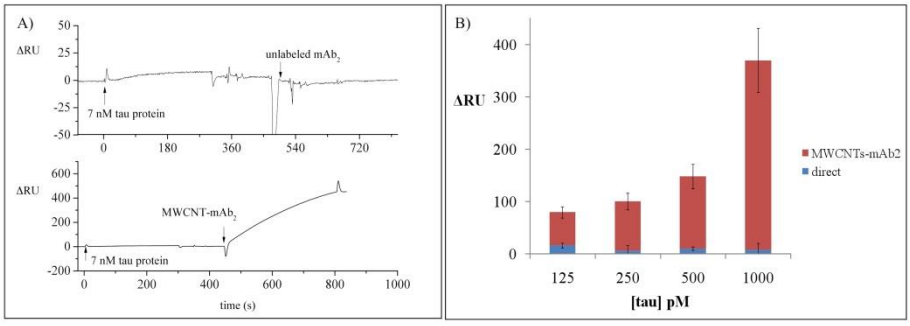


Fig. 3-3: Signal amplification with MWCNTs-mAb_2. A) Comparison of SPR sensorgrams obtained by unconjugated mAb_2 (top) and MWCNT- mAb_2 (bottom), displaying the marked enhancement achieved at 7 nM tau. B) MWCNTs- mAb_2 / tau dependence. Blue bars represent the results for direct assay; red bars reported RU values after interaction of tau-mAb_1 complex with MWCNTs-mAb_2.

To overcome this limitation, a final centrifugation step was performed to select the low-size fraction of NTs [24], and only the supernatant was then used for the injection. By this approach we successfully achieved the enhancement of the SPR signal in a dose-response manner, together with the good regeneration of the tau-MWCNTs-mAb_2 adduct after each measurement. Under the optimized conditions, tau concentration from 125 to 1000 pM were assayed in aCSF, clearly showing a dose-response trend (Fig. 3-3B), which displays an exponential shape. In fact, we observed that the increase of tau bound to mAb_1 induces a deviation from linearity, probably due to local packing of nanostructures at the surface. This likely reflects the complexity of tau-MWCNTs-mAb_2 interactions at the sensor surface, and suggests a key role of the protein in bundles deformation process [25]. This process eventually might favor nanotube-nanotube interactions and produce both deviation from linearity and difficulties in regenerating the biochip, especially for tau concentration higher than 1 nM. As negative control, MWCNTs-mAb_2 were tested on the non-functionalized CM5 surface, showing low absorption (21.20 RU ± 5.27 RU) compared to the signal obtained in the presence of the analyte. Conversely, when MWCNTs-mAb_2 are injected on mAb_1 in absence of tau, valuable adsorption of about 60 RU is recorded, therefore an experimental detection limit of 125 pM is obtained. The variability among different MWCNTs injections (n=6) was estimated by relative error (E_r), resulting about 14% probably due to the complexity of the nanostructures, both in controlling their size distribution and their reproducible functionalization. Although some room for improvement is still possible to effectively apply the present approach to reusable biosensing platforms such as SPR, our results are easily transferable to disposable devices for AD biomarkers detection in CSF, e.g. for point-of-care testing, on which regeneration aspects would be of minor importance.

3.4 Conclusions

Interest on Tau protein is fast increasing in CSF AD biomarkers detection, and there is the urgent need of highly sensitive and specific diagnostic platforms for its quantification, also in combination with the other AD hallmarks. SPR is still poorly exploited for tau detection by immunosensing, displaying sensitivity limits at nM concentration, whereas the clinical requirement is in the pM range. Herein we report the successful use of MWCNTs as mass enhancers in a classic sandwich assay carried out by SPR biosensing. The analytical performances of direct, classic sandwich, and MWCNTs-based amplification strategies are here tested and compared. As a whole, a significant amplification capability (two orders of magnitude) was obtained at the detection limit of the direct assay likely due to both the mass of nanostructures and their large surface area that makes mAb_2 more available for binding. An investigation on dose-response dependence at pM tau concentration seems to highlight that several contributions are involved in the amplification trend, especially increasing tau concentration up to the nanomolar range. Effective regeneration of the biochip surface is achievable in the picomolar range of the analyte only if nanotubes are well dispersed and bundles formation is minimized. Once injected on the sensor surface, the establishment of interactions among MWCNTs and tau strongly influences the overall behavior of the analytical response, and different optimized conditions should be likely adopted to calibrate tau in the nanomolar range. The preparation and use of MWCNTs-mAb_2 conjugates are here optimized to work within the tau picomolar range, and in these conditions the bioassay resulted reversible and with an estimated batch-to-batch variability of 14%. As a whole, in this paper we report the preliminary but encouraging

results on the use of MWCNTs-antibody conjugation for the improvement of the analytical performances of SPR sensing for tau detection in the picomolar range, and may open new roads for innovative and sensitive SPR-based methods.

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Notes

Authors declare no conflict of interest.

References

[1] K. Blennow, H. Hampel, M. Weiner, H. Zetterberg, Cerebrospinal fluid and plasma biomarkers in Alzheimer disease., Nat. Rev. Neurol. 6 (2010) 131–144.

[2] L.M. Shaw, M. Korecka, C.M. Clark, V.M.-Y. Lee, J.Q. Trojanowski, Biomarkers of neurodegeneration for diagnosis and monitoring therapeutics., Nat. Rev. Drug Discov. 6 (2007) 295–303.

[3] F. Hernández, J. Avila, Tauopathies, Cell. Mol. Life Sci. 64 (2007) 2219–2233.

[4] H.V.S. Ganesh, A.M. Chow, K. Kerman, Recent advances in biosensors for neurodegenerative disease detection, Trends Anal. Chem. (2016).

[5] A. Kaushik, R.D. Jayant, S. Tiwari, A. Vashist, M. Nair, Nano-biosensors to detect beta-amyloid for Alzheimer's disease management, Biosens. Bioelectron. 80 (2016) 273–287.

[6] J. Homola, Surface plasmon resonance sensors for detection of chemical and biological species., Chem. Rev. 108 (2008) 462–493.

[7] S. Mariani, M. Minunni, Surface plasmon resonance applications in clinical analysis., Anal. Bioanal. Chem. 406 (2014) 2303–2323.

[8] A. Barrantes, J. Sotres, M. Hernando-Pérez, M.J. Benítez, P.J. de Pablo, A.M. Baró, J. Avila, J.S. Jiménez, Tau aggregation followed by atomic force microscopy and surface plasmon resonance, and single molecule tau-tau interaction probed by atomic force spectroscopy., J. Alzheimer's Dis. 18 (2009) 141–151.

[9] Z. Kristofikova, D. Ripova, K. Hegnerová, J. Sirova, J. Homola, Protein τ-mediated effects on rat hippocampal choline transporters CHT1 and τ-amyloid β interactions., Neurochem. Res. 38 (2013) 1949–59.

[10] M. Vestergaard, K. Kerman, D.-K. Kim, M.H. Ha, E. Tamiya, Detection of Alzheimer's tau protein using localised surface plasmon resonance-based immunochip., Talanta. 74 (2008) 1038–1042.

[11] M. Stewart, Nanostructured Plasmonic Sensors, Chem. Rev. 108 (2008) 494–521.

[12] L.M. Zanoli, R. D'Agata, G. Spoto, Functionalized gold nanoparticles for ultrasensitive DNA detection., Anal. Bioanal. Chem. 402 (2012) 1759–1771.

[13] S. Mariani, S. Scarano, M.L. Ermini, M. Bonini, M. Minunni, Investigating nanoparticle properties in plasmonic nanoarchitectures with DNA by surface plasmon resonance imaging, Chem. Commun. 51 (2015) 6587–6590.

[14] S. Mariani, S. Scarano, J. Spadavecchia, M. Minunni, A reusable optical biosensor for the ultrasensitive and selective detection of unamplified human genomic DNA with gold nanostars, Biosens. Bioelectron. 74 (2015) 981–988.

[15] E.G. Lee, K.M. Park, J.Y. Jeong, S.H. Lee, J.E. Baek, H.W. Lee, J.K. Jung, B.H. Chung, Carbon nanotube-assisted enhancement of surface plasmon resonance signal, Anal. Biochem. 408 (2011) 206–211.

[16] S. Kruss, A.J. Hilmer, J. Zhang, N.F. Reuel, B. Mu, M.S. Strano, Carbon nanotubes as optical biomedical sensors, Adv. Drug Deliv. Rev. 65 (2013) 1933–1950.

[17] C.B. Jacobs, M.J. Peairs, B.J. Venton, Review: Carbon nanotube based electrochemical sensors for biomolecules, Anal. Chim. Acta. 662 (2010) 105–127.

[18] C. Humpel, Identifying and validating biomarkers for Alzheimer's disease, Trends Biotechnol. 29 (2011) 26–32.

[19] K. Ganrot, C.B. Laurell, Measurement of IgG and albumin content of cerebrospinal fluid, and its interpretation, Clin. Chem. 20 (1974) 571–573.

[20] S. Zhang, Hydrogels: Wet or let die, Nat. Mater. 3 (2004) 7–8.

[21] J.-H. Kang, M. Korecka, J.B. Toledo, J.Q. Trojanowski, L.M. Shaw, Clinical utility and analytical challenges in measurement of cerebrospinal fluid amyloid-β(1-42) and τ proteins as Alzheimer disease biomarkers., Clin. Chem. 59 (2013) 903–916.

[22] H. Vanderstichele, M. Bibl, S. Engelborghs, N. Le Bastard, P. Lewczuk, J.L. Molinuevo, L. Parnetti, A. Perret-Liaudet, L.M. Shaw, C. Teunissen, D. Wouters, K. Blennow, Standardization of preanalytical aspects of cerebrospinal fluid biomarker testing for Alzheimer's disease diagnosis: a consensus paper from the Alzheimer's Biomarkers Standardization Initiative., Alzheimers. Dement. 8 (2012) 65–73.

[23] L. Liu, F. Zhang, F. Xi, X. Lin, Highly sensitive biosensor based on bionanomultilayer with water-soluble multiwall carbon nanotubes for determination of phenolics, Biosens. Bioelectron. 24 (2008) 306–312.

[24] Q.P. Feng, X.M. Xie, Y.T. Liu, Y.F. Gao, X.H. Wang, X.Y. Ye, Length sorting of multi-walled carbon nanotubes by high-speed centrifugation, Carbon N. Y. 45 (2007) 2311–2313.

[25] E. Edri, O. Regev, "Shaken, not stable": dispersion mechanism and dynamics of protein-dispersed nanotubes studied via spectroscopy, Langmuir. 25 (2009) 10459–10465.

3.5 Supplementary material

3.5.1 Chemicals and procedures

Reagents and buffers

Human tau protein (isoform 2N4R) was purchased from Enzo Life Sciences (Lyon, France). Primary antibody (mAb_1, monoclonal, clone 39E10 produced in mouse) was from Biolegend (San Diego, U.S.A). It recognizes tau protein in its middle domain (amino acids 189-195), a region shared by all isoforms. Secondary monoclonal antibody (mAb_2, monoclonal, clone Tau12, produced in mouse) was purchased from Merck (Daarmstaad, Germany) and recognizes tau in N terminus part (aa 9-18). Bovine and Human Serum Albumin (BSA and HSA), MWCNTs were purchased from Sigma Aldrich (Milan, Italy). HEPES, EDTA, N-Hydroxy succinimide (NHS), 1-Ethyl-3-(3'-dimethylaminopropyl)carbodiimide (EDAC), ethanolamine (EA) and all the other reagents for surface modification or buffer preparation were commercially available and purified at analytical grade. Buffers and solutions used in the works are listed below:

Running buffer (1X HBS-EP): 10 mM HEPES, 150 mM NaCl, 3 mM EDTA with 0.005% Tween20; pH 7.4.

Immobilization buffer: 10 mM CH_3COONa 3 H_2O; pH 4.0.

Artificial CerebroSpinal Fluid (aCSF):150 mM NaCl, 3 mM KCl, 1.4 mM $CaCl_2$ $2H_2O$, 0.8 mM $MgCl_2$ $2H_2O$, 1.0 NaH_2PO_4 H_2O with 100 mg L^{-1} HSA, pH 7.3) [1].

Conjugation buffer (0.5X HBS-EP): 5 mM HEPES, 75 mM NaCl, 1.5 mM EDTA with 0.005 % Tween20, pH 7.4.

SPR instrumentation and biochips modification

All measurements were performed on a BiacoreX™ provided by GE Healthcare (Uppsala, Sweden) The system consists in a liquid delivery and an optical system, both thermoinsulated and maintained at T=25.00±0.01°C. Optical system was arranged in classical Kretschmann configuration [2] (Kretschmann, Raether; 1968). In this configuration the diode array (at 632.5 nm wavelength) of the instrument interacts with a metal film (i.e. gold of about 50 nm thick) vaporized over a glass prism which serves as wavelength coupler. Commercially available CM5 chips (GE Healthcare, Uppsala, Sweden) are provided with 100 nm layer of carboxymethylated dextran to allow covalent linkage for biomolecules. Primary mAb (mAb_1) was attached to CM5 surface using the following procedure: carboxymethylated dextran surface was activated with 50/200 mM NHS/EDAC aqueous solution (50 µL, 10 min). 200 mg L^{-1} mAb_1 (75 µL, 15 min) were prepared in immobilization buffer and let react with the surface on flow cell 1 (fc_1) of BiacoreX™. Residual active sites of the surface were finally de-activated with 1 M EA solution (pH 8.5) (75 µL, 15 min, 2X). Flow cell 2 (fc_2) underwent the same protocol except for antibody injection and is used as negative reference. The reagents required for all steps were injected manually in the SPR system, and transferred to biochip cells using running buffer as carrier. Reproducible sample volume was insured by the Integrated µ-Fluidic Cartridge (IFC) of the instrument. An external compressor and an automatic syringe pump provided a constant flow rate, maintained at 5 µL min-1for all functionalization steps. SPR signals were expressed as difference of resonance units (ΔRU) with respect of the absolute baseline RU value.

MWCNTs characterization

Carbon nanostructures have been characterized by Transmission Electron Microscopy (TEM), IR spectroscopy and Elemental Analysis (Elem. Anal.). TEM analysis was performed on a Philips STEM CM12 at room temperature, IR spectra were recorded, under nitrogen at room temperature, on a Perkin Elmer FT-IR 881 spectrophotometer, in KBr (pellets). Elemental analysis was carried out on a Perkin Elmer 240, C/H/N analyzer.

Carbon-Nanotubes oxidation and activation process

Commercially available Multi-Walled Carbon Nanotubes (MWCNTs) (507 mg) (CAS: 308068-56-6, Sigma-Aldrich, product code: 724769, lot. MKBH7743V, O.D. x L. = 6-9 nm x 5 μm, carbon>95%) were stirred in sulphonitric mixture (20 mL), (H_2SO_4 96% and HNO_3 65%, 3:1) at 100°C for 30 min. The suspension was then diluted with cold water and filtered on Teflon filter (Wathmann, pore size 0.2 μm). The residue was washed with water to neutral pH. Finally, the solid was dried under vacuum for 12 h affording a black powder (382 mg). Activation of Ox-MWCNTs was performed with NHS/EDAC as described by Jiang and colleagues [3].

Coupling Carbon nanotubes with mAb_2

Oxidized and activated MWCNTs (1 mg) were dispersed in 1 mL of 0.5X HBS-EP and sonicated for 30 min. Afterward, 1 mg L^{-1} mAb_2 was added and incubated under stirring, overnight at room temperature. A centrifugation step (14000 *g*, 10 min, 2X) was realized to remove unbound mAb_2 by discarding the obtained supernatant. Then MWCNT-mAb_2 conjugates were passivated with aqueous 1 M EA solution (pH 8.5) for 2 h at RT. A final centrifugation step (14000 *g*, 10 min) followed by re-suspension in coupling buffer, was performed to wash EA after

passivation. Finally a mild centrifugation step was realized (2000 *g*, 90 seconds) in order to remove evident aggregates from the solution. Only the supernatant was injected in the SPR system to interact with the analyte.

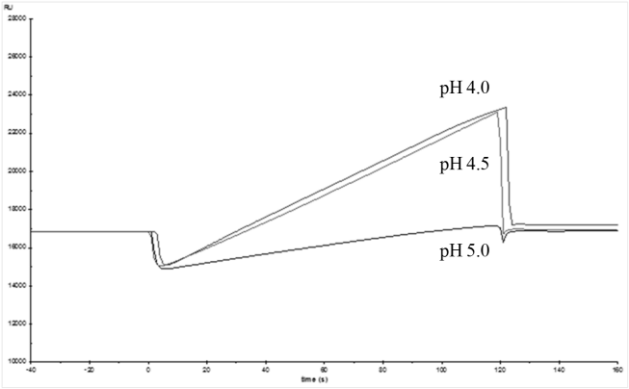

Fig. S3-1: Pre-concentration test for mAb_1. The test was performed dissolving 10 mg L^{-1} mAb_1 in 10 mM $CH_3COONa \cdot 3H_2O$ at pH 4.0, 4.5, and 5.0, as indicated in figure. Antibody solutions were injected for two minutes on non-functionalized CM5 sensor chip to perform the test. Running buffer was maintained at constant flow rate of 10 μL min^{-1}.

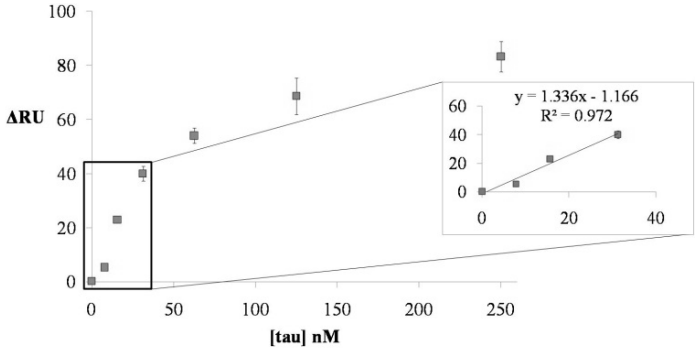

Fig. S3-2: Calibration of the immunosensor for tau protein in 1X HBS-EP. Limit of detection is estimated by the linear plot considering 3σ=6 RU.

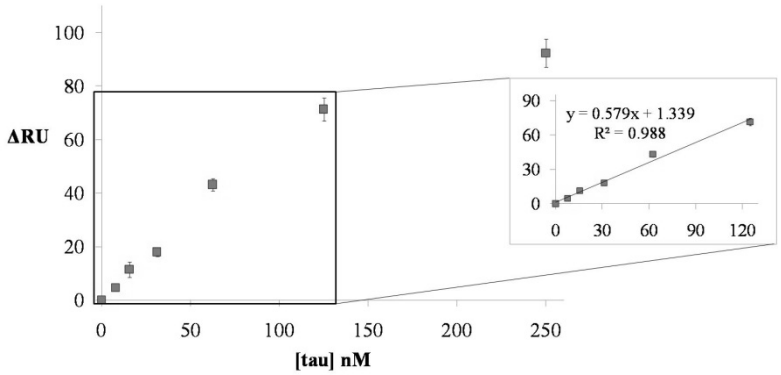

Fig. S3-3: Calibration of the immunosensor in artificial cerebrospinal fluid (aCSF). Limit of detection is estimated by the linear plot considering 3σ=6 RU.

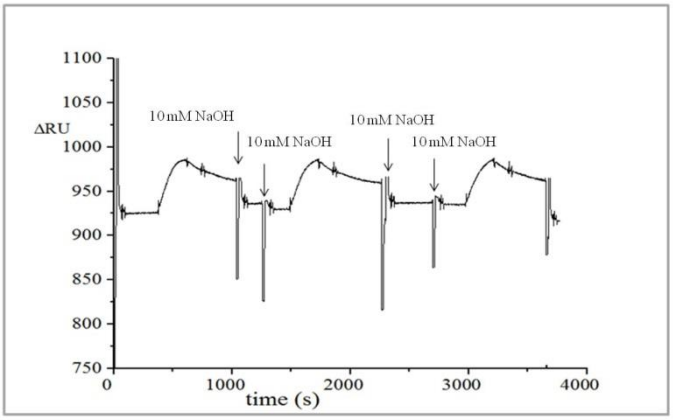

Fig. S3-4: Regeneration of the sensor during three sequential cycles. NaOH injections (10 mM, 24 s) are indicated by arrows.

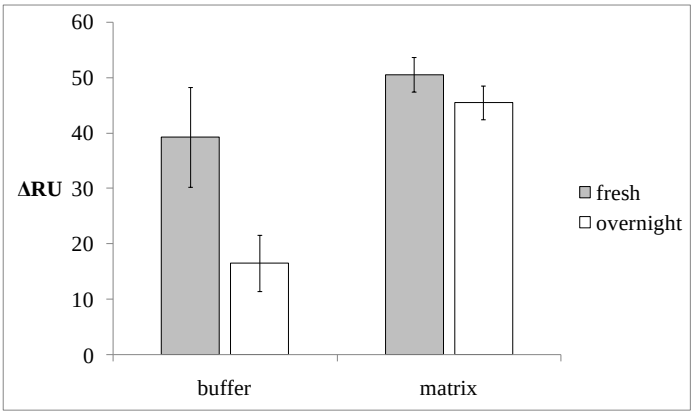

Fig. S3-5: Reflectivity changes inferred from 62.5 nM of tau protein in 1X HBS-EP (buffer) or aCSF (matrix). Grey bars represent fresh tau aliquots injected immediately after preparation, while white bars represent tau protein preserved overnight at 4°C. Measurements were performed in triplicates.

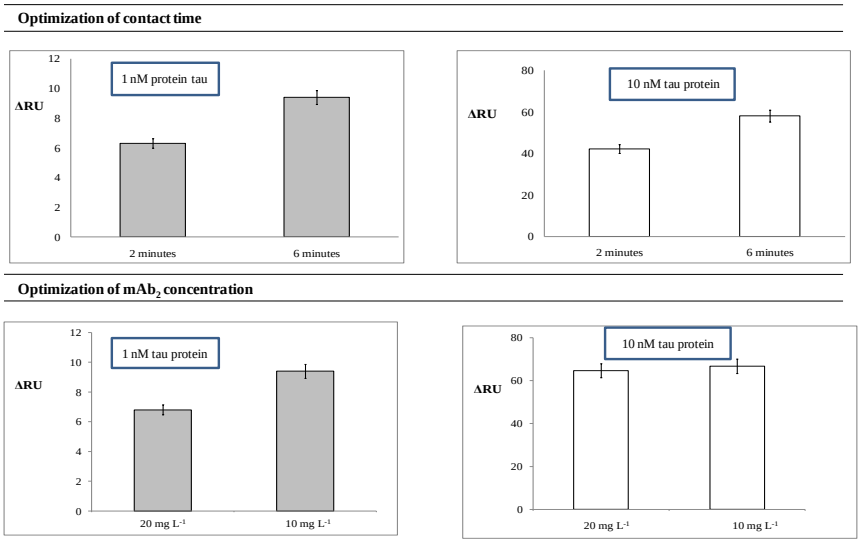

Fig. S3-6: Upper panel: variation of tau-mAb_2 interaction time at constant antibody concentration. 2 and 6 minutes were tested respectively. Lower panel: variation of mAb_2 concentration (20 mg L^{-1} versus 10 mg L^{-1}) at optimal contact time of six minutes.

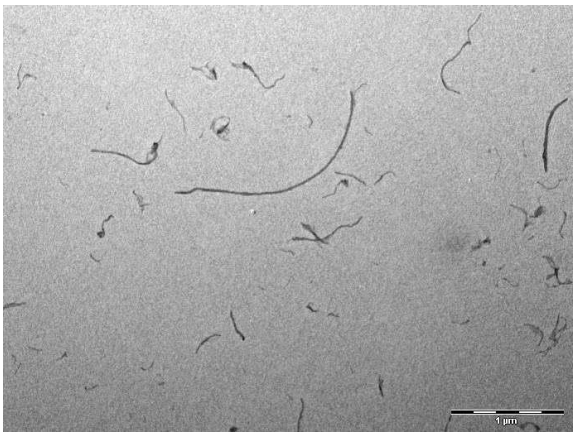

Fig. S3-7: TEM image of oxidized CNT.

Elemental analysis:

a) oxidized MWCNT: IR (KBr): 3422, 2918, 2851, 1696, 1621, 1546, 1211, cm^{-1}.

Elem. Anal. C, 81.34; N, 0.19; H, 0.65. (Oxidized MWCNT)

b) activated O-Succinimide-Ox-MWCNT: IR (KBr): 3446, 3020, 2958, 1699, 1553, 1330 cm^{-1}

Elem. Anal (activated OSu Ox MWCNT): C, 75.80; N, 5.49, H, 2.70.

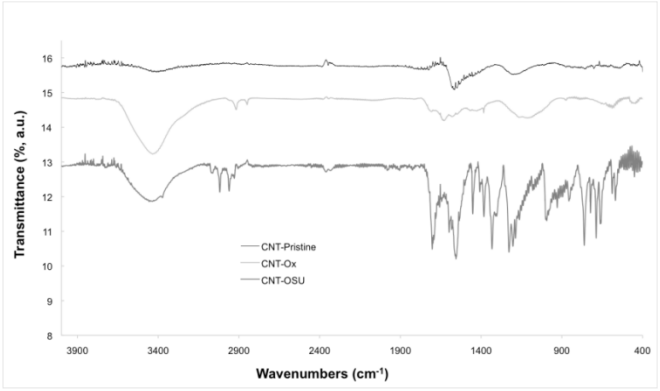

Fig. S3-8: IR spectra of pristine (black line, top), oxidized (red line, middle), and activated with O-succinimide groups (blue line, bottom) nanotube obtained in KBr.

3.5.2 Optimization of parameters for MWCNTs-mAb2 coupling

The behavior of activated-MWCNTs in solution, initially studied on BSA as model system, resulted highly dependent from the sonication pre-treatment performed, in agreement with previous observation of two different physical states for these nanostructures by using different sonication times [4]. The suspension was more stable after 30 minutes sonication in 0.5X HBS-EP, while a thick deposit was observed for MWCNTs sonicated in the same medium for 10 minutes (Fig. S3-9: Steps carried out to optimize CNTs functionalization by varying the sonication time on non-functionalized MWCNTs (1), the immobilization medium used for MWCNT-mAb_2 conjugation (2), and the MWCNTs/mAb_2 ratio (3).

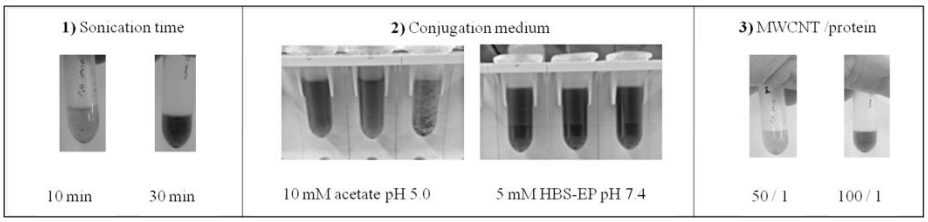

Fig. S3-9: Steps carried out to optimize CNTs functionalization by varying the sonication time on non-functionalized MWCNTs (1), the immobilization medium used for MWCNT-mAb_2 conjugation (2), and the MWCNTs/mAb_2 ratio (3).

Buffer composition and pH strongly affect the handling of MWCNTs during their conjugation to mAb_2. Despite acetate buffer (10 mM, pH 5.0, under mAb_2 isoelectric point) was the first choice to perform amino-coupling reaction and mAb_2 conjugation, we observed a massive aggregation under this condition, probably due to the protonation of carboxyl sites over the nanotubes surface (Fig. S3-9, panel 2, left). On the contrary, the use of HBS-EP buffer (0.5 X, pH 7.4) used for binding assays provided very homogeneous and stable dispersion of MWCNTs (Fig. S3-9, panel 2, right) and was selected for conjugation. Finally, also

the mAb_2 concentration strongly affects the stability of MWCNTs suspension. In particular, MWCNTs/mAb_2 ratios higher than 50:1 led to the rapid aggregation of nanostructures (Fig. S3-9, panel 3), whereas by scaling down the ratio of 100:1 the aggregation can be successfully avoided and it was hence chosen for bioconjugation.

References of supplementary material

[1] L. Liu, F. Zhao, F. Ma, L. Zhang, S. Yang, N. Xia, Electrochemical detection of β-amyloid peptides on electrode covered with N-terminus-specific antibody based on electrocatalytic O2 reduction by Aβ(1-16)-heme-modified gold nanoparticles., Biosens. Bioelectron. 49 (2013) 231–235.

[2] Kretschmann, E.; Raether, H. Z. Radiative Decay of Non Radiative Surface Plasmons Excited by Light Naturforsch, 23, (1968) 2135-2136, 10.1515/zna-1968-1247

[3] K. Jiang, L.S. Schadler, R.W. Siegel, X. Zhang, H. Zhang, M. Terrones, Protein immobilization on carbon nanotubes via a two-step process of diimide-activated amidation, J. Mater. Chem. 14 (2004) 37–39.

[4] E. Edri, O. Regev, "Shaken, not stable": dispersion mechanism and dynamics of protein-dispersed nanotubes studied via spectroscopy, Langmuir. 25 (2009) 10459–10465.

4 Aptamer selection for protein tau

After demonstrating the suitability of Surface Plasmon Resonance in quantification of tau protein by well-known antigen-antibody interaction, the second part of the work was dedicated to the development of the aptamer against tau protein. Two different aptamer selection strategies were designed and compared, both based on Capillary Electrophoresis (CE) as a separation technique. The first approach was based on the conventional CE- SELEX approach, in which separation and amplification by Polymerase Chain Reaction (PCR) were carried out in each selection round, whether the second was instead inspired to the CE-Non-SELEX method. Here the amplification by PCR was realized only once, after all partitioning rounds, on the pool of sequences that showed the highest affinity for the target.. Only CE-Non-SELEX resulted suitable for affinity improvement, possibly because of the non-amplification of the PCR by products, with little affinity for tau protein. Pooled sequences issued of the third round of CE-Non-SELEX, were sequenced by Next Generation Sequencing (NGS) based on the ion-torrent machine. Thanks to this, thousands of potential aptamers were obtained, thus data were further analyzed by bioinformatics tools before choosing the isolated sequences for experimental screening. After this step five isolated DNA strands, were collected and their affinity for tau was evaluated by Fluorescence Anisotropy (FA) and SPR. Both seemed to confirm that three aptamers presented higher affinity for the target, compared to the initial library. As a whole in this part of the work we concluded that CE-Non-SELEX was superior to SELEX in selecting aptamers for tau protein. The large pool of sequences obtained, despite promising, seem only very partially explored, thus more in depth analysis of the pool may give access to aptamer that

would be more potent than those analyzed experimentally during this work, leaving great room for improving the technology.

A manuscript from the results reported here is in preparation with the following title:

"CE-SELEX and CE-Non-SELEX comparison for the selection of an aptamer directed against the tau protein."-*Manuscript in preparation*

4.1 Introduction

After the first selection in 1990 [1,2], aptamers have been widely employed either for therapeutic purposes, or for bioanalytical devices. Such shorts single stranded DNA (ssDNA) or RNA sequences have properties that make them suitable for competing with the most popular class of receptors *i.e.* antibodies [3,4]. For instance, one of their major advantages is related to the production process, which is carried out *in vitro* by the so-called SELEX (from Systematic Evolution of Ligands by EXponential enrichment), with reduced batch-to-batch variability [5]. Among SELEX methodologies, capillary electrophoresis- SELEX (CE-SELEX) [6–8] presents some significant advantages, such as very low sample consumption, no need of target immobilization, and absence of negative selection steps [6]. A slightly modified version of CE-SELEX is also reported and named CE-Non-SELEX. While in conventional SELEX each round is followed by Polymerase Chain Reaction (PCR) amplification, in CE-Non-SELEX selection cycles are carried out without amplification steps between them. After separation, the recovered DNA is incubated with a new amount of the target and immediately re-injected into the separation system, considerably speeding-up the selection process, and leading to the aptamer selection in about 1 h [9]. Despite the above-mentioned advantages, to the best of our knowledge very few examples of aptamer selection by CE-Non-SELEX are reported [10,11].

With this in mind, the selection of aptamers against tau protein was approached by both methodologies, in order to compare the outcomes of the two processes. Human tau, together with amyloid beta peptide, is one of the currently accepted biomarker in CerebroSpinal Fluid (CSF) for Alzheimer's disease (AD) early diagnosis [12]. Up to now majority of works that investigated interaction among tau and nucleic acids reported ability of the protein to interact with double strands DNA or RNA only

[13,14]. However more recently ssDNA-tau affinity complex was highlighted, making tau a potential candidate for aptamer selection [15,16]. After the most affine ssDNA sequence described by Krylova and colleagues [16] was used as capturing agent for a mixed DNA-antibody assay on SPR [17], Kim and colleagues described a selection process for anti-tau aptamer, in 12 selection rounds. [18]. Here is reported the selection of aptamer candidates for tau protein after only three rounds of CE-Non-SELEX. Two techniques were used to evaluate the affinity of isolated sequences: Fluorescence Anisotropy (FA), and Surface Plasmon Resonance [19,20]. A critical comparison between the CE-SELEX and CE-Non-SELEX methods showed some weakness and strengths of the two selection approaches, leaving room for possible improvement in the near future.

4.2 Materials and Methods

4.2.1 Materials

Human tau protein longest isoform (tau441) was purchased from Enzo Life Science (Lyon, France), supplied lyophilized and reconstituted as recommended by the seller. The linear peptide, LinR3 (representing amino acid G271-G323 of tau441) was kindly provided by I2BM group. Human Serum Albumin (HSA), mesityloxide (MO), trizima base (tris), glycin (gly) and all the reagents for acrylamide gel preparation were from Sigma Aldrich (St Quentin Fallavier, France). N-hydroxysuccinimide (NHS) was from Fluka (Milan, Italy); sodium acetate was from Carlo Erba reagents (Milan, Italy);1-ethyl-3-(dimethylaminopropyl) carbodiimide (EDAC) was fromMerck (Darmstadt, Germany); 4-(2-Hydroxyethyl)piperazine-1-ethanesulfonic acid (HEPES), ethylenediaminetetraacetic acid(EDTA),

ethanolamine hydrochloride (EA) used for SPR experiments were all from Sigma Aldrich (Milan, Italy). Other chemicals for buffer preparation were commercially available and purified at analytical grade. The 77-nucleotide (77-nt) fluorescently labeled single-stranded DNA (ssDNA) library (5'-carboxy fluorescein (FAM)-GCCTGTTGTGAGCCTCCTGTCGAA-30 random nucleotides-TTGAGCGTTTATTCTTGTCTCCC-3'), unlabeled reverse primer, (5'-ACTGACTGACTGACTGACTA-6C3-GGGAG ACAAGAATAAACGCCAA and isolated aptamers were from Eurofins Genomics (Ebersberg, Germany). Labeled (5'-FAM-) and unlabeled forward primer (5'-GCCTGTTGTGAGCCTCCTGTCGAA) were from Eurogentec (Liege, Belgium).GeneAmp 10X PCR buffer II, AmpliTaq Gold DNA polymerase and SYBR® green PCR Kit were provided were from Applied Biosystems(Foster city, California, USA).

Capillary Electrophoresis apparatus – All separations were carried out using Beckman Coulter P/ACE MDQ system (Fullerton, California, USA) with exchangeable UV absorbance and laser-induced fluorescence (LIF) detectors (λ_{ex} 488 nm and λ_{em} 520 nm) equipped with a fused silica capillary for all experiments. The sample was injected applying 0.7 psi pressure at the inlet of the capillary and injection times varied depending on the experiment. The fused-silica capillaries were conditioned by performing the following washing steps at 20 psi: 1 M NaOH for 5 min, water for 5 min and migration buffer (which composition varies depending on the experiment) for 30 min. Further washing process was performed between each run at 20 psi with 1 M NaOH (2 min), water (2 min) and migration buffer (5 min) to improve repeatability of peak migration time. The voltage applied during separation was 20 kV for all experiments.

4.2.2 Preliminary steps

Tau protein mobility identification- Tau protein (15 µM) was dissolved in three different buffers (table s4-1) varying pH values (7.4; 5.5; 3.0) to verify electrophoretic mobility ($\mu_{\pm}$) under different conditions. ElectroOsmotic Flow (EOF) was monitored using a 1 % (w/w) mesityloxide (MO) solution. Since ionic strength, temperature and migration voltage significantly affect the separation [21], all these parameters remained unchanged during the analysis. Electrophoretic runs were performed on a bare silica capillary of 60 cm (total length) with an internal diameter of 50 µm (Polymicro Technologies Inc., Phoenix, Arizona, USA). Inlet was positively charged (normal polarity) and the distance from the detecting window was 50 cm. Protein was detected by UV at 200 nm wavelength. Electrophoretic mobility of the species was calculated according to equation 1 to 3 (in supplementary materials)

Quantitative PCR (qPCR) or RT-PCR (real time PCR) - Experiments were carried out on collected samples, issued from CE separation, using Applied Biosystem StepOne™ Real Time - PCR instrument (Applied Biosystem, Foster city, California, USA). Amplification was carried out following previously published protocol [22]. Briefly 2 µL of collected ssDNA were mixed with 18 µL of SYBR® green PCR Kit (final volume 20 µL). Unlabeled forward primer and reverse primer were kept at 10^{-5} M. Thermal cycle consists in initial denaturation at 95 °C (10 minutes), followed by 36 amplification cycles during which 15 seconds at 90 °C were alternated with 30 seconds annealing step at 60 °C. Threshold cycles (C_t) were determined for each sample, and averaged on three independent measurements.

4.2.3 Aptamer selection for tau protein

CE-SELEX - Separation was optimized using TGK as migration buffer (25 mM Tris, 192 mM glycine, 5 mM K_2HPO_4, pH 8.34). DNA library was heated at 80°C for 5 minutes and left at room temperature for 10 minutes prior to tau addition. After 20 minutes incubation, at room temperature, in TGK buffer with 1 mM $MgCl_2$, circa 30 nL (calculated by Poiseuille equation) of the mixture (total volume 30 µL) were injected. The capillary used for conventional selection was 60 cm length from inlet to the bottom (effective length from inlet to detector was 50 cm) with an inner diameter of 50 µm and an outer diameter of 360 µm (Polymicro Technologies Inc., Phoenix, Arizona, USA). Six rounds were carried out, during which the sample was collected repeatedly (up to 8 times) in 100 µL of migration buffer. Concentration of tau protein and library ratio were modified along rounds as reported in table 4-1, and finally amplified by PCR.

table 4-1: tau and DNA concentration along CE-SELEX rounds.

round	[tau]nM	[ssDNA] µM	DNA/ tau ratio
1	7.50	40	5
2	0.25	1.25	5
3	0.13	1.25	10
4	0.13	1.25	10
5	0.06	1.20	20
6	0.04	1.10	30

CE-Non-SELEX - The capillary for electrophoretic separation was 80 cm in length (70 cm from inlet to detection window) with an inner diameter of 75 µm (Polymicro Technologies Inc., Phoenix, Arizona, USA).The DNA library (40 µM) was heated at 80°C for 5 min and left at room temperature for 10 min. Tau (7.50 µM) was then added to the library for the first round of selection. The mixture was incubated at room temperature for 20

minutes in incubation buffer (10 mM Tris, 20 mM NaCl, 1 mM $MgCl_2$; pH 8.34). For each selection round, DNA sequences, target protein and incubation buffer were combined in 40 µL total volume. Tau concentrations were 750 nM and 75 nM for rounds 2, and 3, respectively. The equilibrated sample was injected for 40 seconds and detected using UV to monitor the separation. During a selection round, the eluate was collected into 30 µL migration buffer (50 mM Tris, pH 8.34) until the unbound DNA peak began to elute. During the first round approximately 187 nL were injected, containing 10^{12} sequences of ssDNA. Subsequent rounds of selection used collected DNA, from the previous round as the input DNA. Immediately after migration, the collected mixture was incubated with a new aliquot of the target and injected again for the new selection cycle. Only the last round is amplified by PCR and sequenced.

PCR amplification and ssDNA recovery - ssDNA candidates were generated using a reverse primer and a 5'-FAM-labeled forward primer during each round of the selection procedure. The reverse primer is made heavier with a succession of six C3 links extended with a 5' DNA stretch of 20 nucleotides. During PCR, this six C3 region and the 20-nucleotide stretch cannot be amplified by the Taq DNA polymerase. A PCR product with two strands of unequal length is consequently synthesized. Each strand is then easily purified on a denaturing polyacrylamide gel. All PCR were performed using a Biometra cycler from Labgene (Archamps, France). Master mix was made by combining 886 µL nuclease-free water, 64 µL deoxyribonucleotide triphosphate (dNTPs) (25 mM of each) (Invitrogen, Cergy-Pontoise, France), 200 µL each of forward and heavier reverse primers (10^{-5} M), 240 µL $MgCl_2$ (25 mM), and 200 µL GeneAmp 10X PCR buffer II (500 mM KCl and 100 mM Tris-HCl, pH 8.3). After mixing, 10 µL (5 U/µL) of the AmpliTaq Gold DNA polymerase was

added. To finish, 200 µL of DNA collected during selection were added. This mixed solution was divided equally over thin-walled tubes that were subjected to PCR. The thermal cycling regime was: initial denaturation for 10 min at 95°C, and then cycling for 60 s at 95°C, 60 s at 60°C and 90s at 72°C for 14 cycles. After a Nanosep® 3K purification (Pall, Washington, New York, USA), the samples, which contained different amounts of amplified products, were resolved on a 12% acrylamide gel (see supplementary material for preparation and apparatus) at an applied voltage of 300 V. The band corresponding to the selected aptamers migrates with the same velocity of the library (used as a witness) and was visualized by UV-shadow method at 254 nm. After that, it was cut and eluted for 1 hour at 65°C and 1 hour at 4°C, in 1 mL of the extraction buffer (100 mM Tris-HCl, 500 mM NaCl, 1 mM EDTA, pH 7.4). To remove acrylamide, the extracted product was transferred into a Nanosep® 3K device, the retention membrane was replaced by glass wool and the system was centrifuged during 10 min at 14,000 rpm with MIKRO 220R centrifuge (r=87 mm) (Hettic centrifuges, Tuttlingen, Germany). Filtrate was removed, transferred in another native Nanosep® 3K device and centrifuged for 90 min at 5,000 rpm at 15°C. The material retained on top of the porous membrane was washed with 100 µL of water and centrifuged again. Finally ssDNA amount was quantified by UV absorbance at 260 nm.

4.2.4 High Throughput Sequencing

After purifying PCR products with MinElute PCR Purification Kit Qiagen (70 bp to 4 kb) and elution in very small volumes (10 µL), Next Generation Sequencing (NGS) was used to determine aptamers sequences. According to the protocol, DNA concentrations were adjusted to 200nM (Qubit® dsDNA BR Assay Kit and Qubit® 2.0 Fluorometer) and a library

was built using AB Library Builder System and Ion Xpress Barcode. Clonal amplification was performed using the Ion One Touch 2 system and the Ion PGM Template OT2 200 kit (all materials were from Life Technologies SAS, Saint Aubain, France).

The sequencing step was performed using the Ion PGM™ Sequencing 200 Kit v2, the Ion 316 Chip and the PGM instrument, Personal Genome Machine (PGM™) System (Life Technologies SAS, Saint Aubin, France). The PGM technology is based on the detection of releasing hydrogen ion during nucleotide incorporation in DNA template sequences [23]. In case of all chip micro-wells, the incorporated dNTP and the release of a proton triggers an ion sensor and an electrical signal indicates that a reaction has occurred. Automated read datasets were provided by Ion Torrent software 7.0.3 as FASTQ format. Obtained sequences were further analyzed by two software: PATTERNITY-seq (v 1.0) and MEME (Multiple Em for Motif Elicitation, where Em stand for the algorithm used for motif discovery) open source suite for motif discovery [24,25].

4.2.5 Affinity analysis of DNA sequences

CE-LIF - affinity study was carried out on pooled ssDNA to evaluate evolution of selection approaches. To this aim, areas under the curve (AUC) of the species in the electropherogram was calculated. Depending on the selection process, 60 cm or 80 cm length (from the inlet to the end) fused silica capillaries were used (internal diameter 50 or 75 µm respectively). Migration and incubation buffers were the same of CE-SELEX or CE-Non- SELEX. Equilibrium mixture (EM) was composed of 100 nM 5'-FAM-ssDNA (original library or selected pools), heated for 5 minutes at 80 °C prior to be incubated with tau protein (3 to 9 µM) for 30 minutes. EM was injected by applying 0.7 psi pressure at the inlet of the capillary for 20 s. Detection of the species was insured by LIF detector.

FA - Fluorescence Anisotropy (FA) measurements were performed on Tecan Infinite F500 microplates reader (Männedorf, Switzerland). 384-wells black microplates (Grenier Bio-One, Courtaboeuf, France) were used for the minimal sample volume required for the analysis (10 µL). 10 nM of 5'-FAM-ssDNA (library or isolated sequences) were dissolved in incubation buffer (10 mM Tris, 20 mM NaCl, 1 mM $MgCl_2$; pH 8.34) and underwent the same treatment carried out during selection. After 30 minutes at room temperature, tau protein (20 - 700 nM) or LinR3 peptide (0.4 – 14 µM only for selected aptamers) were added to the DNA solution and immediately analyzed. Excitation was set up at 485 ± 20 nm and emitted light was collected at 525 ± 25 nm. Fluorescence anisotropy was directly calculated by the instrument software, and the signal was expressed as difference ($\Delta r=r-r_0$) between that obtained in presence of the target (r) and that of the fluorescently labeled ssDNA alone (r_0).

SPR – BIAcore X™ (GE Healthcare, Uppsala, Sweden) was used for all experiments. Tau protein was immobilized on a CM5 sensor chip (GE Healthcare, Uppsala, Sweden) by amino-coupling reaction [26]. To this aim, the chip surface was activated by the injection of 50 mM/200 mM solution of NHS/EDAC (10 minutes, 5 µL min^{-1}), followed by the injection (30 minutes, 2 µL min^{-1}) of 100 mg L^{-1} tau protein dissolved in 10 mM acetate buffer (pH 4.0). In the end EA (1 M, pH 8.5) was used to block residual active sites (15 minutes, 5 µL min^{-1}). The five selected aptamers (unlabeled, sequences listed in the next section) were injected for 5 minutes in the system at concentrations ranging from 1 to 10 µM, and resulting sensorgrams were analyzed by BIAevaluation 3.1 software. During the analysis HBS-EP (10 mM HEPES, 150 mM NaCl, 3 mM EDTA, with 0.005% Tween 20; pH 7.4) was used both as running buffer

and for aptamer dilutions. The flow rate was maintained at 5 μL min^{-1} and temperature at 25 °C.

4.3 Results and discussion

4.3.1 Preliminary steps for optimization of selection conditions

Electrophoretic tau mobility determination

Aptamer selection by CE-SELEX or CE-Non-SELEX requires optimal electrophoretic separation among the target and the ssDNA library [27]. To this aim identification of tau protein was necessary, however at 200 nm wavelength several peaks were detected in the electropherogram (Fig. S4-1). Usually proteins are specifically detected at 280 nm wavelength (Soret's band), however tau absorbance in this region is very low because of the little amount of aromatic amino acid residues which leads to low molar absorptivity coefficient (7450 cm^{-1}) [28,29].

Using small-diameter capillary tubes, no tau was detected, thus monitoring of electrophoretic mobility (μ_{eff}) of peak 1 and 2 (Fig. S4-1, panel a and b) was chosen as criteria to identify tau. Peak 2, showing higher absorption at 200 nm, showed an electrophoretic mobility value strongly dependent from the pH of the buffer solution ($\mu_{eff}^{pH\ 3.0}$= 9.47 x 10^{-9}; $\mu_{eff}^{pH\ 5.5}$= 1.52 x 10^{-10}; $\mu_{eff}^{pH\ 7.4}$= -5.93 x 10^{-10} m^2 x V^{-1} x s^{-1}). This behavior is consistent with protein properties, since the net charge depends on their isoelectric point. Unlike species represented by peak 2, peak 1 showed a constant electrophoretic mobility, and eventually disappeared at pH 3.0, probably because fully undissociated under these conditions (Fig. S4-1, panel c).

Once the peak of tau protein was identified, the separation conditions both for CE-SELEX and CE-Non-SELEX were optimized and the collection windows were defined (Fig. 4-1).

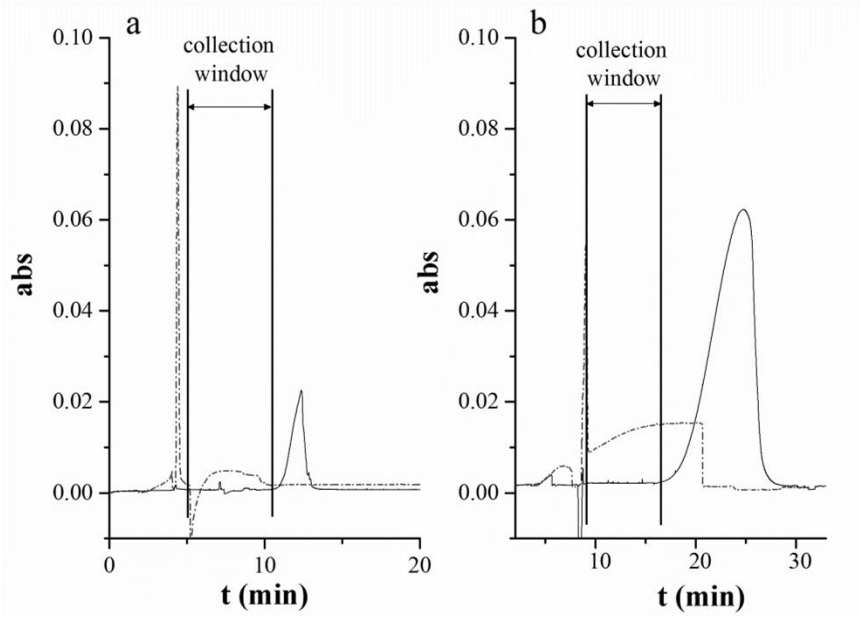

Fig. 4-1: Collection window for CE-SELEX (panel a) and CE-Non-SELEX (panel b). Tau protein peak (dashed line) migrated before the beginning of collection window, while the ssDNA library (solid line) was outside the boundaries of collected sample. Both electropherograms were recorded at 20 kV, T 25°C and detected by UV at 200 nm.

4.3.2 Optimization of PCR rounds and target concentration

Starting target concentration is one of the key points for successful selection. As a rule of thumb the target should be incubated with the library at concentration two orders of magnitude lower than initial K_d [27] but, in this case, little amount of stable affinity complex was observed when 750 nM of tau were injected. A more in depth analysis to evaluate presence of DNA-tau complex was realized by qPCR, carried out after CE separation under optimized conditions. A higher amount of dsDNA is recovered from PCR if the DNA-tau adduct is present. Nevertheless, at 750 nM tau concentration, C_t (threshold cycles) for control (library alone) and positive sample were the same (Fig. 4-2, solid line versus dashed-dot-line respectively).

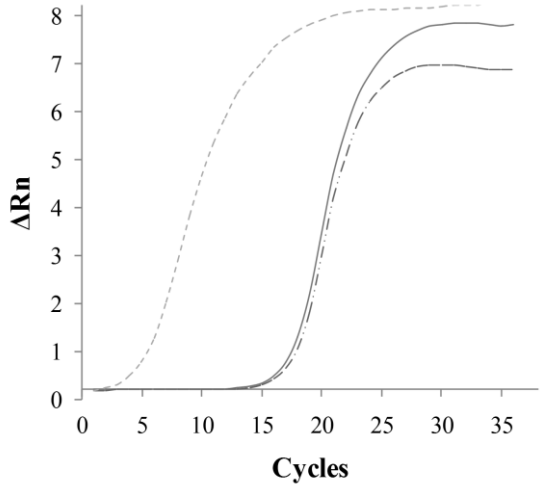

Fig. 4-2: Comparison of qPCR experiments realized after capillary electrophoretic separation of 750 nM tau-ssDNA (red, dashed-dot line) and 7.5µM tau-ssDNA (green, dashed line). 40 µM of ssDNA were used for all experiments. Library alone was also amplified by qPCR as control (blue, solid line).

To overcome this limit, the increase of tau concentration during the CE separation was considered. To this aim, two strategies were tested. The first one consisted in increasing the number of collections and the injected volume for each separation, while in the second approach tau concentration, incubated with the library, was increased from 750 nM to 7.50 µM. Using the first approach, electrophoretic migration time of tau protein and library showed poor repeatability after few collections (data not shown). The second approach showed only little influence on the migration time of the species and, more importantly, C_t were significantly lowered as compared with previous experimental set-up (6.26 ± 0.04 versus 17.57 ± 0.44). These operative conditions revealed a significantly higher amount of tau-ssDNA complex, and were therefore chosen for selection.

4.3.3 Aptamer selection: CE-SELEX versus CE- Non -SELEX

After optimization of the initial conditions, the selection was carried out. The affinity of the selected pool was constantly monitored by measuring the area under the curves (AUC) in the electropherogram, using LIF detector. Only CE-Non-SELEX approach showed significant increase of AUC of the affinity complex (Fig. 4-3a), whether no evolution was found for CE-SELEX (Fig. 4-3b) .

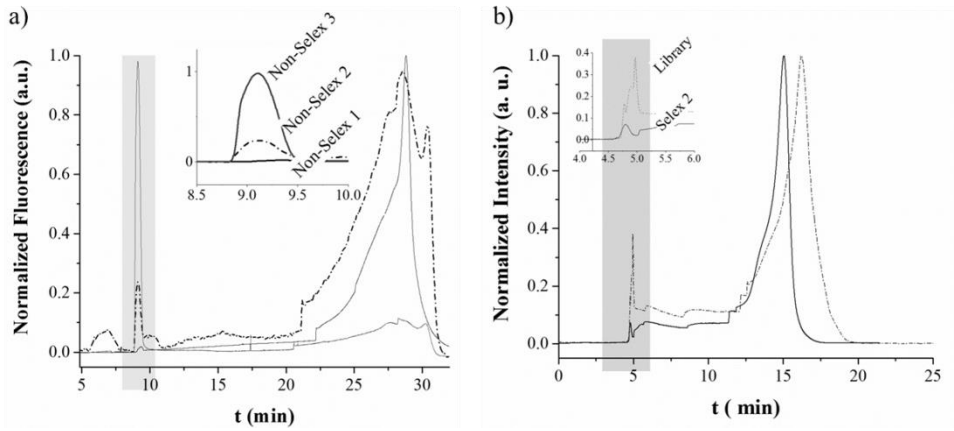

Fig. 4-3: Affinity complex of the two selection methods. a) CE-Non-SELEX; stable affinity complex of the three rounds (indicated in the inset) is collected at 9.8 minutes migration time. Areas under the curves of library and first selection round were closely related, thus only the latter is reported. b) CE-SELEX showed no evolution; stable complex formation (in the inset) is better for library (dotted line) than after two rounds of conventional selection (solid line).

The reasons leading to affinity improvement by CE-Non-SELEX might be related to the absence of the amplification step. Indeed, for the CE-SELEX approach, the presence of PCR by-products was suggested by two different experiments. A significant shift of melt temperature (T_m) was observed by qPCR after six rounds of selection by CE-SELEX, showing a T_m of 68°C which was considerably lower than that observed for the initial library (T_m = 75°C) (Fig. S4-1). Even though such shift may be addressed to the increased structural homogeneity of the selected pool compared to

initial library, this possibility seemed to be remote. Schütze and colleagues reported an increment of T_m as long as the selection proceeded [30]. Here T_m reduction could most likely be addressed with the formation of PCR by-products due to primers dimerization, primer-primer (forward with reverse) interactions, or to the presence of impurities in the original library [31,32]. Presence of PCR by-products was also suggested from the amplification of a negative control sample in which the library was injected alone into the capillary and collected in the same conditions of CE-SELEX. No ssDNA should have been recovered from the collection window, however a broad peak, running with same migration time of the library, was observed in the electropherogram (Fig. S4-3). By- products formation is a well-known problem of SELEX. The use of CE for separation might even worsen such issue because, when compared with other approaches, lower amount of ssDNA is injected into the capillary, requiring very high efficiency of the amplification step. When the accumulation of the desired amplicons is over, presence of primer excess in solution could significantly reduce efficiency, resulting in the undesired amplification of secondary products [33,34].

4.3.4 Sequencing results

After selection, the ion torrent sequencing gave rise to 4792 DNA strands which are all possible aptamer candidates. PATTERNITY-Seq, developed by Ducongé's group, was used for identification of most abundant families within the whole DNA pool. This in-house developed software, was designed to highlight DNA families by finding similarities among DNA sequences using Levenshtein distance [35]. The tool was conceived for screening the sequences of any selection round, in order to highlight the presence of DNA similarities along cycles. Nevertheless the software can be used also to analyze the data of a single round of selection. Here only

round three was analyzed, since the affinity test showed the best binders were in that pool. High diversity of the sequence space was revealed, with the most abundant family representing only 0.09% of the whole pool. Such lack of enrichment might be explained by a multitude of factors. First of all the homogenous nature of CE separation used for selection greatly increased the degree of freedom for tau-ssDNA interaction. Usually considered as an advantage of the technique, in this case this feature could have contributed to the diversity obtained during the sequencing. Secondly, CE-Non-SELEX also limited the enrichment of the pool because of the lack of repeated PCR amplifications.

Together with this method, a more traditional approach was used to obtain a limited amount of sequences for experimental screening of the affinity. The entire DNA set was screened again using MEME-suite software, based on a different algorithm to seek for conserved motifs. Considering the limitations of this online open source utility, only a limited amount of DNA strands was uploaded on the website (in FASTA format), after the removal of constant region, and elimination of sequences shorter than 30nt (random region). MEME confirmed the results obtained previously, since no highly conserved motifs were found. Nevertheless five different blocks were isolated by grouping the aptamers depending on the number of single nucleotides (counting number of A,G etc.) present within each sequence. From each one of this block, five oligonucleotides were chosen for affinity determination (table 4-2)

table 4-2: Isolated ssDNA strands selected as possible anti-tau aptamer candidates. Only random region is reported.

Aptamer identifier	Truncated primer Aptamer sequence	Features
3146	CCTTTGGGGTGGCTTGACGAAGAAAGTAGT	Majority of G
3914	CGGTTCTTAAGGCGTCCGTCTTCATTTGTT	T with G and C; Stem-loop at 5'-end
4133	CCTGTCAGGTCTTTGACGAGGCTTTTCTTC	T with G and C; linear 5'-end
4618	CATGTTAATATTCCATCACCGACTTCTTTC	Majority of T
433	GGTGTCGACACCAGCCTTTAACCCTGTGTC	Equal distribution of all nucleotides

4.3.5 Affinity results of isolated sequences

Fluorescence Anisotropy

For affinity evaluation of the five isolated aptamers (including primer-binding regions) we initially chose an homogeneous signaling method to replicate the condition of selection process. Among tested sequences, three aptamers (sequences 4133, 3146, 433) showed higher affinity than the library for the target, whether low binding capabilities were found for sequences 3914 and 4618 (Fig. 4-4, panel a and b).

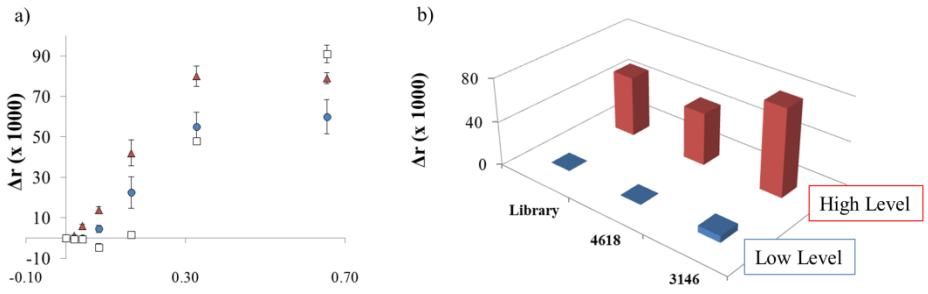

Fig. 4-4: Fluorescence anisotropy variations for aptamers 3146 (red triangles, panel a), 4618 (white squares, panel a) and the library (blue circles, panel a) incubated with tau protein (0.02 – 0.7 µM). Panel b shows same data for two concentrations of the target: 0.04 µM (low level) and 400 µM (high level).

All selected aptamers showed similar affinity for tau protein model (peptide LinR3, data not shown), revealing a role in improving selectivity for the amino acid residues present in the whole protein and absent in the LinR3 model. Human Serum albumin, chosen as negative control, did not interact with any of the selected aptamers, showing good specificity toward physiological proteins.

SPR

Tau protein immobilization was carried out at surprisingly acidic pH (4.0) with respect to the isoelectric point reported in literature (pI 8.6) [28]. Such immobilization pH was chosen with the help of preconcentration test (Fig. S4-4) carried to maximize amino-coupling efficiency, which gave rise to an average signal of 5190 ± 2 RU. The five aptamers, including the primer-binding regions, were then injected at same concentration level (1 µM) to compare affinity with tau under the same conditions. In Fig. 4-5 sensorgrams of the five sequences are reported.

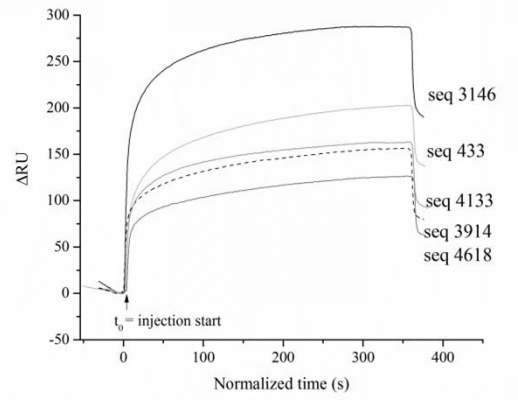

Fig. 4-5: Sensorgrams of five isolated sequences injected at 1 µM.

Despite the results reported here are only preliminary, they seemed to confirm those obtained by fluorescence anisotropy experiments. Aptamers

3146, 433, and 4133 showed higher signal as compared to the other two sequences. Aptamer 3146 was chosen for kinetic experiments. A 10 µM solution of DNA was initially injected and seemed able to saturate whole active sites present on the sensor surface. Recovered signal (2570 ± 2 RU) was indeed compatible with theoretical R_{max} (2720). Since R_{max} value might be obtained at species concentration equal to 100 K_d, we could expect affinity values for the aptamer is in the nM region. However some experimental problems were highlighted. After few injections the repeatability of the signal was significantly worsened, resulting in difficult interpretation of the sensorgrams recorded at low aptamer concentrations.

4.4 Conclusion

In this paper, we reported a fast method for selecting aptamer for tau protein. Since the initial results obtained by conventional CE-SELEX were disappointing, an analysis of the factors leading to the lack of evolution was made. In the end amplification step results as a major obstacle to allow affinity to be improved. Reducing PCR steps in CE-Non-SELEX led to significant affinity increase in only three rounds, and at the same time, it considerably reduced the time demanded to realize the whole process. Despite highly stable DNA-tau complex was obtained with this approach, a very high diversity of the selected pool was highlighted by the bioinformatics tool developed by Ducongé's group. Absence of highly predominant families was obtained from PATTERNITY-Seq, revealing a lack of exponential enrichment using only one PCR step. To overcome this limitation, some strategies such as emulsion PCR has been reported to improve amplification efficiency [34], and might be considered for future development. Nevertheless the affinity of five isolated aptamers was tested by Fluorescence Anisotropy (FA) and Surface Plasmon Resonance (SPR),

showing similar results. The Aptamers 3146, 4133, and 433 clearly showed affinity improvements compared to the initial library, with a dissociation constant estimated around nM range by SPR. In conclusion, here we highlighted the importance of PCR in amplification when highly heterogeneous libraries are used as is the case aptamer selection. CE-Non-SELEX clearly improved the selection of anti-tau aptamers, revealing affinity in nM range, similarly to several aptamers present in scientific literature. Despite tau concentration is in pM range in Cerebrospinal fluid, the work seems promising to highlight critical areas for future improvement, leading to aptamers application to AD diagnosis.

References

[1] C. Tuerk, L. Gold, Systematic evolution of ligands by exponential enrichment: RNA ligands to bacteriophage T4 DNA polymerase, Science. 249 (1990) 505–510.

[2] A.D. Ellington, J.W. Szostak, In vitro selection of RNA molecules that bind specific ligands, Nature. 346 (1990) 818–822.

[3] M. Mascini, I. Palchetti, S. Tombelli, Nucleic acid and peptide aptamers: Fundamentals and bioanalytical aspects, Angew. Chem. Int. Ed. 51 (2012) 1316–1332.

[4] R. Stoltenburg, C. Reinemann, B. Strehlitz, SELEX--a (r)evolutionary method to generate high-affinity nucleic acid ligands., Biomol. Eng. 24 (2007) 381–403.

[5] M. Famulok, G. Mayer, Aptamers and SELEX in chemistry & biology, Chem. Biol. 21 (2014) 1055–1058.

[6] S.D. Mendonsa, M.T. Bowser, In vitro evolution of functional DNA using capillary electrophoresis., J. Am. Chem. Soc. 126 (2004) 20–21.

[7] R.K. Mosing, S.D. Mendonsa, M.T. Bowser, Capillary electrophoresis-SELEX selection of aptamers with affinity for HIV-1 reverse transcriptase., Anal. Chem. 77 (2005) 6107–6112.

[8] S.D. Mendonsa, M.T. Bowser, In Vitro Selection of Aptamers with Affinity for Neuropeptide Y Using Capillary Electrophoresis, J. Am. Chem. Soc. 127 (2005) 9382–9383.

[9] M. Berezovski, M. Musheev, A. Drabovich, S.N. Krylov, Non-SELEX selection of aptamers., J. Am. Chem. Soc. 128 (2006) 1410–1411.

[10] X. Yu, Y. Yu, A mathematical analysis of the selective enrichment of NECEEM-based non-SELEX, Appl. Biochem. Biotechnol. 173 (2014) 2019–2027.

[11] J. Ashley, K. Ji, S.F.Y. Li, Selection of bovine catalase aptamers using non-SELEX., Electrophoresis. 33 (2012) 2783–2789.

[12] K. Blennow, H. Hampel, M. Weiner, H. Zetterberg, Cerebrospinal fluid and plasma biomarkers in Alzheimer disease., Nat. Rev. Neurol. 6 (2010) 131–144.

[13] Q. Hua, R.Q. He, N. Haque, M.H. Qu, A. Carmen, I. Grundke-Iqbal, K. Iqbal, A. del Carmen Alonso, Microtubule associated protein tau binds to double-stranded but not single-stranded DNA., Cell. Mol. Life Sci. 60 (2003) 413–421.

[14] T. Kampers, P. Friedhoff, J. Biernat, E.M. Mandelkow, E. Mandelkow, RNA stimulates aggregation of microtubule-associated protein tau into Alzheimer-like paired helical filaments., FEBS Lett. 399 (1996) 344–349.

[15] S. Camero, M.J. Benítez, A. Barrantes, J.M. Ayuso, R. Cuadros, J.J. Avila, Tau protein provides DNA with thermodynamic and structural features which are similar to those found in histone-DNA complex., J. Alzheimer's Dis. 39 (2014) 649–660.

[16] S.M. Krylova, M. Musheev, R. Nutiu, Y. Li, G. Lee, S.N. Krylov, Tau protein binds single-stranded DNA sequence specifically-the proof obtained in vitro with non-equilibrium capillary electrophoresis of equilibrium mixtures., FEBS Lett. 579 (2005) 1371–1375.

[17] S. Kim, A.W. Wark, H.J. Lee, Femtomolar Detection of Tau Proteins in Undiluted Plasma Using Surface Plasmon Resonance, Anal. Chem. 88 (2016) 7793–7799.

[18] J.H. Kim, E. Kim, W.H. Choi, J. Lee, J.H. Lee, H. Lee, D.E. Kim, Y.H. Suh, M.J. Lee, Inhibitory RNA Aptamers of Tau Oligomerization and Their Neuroprotective Roles against Proteotoxic Stress, Mol. Pharm. 13 (2016) 2039–2048.

[19] I. Lamberti, S. Scarano, C.L. Esposito, A. Antoccia, G. Antonini, C. Tanzarella, V. De Franciscis, M. Minunni, In vitro selection of RNA aptamers against CA125 tumor marker in ovarian cancer and its study by optical biosensing, Methods. 97 (2015) 58–68.

[20] G. Gokulrangan, J.R. Unruh, D.F. Holub, B. Ingram, C.K. Johnson, G.S. Wilson, DNA aptamer-based bioanalysis of IgE by fluorescence anisotropy, Anal. Chem. 77 (2005) 1963–1970.

[21] H. Whatley, Basic Principles and Modes of Capillary Electrophoresis, in: J.R. Petersen, A.A. Mohammad (Eds.), Clinical and Forensic Applications of Capillary Electrophoresis, Humana Press, Totowa, NJ, 2001: pp. 21–58.

[22] E. Fiore, E. Dausse, H. Dubouchaud, E. Peyrin, C. Ravelet, Ultrafast capillary electrophoresis isolation of DNA aptamer for the PCR amplification-based small analyte sensing., Front. Chem. 3 (2015) 49.

[23] M. Quail, M. Smith, P. Coupland, T.D. Otto, S.R. Harris, T.R. Connor, A. Bertoni, H.P. Swerdlow, Y. Gu, A tale of three next generation sequencing platforms: comparison of Ion torrent, pacific biosciences and illumina MiSeq sequencers, BMC Genomics. 13 (2012) 1–13.

[24] T.L. Bailey, M. Boden, F.A. Buske, M. Frith, C.E. Grant, L. Clementi, J. Ren, W.W. Li, W.S. Noble, MEME suite: tools for motif discovery and searching, Nucleic Acids Res. 37 (2009) 202–208.

[25] N.N. Quang, C. Bouvier, B. Lelandais, F. Ducongé, Improvement of aptamers using PATTERNITY-seq (high-throughput analysis of sequence patterns and paternity relationship between them)- International congress of Aptamer - oral communication, (2016) June 24th-25th, Bordeaux, France.

[26] E. Bulukin, V. Meucci, M. Minunni, C. Pretti, L. Intorre, G. Soldani, M. Mascini, An optical immunosensor for rapid vitellogenin detection in plasma from carp (Cyprinus carpio), Talanta. 72 (2007) 785–790.

[27] M. V Berezovski, M.U. Musheev, A.P. Drabovich, J. V Jitkova, S.N. Krylov, Non-SELEX: selection of aptamers without intermediate amplification of candidate oligonucleotides., Nat. Protoc. 1 (2006) 1359–1369.

[28] S. Jeganathan, M. Von Bergen, E.M. Mandelkow, E. Mandelkow, The natively unfolded character of Tau and its aggregation to Alzheimer-like paired helical filaments, Biochemistry. 47 (2008) 10526–10539.

[29] Y. Furukawa, K. Kaneko, N. Nukina, Tau protein assembles into isoform- and disulfide-dependent polymorphic fibrils with distinct structural properties, J. Biol. Chem. 286 (2011) 27236–27246.

[30] T. Schutze, B. Wilhelm, N. Greiner, H. Braun, F. Peter, M. Morl, V.A. Erdmann, H. Lehrach, Z. Konthur, M. Menger, P.F. Arndt, J. Glkler, Probing the SELEX process with next-generation sequencing, PLoS One. 6 (2011) e29604.

[31] T. Kanagawa, Bias and artifacts in multitemplate polymerase chain reactions (PCR), J. Biosci. Bioeng. 96 (2003) 317–323.

[32] F. Tolle, J. Wilke, J. Wengel, G. Mayer, By-product formation in repetitive PCR amplification of DNA libraries during SELEX., PLoS One. 9 (2014) e114693.

[33] M.U. Musheev, S.N. Krylov, Selection of aptamers by systematic evolution of ligands by exponential enrichment: addressing the polymerase chain reaction issue., Anal. Chim. Acta. 564 (2006) 91–96.

[34] R. Yufa, S.M. Krylova, C. Bruce, E.A. Bagg, C.J. Schofield, S.N. Krylov, Emulsion PCR significantly improves nonequilibrium capillary electrophoresis of equilibrium mixtures-based aptamer selection: Allowing for efficient and rapid selection of aptamer to unmodified ABH2 protein, Anal. Chem. 87 (2015) 1411–1419.

[35] O. Milenkovic, N. Kashyap, On the design of codes for DNA computing, Springer Berlin Heidelberg, 2006.

4.5 Supplementary materials

- Protocols
- Results
- Equations

4.5.1 Acrylamide gel preparation protocol

Gel for single strand-DNA recovery after PCR amplification was prepared as follows: 31.5 mL of 10X TBE buffer (1X: 44.5mMTris-Borate, 1mM EDTA; pH 8.3) was mixed with 13.5 mL of 40% acrylamide solution (final volume 45 mL). 21.6 g Urea were then added to the solution (final concentration 8M) and served as denaturing agent for dsDNA issued of PCR [1]. After Urea was completely dissolved, 450 µL of 10 % ammonium persulfate solution (APS) and 45 µL of 1,2-Bis(dimethylamino)ethane (TEMED) were added. The mixture was immediately poured within two glass plates and a comb was placed on top of the plates, serving as a mold for the wells in which the samples were deposited. Polymerization lasted 45 minutes, after which 300 V were applied for 10 minutes using ENDURO™ power supplies (Labnet International, Edison, USA). 40 to 80 µL of DNA (dsDNA from PCR, primer mixture, and original library) were then mixed with equal volume of gel loading and added on the well. Separation lasted at least one hour after which the species were revealed by UV detection (254 nm). All reagents used for gel electrophoresis separation were from Sigma (St. Quentin Fallavier, France)

4.5.2 Results

Electrophoretic mobility of tau protein

Electrophoretic mobility of tau protein was evaluated in the three different buffers reported in table s4-1

table s4-1: buffer composition for protein tau mobility test.

pH	Composition	Ionic strength
7.4	50 mM Tris, 50 mM NaCl	92 mM
5.5.	92 mM CH_3COOK	
3.0	63 mM K_2HPO_4, 28 mM NaCl	

Electropherograms of the tau protein are reported in Fig. S4-1.

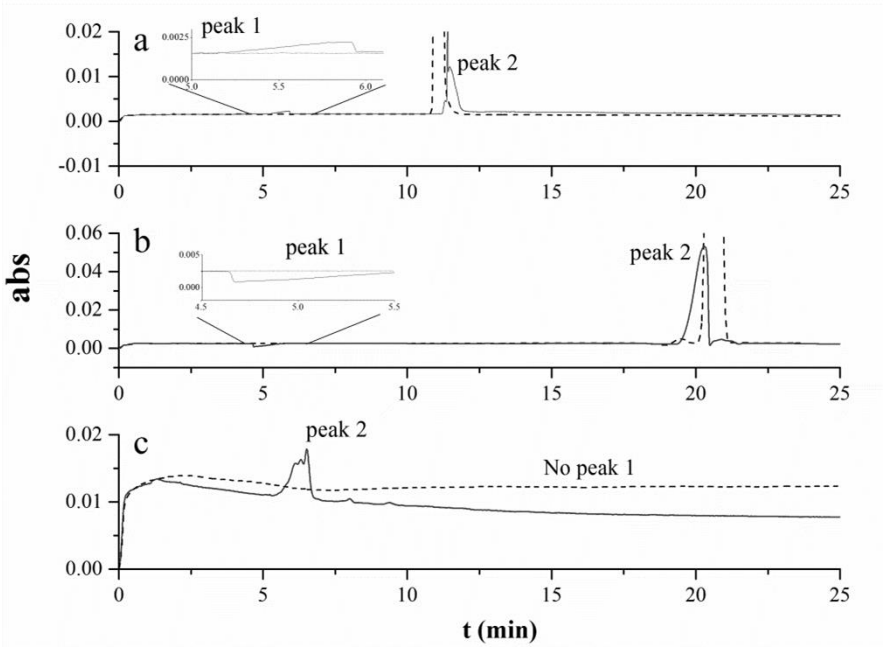

Fig. S4-1: UV absorption of tau protein (15 µM) at pH 7.4 (panel a), pH 5.5 (panel b), and pH 3.0 (panel c). Solid line represents tau samples, while dashed line is for reference compound (mesytiloxide)

Analysis of melt temperature

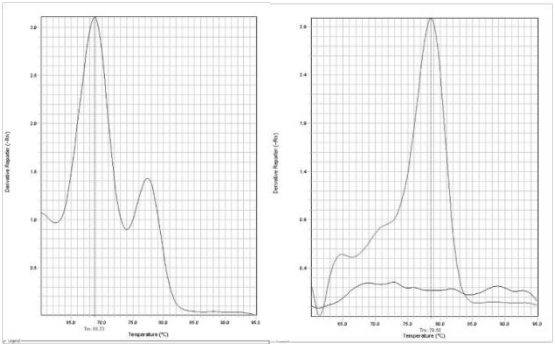

Fig. S4-2: Melt Curves of the DNA pool after round 6 of CE-SELEX process (left panel) compared with original library (right panel). In left panel two populations seemed to be present.

Electrophoretic migration of library and control sample.

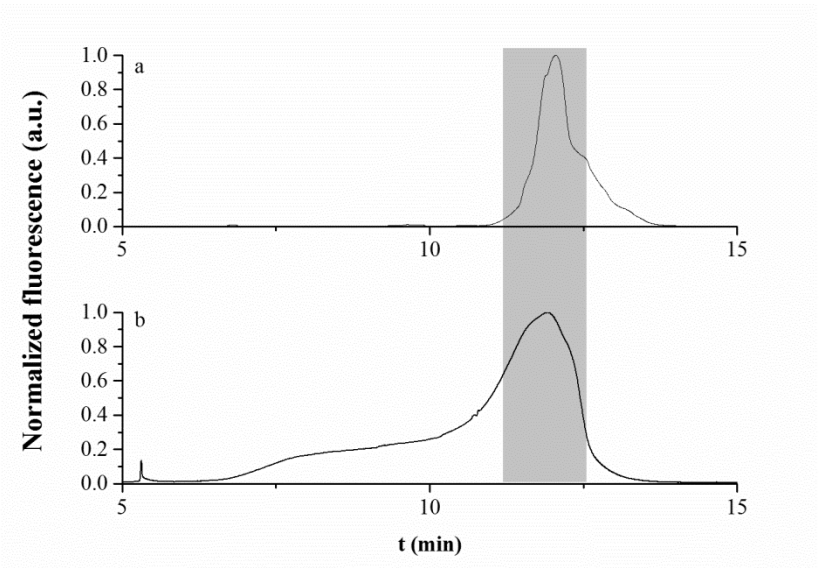

Fig. S4-3: Electropherograms of ssDNA library (100 nM, panel a) and control sample, recovered after PCR amplification, in absence of target (estimated concentration, 655 nM, panel b). Intensity of both electropherograms were normalized on the peak at 12 min (migration time). TGK was used as background electrolyte, temperature was kept at 25°C.

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Surface Plasmon Resonance: pre-concentration test

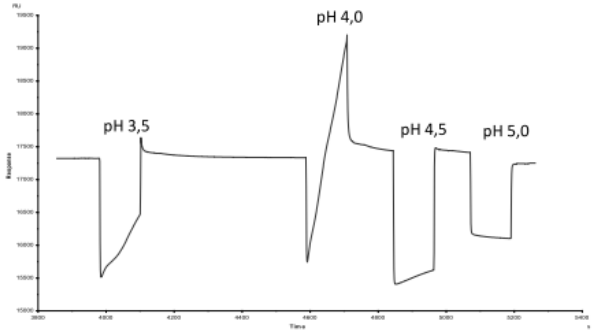

Fig. S4-4: Pre concentration test for tau protein immobilization. For all samples tau was dissolved at 10 mg L^{-1} in acetate buffer (10 mM).

4.5.3 Theoretical models

Electrophoretic mobility calculation

The equations below (equation 1 - 3) were used to evaluate the mobility of tau protein in the conditions reported in section 3.1.1 of the paper.

$$\mu_{app\pm} = \frac{Ld/t_{\pm}}{V/Lt} \qquad \text{eq (1)}$$

$$\mu_{eo} = \frac{Ld/t_{neutral}}{V/Lt} \qquad \text{eq. (2)}$$

$$\mu_{\pm} = \mu_{app\pm} - \mu_{eo} \qquad \text{eq (3)}$$

Where μ identified electrophoretic mobility of charged species ($\mu_{\pm}$) or neutral electroosmotic flow marker (μ_{eo}), Lt, Ld are total length of the capillary and lentgh from inlet to detection window respectively (Here 60 cm, and 50 cm), and V is the separation voltge (20 kV).

References

[1] C. Simard, R. Lemieux, S. Cote, Urea substitutes toxic formamide as destabilizing agent in nucleic acid hybridizations with RNA probes., Electrophoresis. 22 (2001) 2679–2683.

5 General Conclusions and future perspectives

In this thesis tau protein detection was faced from two different aspects. Initially commercially available antibodies were employed to develop the immunoassay, then, using advanced separation and sequencing technologies, selection of novel aptamer receptors was realized. In both these aspects versatility of Surface Plasmon Resonance (SPR) results crucial both for quantification of tau and to monitor affinity of the selected aptamers toward the target.

In immunosensing approach the use of a primary antibody only (direct assay) did not allow to detect the analyte at pM levels usually founds in Cerebrospinal fluid of healthy subjects and those affected by Alzheimer's disease. Nevertheless sensor was able to work both in buffer solution and in simulated matrix, encouraging further developments. Hence amplification strategy based on multi walled carbon nanotubes (MWCNTs) was developed. Such nanostructures were used as label for a secondary monoclonal antibody to develop a sandwich-like assay. Using MWCNTs pM levels were reached, and the sensor surface was easily regenerated in this concentration range. Since Carbon nanotubes can interact among them forming bundles, we addressed the regeneration and repeatability worsening observed at nM tau level to non-covalent interaction between tubes. Possible improvement of both regeneration and repeatability issues may come from reducing length-dispersion commonly associated with MWCNTs [1], by selecting mono-disperse (or at least less disperse) fractions of the nanostructures prior to antibody conjugation.

Otherwise, nanotubes could be fixed onto the sensing surface of the SPR. In fact graphene sheets have been recently reported as SPR signal enhancers for the exceptional electrons mobility associated with the planar

hybridization of carbon atoms [2,3]. Even though carbon nanotubes are oxidized (thus they hold defects that decrease total conductivity) to provide functional groups for biomolecules attachment, some properties of graphene retained. Moreover increasing superficial area of the sensor could greatly improve sensitivity by providing additional capturing capability to the sensing elements (*i.e.* antibodies, aptamers etc.).

With respect of aptamer selection, despite Kim and colleagues [4] isolated few aptamer candidates against tau, the selection they set up was characterized by a higher number of selection rounds (12) compared to the method developed in this thesis. Here we reported the selection in only three rounds of CE-Non-SELEX, which allowed the affinity to be significantly improved, compared with initial library. The sample was enriched by carrying out a single PCR amplification step on the sequences resulting from the third round of selection. Since little sample volumes are used during CE, the PCR amplification steps needs to be very efficient to avoid amplification of by-products, that are detrimental for affinity improvement. Although here the elimination of PCR among different selection rounds allowed the aptamers to be selected, further development are desirable for the optimization of the amplification step. Next Generation Sequencing (NGS), was chosen instead of conventional cloning-sequencing process to keep maximum amount of information about the selected sequences. This results in the identification of thousands aptamers, all potentially good for binding the target. Although this might be seen as a limitation because of the difficult interpretation of the data, the high information content stored in the dataset could be also interpreted as one of the main strength of the aptamer technology. Commercially available antibodies have, in fact, limited room for improvement with respect of enhancing affinity of the receptor, and they can be considered as a mature technology. With the advent of NGS, an

enormous amount of sequences can be investigated simultaneously and even rare, highly potent aptamers can be selected [5]. Recently the importance of sequence-specific interaction with the target was also questioned by applying Quantitative Structure Activity Relationship (QSAR) model to aptamer for influenza virus [6].

As a whole despite immunochemistry still play the most important role in diagnostic, the great potential that bioinformatics analysis enable leave margin for improving aptamer selection toward protein tau and in many other cases.

In conclusion, in the next years significant advances in nanotechnology can be foreseen to improve detection by conventional immunoassays, whether growing applications of bioinformatics tool are likely to associated with aptamer technology, to extend the possibility over the use of such receptors in all the field in which they could rival with antibodies.

References

[1] S. Fedeli, P. Paoli, A. Brandi, L. Venturini, G. Giambastiani, G. Tuci, S. Cicchi, Azido-Substituted BODIPY Dyes for the Production of Fluorescent Carbon Nanotubes, Chem. A Eur. J. 21 (2015) 15349–15353.

[2] H.H. Nguyen, J. Park, S. Kang, M. Kim, Surface plasmon resonance: A versatile technique for biosensor applications, Sensors. 15 (2015) 10481–10510.

[3] S. Szunerits, N. Maalouli, E. Wijaya, J.P. Vilcot, R. Boukherroub, Recent advances in the development of graphene-based surface plasmon resonance (SPR) interfaces, Anal. Bioanal. Chem. 405 (2013) 1435–1443.

[4] J.H. Kim, E. Kim, W.H. Choi, J. Lee, J.H. Lee, H. Lee, D.E. Kim, Y.H. Suh, M.J. Lee, Inhibitory RNA Aptamers of Tau Oligomerization and Their Neuroprotective Roles against Proteotoxic Stress, Mol. Pharm. 13 (2016) 2039–2048.

[5] M. Blind, M. Blank, Aptamer Selection Technology and Recent Advances, Mol. Ther. Acids. 4 (2015) e223.

[6] B. Musafia, R. Oren-Banaroya, S. Noiman, Designing anti-influenza aptamers: Novel quantitative structure activity relationship approach gives insights into aptamer - Virus interaction, PLoS One. 9 (2014) e97696.

Appendix I. Riassunto tesi dottorato - Italiano

Introduzione

La malattia di Alzheimer (AD) è stata scoperta da Alois Alzheimer nel 1906, grazie all'osservazione di placche amiloidi e aggregati neurofibrillari localizzati nell'encefalo di August Deter, donna di 50 anni che è il primo caso accertato di AD. Secondo il *World Alzheimer Report*, circa 46 milioni di individui sono affetti da demenza a livello globale tra i quali si stima una prevalenza dei casi di Alzheimer che si attestano a circa il 60-70% dei soggetti totali. Nel corso degli anni numerosi studi hanno delucidato la composizione delle placche amiloidi e degli aggregati neurofibrillari. In particolare, per quanto riguarda le prime, la loro composizione è stata svelata nel 1985, rivelando alte concentrazioni di peptide Aβ, mentre per gli aggregati neurofibrillari (1986), la loro composizione ha mostrato essenzialmente la presenza di proteina tau. Allo stato attuale, nessuna metodica diagnostica tra quelle esistenti si è rivelata efficace per una diagnosi precoce della malattia di Alzheimer (che sarebbe fondamentale per poter produrre un intervento nelle prime fasi della patologia). Tra le varie tecniche che possono supportare la diagnosi *in vivo* possiamo elencare: le tecniche di imaging come la risonanza magnetica per immagini (MRI dall'acronimo inglese), la tomografia ad emissione positronica (PET) o le tecniche basate su saggi immunochimici.

Questi ultimi si basano sulla variazione dei livelli (riportati in Tabella 1) dei biomarcatori di core nel liquido cerebrospinale (CSF usando l'acronimo inglese), che allo stato attuale sono: il peptide Aβ, la proteina tau (il totale delle isoforme) e la proteina tau fosforilata.

Tabella 1: Livelli di concentrazione e relativi cut-off, ottenuti mediante saggio ELISA, per i biomarcatori di core dell'Alzheimer.

Biomarker	Healthy subject (ng L^{-1})	AD subject (ng L^{-1})
$A\beta_{1-42}$	794±20 (184.3 pM)	<500 (<116.3 pM)
Total tau protein (21-50 years)	136±89 (3.0 pM)	n.d.
Total tau protein (51-70 years)	243±127 (5.3 pM)	>450 (>9.8 pM)
Total tau protein (>71 years)	341±171 (7.4 pM)	>600 (>13.1 pM)
Phosphorilated tau protein (181)	23±2 (0.5 pM)	>60 (>1.3 pM)

Essi derivano dalla cosiddetta *Amyloide cascade hypothesis* (rappresentata in Figura 1), sviluppata tra gli anni novanta e 2002 e che pone l'aggregazione dell'Aβ precedente quello della proteina tau.

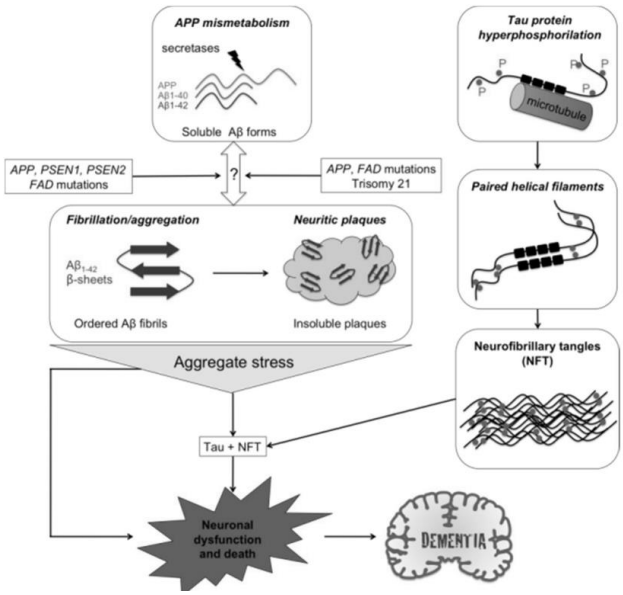

Figura 1: Schema rappresentante il pattern e il coinvolgimento seguiti da proteina tau e peptide amiloide secondo l'ipotesi della cascata amiloide.

Successivi sviluppi nello studio dei biomarcatori hanno poi descritto l'esistenza di specie oligomeriche che, data la loro maggiore diffusibilità, sembrano notevolmente più tossiche degli aggregati.

Nonostante alcuni lavori abbiano descritto l'analisi di queste o altre specie in CSF od in altre matrici biologiche, le attuali tecniche immunochimiche

di routine sono basate sul dosaggio multiplo in CSF dei biomarcatori di core (Aβ, tau-totale e tau iperfosforilata). L'utilizzo di questa combinazione di biomarcatori ha dimostrato un'elevata sensibilità clinica e specificità nel discriminare tra soggetti sani e soggetti affetti da patologia di Alzheimer, mentre minori performances sono state riscontrate nella discriminazione tra i diversi tipi di demenza. Nelle sezioni successive le caratteristiche delle tecniche immunochimiche convenzionali e non, e quelle basate su recettori innovativi, saranno presentate.

Tecniche immunochimiche adottate in analisi di routine

Sviluppati in seguito all'introduzione degli anticorpi commerciali diretti contro i biomarcatori di core e utilizzati lungamente nella ricerca biomedica, il saggio ELISA (*Enzyme Linked Immunosorbent Assay)* e il saggio xMAP, sono ormai adottati nella pratica ospedaliera (dopo l'introduzione nel 2007 delle linee guida pubblicate dal NINCDS-ADRDA[1]) allo scopo di supportare la diagnosi di probabile malattia di Alzheimer. Nonostante gli elevatissimi standard raggiunti in termini di ripetibilità analitica e le ottime prestazioni in termini di sensibilità, con limiti di rivelabilità (LOD) nell'ordine del picomolare, queste due tecniche presentano ancora diversi limiti.

La variabilità interlaboratorio può infatti raggiungere valori elevati, espressi attraverso il coefficiente di variazione (CV%), che possono raggiungere il 30%. Inoltre, l'utilizzo di marcatori fluorescenti o colorimetrici e di recettori come gli anticorpi (affidabili, ma soggetti ad una variabilità intrinseca dovuta al sistema di produzione dei vari lotti)

1 NINCDS-ADRDA: National Institute of Neurological and Communicative Disorders and Stroke and the Alzheimer's Disease and Related Disorders Association.

può fortemente limitare l'applicazione dei saggi nella routine. Per questo sono state sviluppate tecniche alternative, basate, ad esempio, sull'uso di biosensori.

Biosensori di affinità per la diagnosi della malattia di Alzheimer

I principali biosensori di affinità (il cui principio sarà elucidato nella sezione successiva), che sfruttano anticorpi mono- o poli- clonali, utilizzati per la diagnosi *label-free* dell'Alzheimer, sono elencati in Tabella 2.

Tabella 2: Tecniche immunochimiche innovative per la diagnosi *label-free* della malattia di Alzheimer. Abbreviazioni: SPR: risonanza plasmonica di superficie; LSPR: SPR localizzata; SWV: voltammetria con potenziale ad onda quadra; EIS: spettrometria di impedenza; CNT-FET: transistor basati sull'utilizzo di nanotubi di carbonio; ADDL: oligomeri derivanti dal peptide amiloide; mAb: anticorpo monoclonale.

Transduction principle	(1)	Target	LOD	Medium	Detection strategy	Antibody
Optical	SPR	$A\beta_{1-40}$	0.3 fM	buffer	sandwich	Fragmented mAb
		$A\beta_{1-40}$ $A\beta_{1-42}$	20.0 pM	CSF	sandwich	mAb
		tau (captured) $A\beta_{1-42}$ (flowed)	3.0 nM	buffer	sandwich	mAb
		$A\beta_{1-42}$	0.2 µM	buffer	direct	mAb
		ADDLs	280 nM	buffer	sandwich	mAb
		ADDLs	n.d.	buffer	direct	mAb
	LSPR	ADDLs	10.0 pM	human brain cells and CSF	sandwich	mAb
		Tau	0.2 pM	buffer	direct	mAb, protein G oriented
Electrochemical	SWV	$A\beta_{1-40}$ $A\beta_{1-42}$	20.0nM	rat CSF	direct	mAb
	EIS	$A\beta_{1-42}$	0.57 nM	buffer	direct	mAb, protein G oriented
	CNT-FET	$A\beta_{1-42}$	2 nM	serum	direct	mAb

Senza entrare nel dettaglio delle tecniche sviluppate, esse, come riassunto in tabella, permettono l'ottenimento di limiti di rivelabilità comparabili o superiori rispetto alle tecniche di routine. L'assenza di marcatore e la

possibilità di leggere l'interazione in tempo reale (nella maggioranza delle tecniche presentate) ne aumentano la versatilità di utilizzo, semplificando anche l'interpretazione del dato ottenuto. Possibilità di miniaturizzazione strumentale elevate sono inoltre associate all'utilizzo di tecniche come LSPR, e le tecniche elettrochimiche, rendendo entrambe altamente adattabili ad un utilizzo su campo (nel caso di analisi su matrici il cui prelievo sia meno invasivo rispetto al CSF).

La sensibilità delle tecniche immunochimiche può essere enormemente migliorata attraverso l'utilizzo di approcci tipo sandwich e grazie all'utilizzo di marcatori di varia natura (schematizzati in Figura 2).

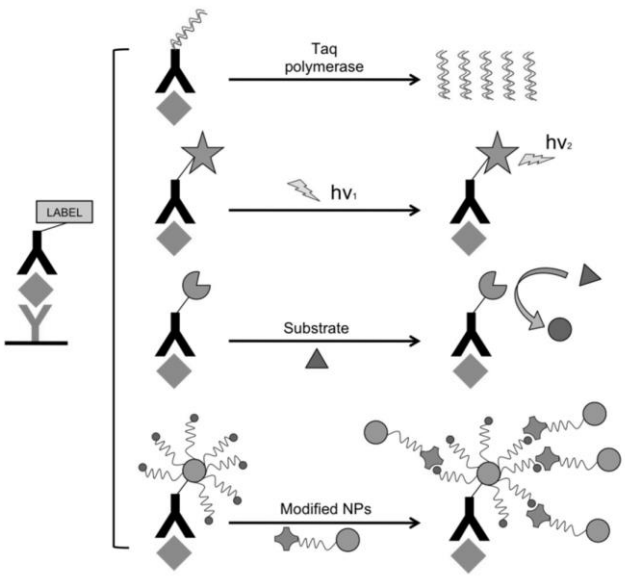

Figura 2: Rappresentazione (non in scala dimensionale) delle classi di marcatori usati nei saggi bioanalitici per l'analisi dei marcatori di core dell'Alzheimer. Dall'alto verso il basso: DNA, fluorofori, enzimi catalitici e nanoparticelle metalliche.

Nel caso del DNA, questo ha trovato applicazione nei metodi che permettono la sua amplificazione attraverso Reazione Polimerasica a Catena (PCR dall'inglese) o che sfruttano la possibilità di predirne gli appaiamenti tra le basi. La prima proprietà viene utilizzata ad esempio nell'immuno-PCR, in cui uno degli anticorpi usati per il riconoscimento dell'analita viene marcato con una sequenza oligonucleotdica, sottoposta a PCR per aumentare la sensibilità del saggio. Elegante esempio dell'abbinamento tra le capacità del DNA e nanoparticelle metalliche viene riportato nel saggio definito *biobarcode,* grazie al quale la sensibilità dell'analisi si è spinta fino a determinare gli oligomeri del peptide amiloide a livelli nell'ordine dell'attomolare.

In altri esempi le nanoparticelle metalliche hanno trovato applicazione nell'amplificazione di fenomeni come la diffusione Raman o in altri metodi ottici, mentre i fluorofori organici sono stati sfruttati per lo studio di risonanza Foster (FRET) o per misure tradizionali dell'intensità di fluorescenza.

I *label* enzimatici, come la fosfatasi alcalina, sono più adatti ad un utilizzo nei metodi elettrochimici (elettrodi convenzionali o stampati SPE[2]), di cui si trovano però un numero inferiore di esempi in letteratura.

In conclusione, per le tecniche emergenti una maggiore attenzione allo sviluppo di metodiche che prevedano l'utilizzo in matrice biologica sembra necessario. L'utilizzo di un marcatore o perlomeno lo sviluppo di saggi di tipo sandwich sembra favorito e, allo stato attuale, la maggioranza dei metodi è rivolta verso l'analisi di specie derivanti dall'APP[3] (ADDLs, Aβ). Da quest'ultima considerazione si può comprendere come sia auspicabile uno sviluppo di tecniche per il dosaggio della proteina tau.

[2] SPE: Screen Printed Electrode
[3] APP: amyloid precursor protein

Recettori biomimetici

Alla famiglia dei recettori biomimetici vengono catalogati tutti quei recettori che hanno proprietà simili (o in alcuni casi migliori) dei recettori di tipo biologico (anticorpi, enzimi, ecc.) e che provengono da un processo di sintesi chimica effettuato *in vitro* e che ne permetta la produzione controllata e riproducibile. In linea generale, gli aptameri, i peptidi, i peptoidi (N-sostituite oligo-glicine) e i MIP[4] sono sicuramente i recettori sintetici più usati per la rilevazione di target proteici.

Nel campo della diagnostica molecolare della malattia di Alzheimer (AD) sono numerosi gli esempi di peptidi utilizzati per la diagnosi, in particolare grazie alla loro intrinseca maggior flessibilità rispetto a recettori proteici strutturati, come ad esempio gli anticorpi. Questa caratteristica è stata utilizzata nei metodi di impedenza, che sfruttano il processo di trasferimento di carica dal peptide (marcato con un mediatore elettrochimico, come ad esempio il ferrocene) alla superficie dell'elettrodo. Nel momento dell'interazione analita-recettore, la conformazione del peptide varia, e allo stesso tempo si osserva una variazione del segnale analitico.

Per quanto riguarda gli aptameri ed i MIP, ad oggi non si riportano applicazioni diagnostiche per questi recettori, anche se un crescente sviluppo viene registrato a partire dal 2002. Entrambi le classi di recettori sono note per garantire un riconoscimento selettivo dei target attraverso la particolare struttura tridimensionale assunta nell'interagire con la molecola d'interesse. Alcuni autori hanno descritto, per gli aptameri, una sostanziale mancanza di selettività verso le specie accomunate da un processo aggregativo guidato dalla formazione di foglietti beta (proteine

[4] MIP: Molecularly imprinted polymer

prioniche, sinucleina-alfa, peptide amiloide, tau ecc.). Tuttavia come descritto nelle successive sezioni della tesi, i risultati ottenuti nel corso di questo lavoro mostrano un significativo miglioramento dell'affinità delle sequenze isolate rispetto a quella misurata per il pool di sequenze iniziale, suggerendo perciò un meccanismo di selettività verso il target.

Altri recettori sintetici sono stati sviluppati e utilizzati in saggi di tipo elettrochimico garantendo limiti di rivelabilità nell'ordine del picomolare.

In conclusione, lo stato dell'arte indica come, nonostante la strada verso una diagnosi precoce dell'AD resti ancora una sfida difficile da affrontare, gli sviluppi in varie aree della sensoristica e della bioanalitica possano offrire soluzioni in questo senso. La strada però non passerà solamente dallo sviluppo analitico in quanto miglioramenti delle tecniche di campionamento, lo sviluppo di matrici di riferimento, l'adozione di protocolli standardizzati e, non ultima, l'aumento di conoscenza degli aspetti biochimici associati con lo sviluppo della patologia, ricopriranno sicuramente un ruolo chiave verso la diagnosi e la cura dell'Alzheimer.

Principi di Risonanza Plasmonica di superficie e di selezione degli aptameri

In questo lavoro la risonanza plasmonica di superficie (SPR) e l'elettroforesi capillare (CE) hanno rivestito un ruolo importante per ottenere i risultati sperimentali descritti a partire dalla successiva sezione. Qui ne vengono descritti i principi fondamentali, facendo riferimento alla fisica legata allo sfruttamento delle caratteristiche dell'SPR e alle tecniche di selezione sviluppate partendo dalla CE come tecnica separativa.

Principi di risonanza plasmonica di superficie

La risonanza plasmonica di superficie (SPR) è uno dei fenomeni fisici più sfruttati per la costruzione di biosensori di affinità. In generale un biosensore viene definito come uno strumento capace di trasdurre l'interazione di tipo biologico, in un segnale elettrico, ottico o termico, analiticamente rilevabile. Nonostante in letteratura siano riportati diversi esempi di biosensori sviluppati in soluzione omogenea, la configurazione standard dei biosensori prevede un contatto fisico tra un recettore e una superficie come mostrato in Figura 3.

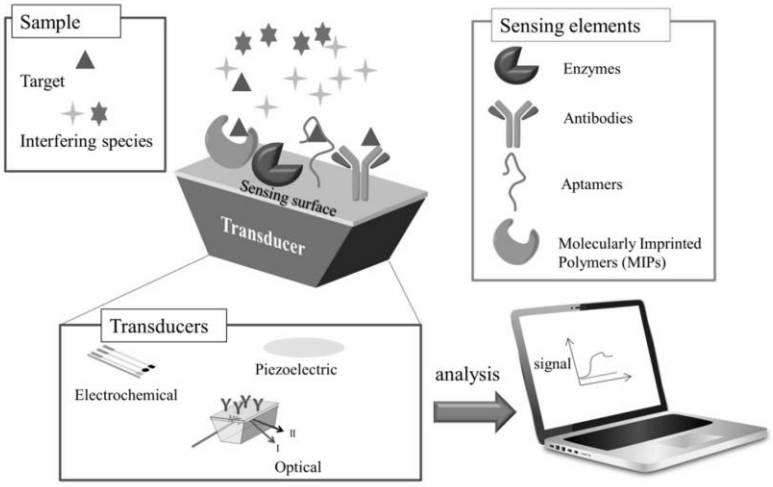

Figura 3: Principi ed elementi principali di un biosensore.

La trasduzione attraverso SPR è garantita dall'accoppiamento tra i polaritoni di superficie all'interfaccia tra un dielettrico e un metallo, e una radiazione a lunghezza d'onda tale da essere messa in risonanza con gli elettroni del metallo.

Sotto particolari condizioni, dipendenti dai materiali utilizzati e dalla configurazione spaziale degli elementi componenti il sensore (prisma, sorgente della radiazione, spessore del film metallico all'interfaccia), gli elettroni vengono eccitati collettivamente dando luogo ad un'onda evanescente che penetra il metallo raggiungendone la faccia a contatto con il dielettrico. Grazie a ciò gli eventi che interessano il dielettrico stesso, provocando la variazione dell'indice di rifrazione, sono rilevati con estrema sensibilità in una profondità di campo di circa 500 nm nel caso in cui il film metallico sia l'oro.

La trasduzione in tempo reale permette numerosi vantaggi rispetto ai saggi ELISA, come ad esempio: a) la possibilità di monitoraggio delle proprietà cinetiche (e termodinamiche) dell'interazione biomolecolare; b) l'esistenza di procedure di rigenerazione, che permettono il riutilizzo del

sensore per numerosi cicli; c) la possibilità di amplificare il segnale attraverso l'utilizzo di nanostrutture che permettano l'accoppiamento plasmonico tra la superficie e le nanostrutture stesse.

Nei paragrafi successivi le proprietà dell'SPR utilizzate nel lavoro saranno riportate dettagliatamente, mettendo in mostra la versatilità della tecnica nei diversi ambiti della bioanalitica.

SELEX e Non-SELEX: vantaggi e inconvenienti delle tecniche per la selezione degli aptameri

La tecnica del SELEX[5] è stata sviluppata nel 1990 da due lavori indipendenti. I principali step della selezione sono la sintesi chimica della library di sequenze a DNA (singolo filamento) o RNA (*step 1)*, a cui fa seguito l'incubazione con il target d'interesse *(step 2)*. Grazie all'utilizzo di diverse tecniche di separazione, solo le sequenze affini vengono isolate e amplificate attraverso PCR (*step 3)*. Dopo la rimozione dello strand complementare *(step 4)*, le sequenze affini vengono ri-iniettate in modo che il ciclo possa ricominciare. In funzione delle condizioni di selezione e delle metodiche di separazione, il numero di cicli di selezione può raggiungere i 15 rounds. La Figura 4 riassume i passaggi descritti precedentemente.

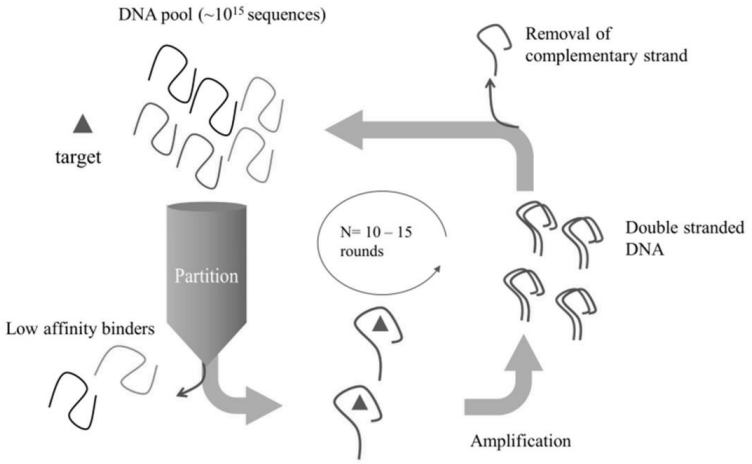

Figura 4: Passaggi principali della tecnica SELEX per l'identificazione di aptameri.

[5] SELEX: Systematic Evolution of Ligands by EXponential enrichement

In questo lavoro si è sfruttato l'utilizzo della CE per la sua elevata efficienza separativa. A differenza di altre tecniche separative, nel cosiddetto CE-SELEX l'immobilizzazione del target non è necessaria, rendendo quest'ultimo completamente accessibile da parte della library durante il processo di riconoscimento molecolare. Inoltre grazie alla CE, è possibile osservare, utilizzando una sola tecnica, l'evoluzione dei vari cicli di selezione, in quanto è possibile stimare l'affinità dei pool selezionati, misurando l'area sottesa ai picchi presenti nell'elettroferogramma.

Tali considerazioni sono altresì valide per la tecnica che prende il nome di CE-Non-SELEX, in cui i vari cicli di partizione non sono intervallati da amplificazione PCR, che viene invece realizzata solo sull'ultimo pool selezionato. Ciò riduce notevolmente i tempi di lavoro, da alcune settimane a poche ore. Tuttavia il campione risulta poco arricchito, diventando di più difficile interpretazione a causa di una popolazione di sequenze di DNA altamente variegata.

Scopo del lavoro

Scopo di questo lavoro è quello di studiare lo sviluppo di un metodo analitico basato su SPR per il rilevamento della proteina tau. Inizialmente lo sviluppo ha riguardato l'utilizzo di anticorpi commerciali, che sono poi stati marcati con nanostrutture per incrementare il segnale analitico.

Parallelamente, recettori di concezione innovativa sono stati selezionati attraverso elettroforesi capillare. Due diversi metodi di selezione sono stati confrontati (CE-SELEX e CE-Non-SELEX) in modo da comprendere quale dei due si adatti meglio allo sviluppo di aptameri per la proteina tau. In questo caso l'SPR è stato ideale per connettere entrambi gli aspetti del lavoro, che ha dimostrato, una volta di più, le potenzialità della tecnica in tutti i campi della bioanalitica.

Immunosensore per la proteina tau

L'immunosensore per l'analisi della proteina tau è stato sviluppato grazie ad una collaborazione con il gruppo del prof. Cicchi, che ha fornito i nanotubi di carbonio utilizzati per l'amplificazione del segnale (Figura 5)

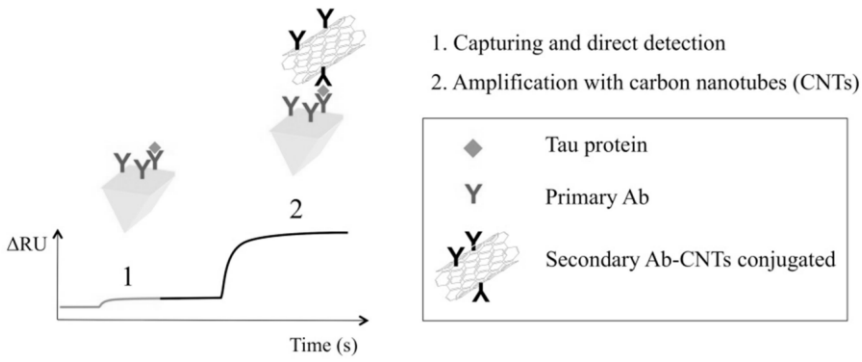

Figura 5: Schema del saggio immunochimico. Step 1: saggio diretto in cui l'anticorpo primario è immobilizzato sulla superficie dà il riconoscimento molecolare necessario per la cattura del target. Step 2: iniezione dell'anticorpo secondario, con nanotubi di carbonio come amplificatori del segnale.

L'utilizzo di queste nanostrutture per l'amplificazione del segnale SPR è stato precedentemente riportato per l'analisi dell'EPO[6], risultando come efficace per l'aumento della sensibilità verso livelli fisiologici di tale analita. Queste strutture possono essere proposte come alternativa alle nanostrutture metalliche, più comunemente usate in SPR. Tra le caratteristiche che permettono l'amplificazione del segnale si possono annoverare un aumento dell'area superficiale a disposizione dell'anticorpo secondario e analita per interagire tra loro, nonché un forte spostamento dell'indice di rifrazione causato dalla particolare geometria delle nanostrutture.

[6] EPO: eritropoietina, ormone proteico di 34 KDa coinvolto nel processo di ematopoiesi.

Risultati e discussione

La proteina tau è stata iniettata ad una concentrazione compresa tra 125 pM e 250 nM. In prima istanza lo sviluppo ha riguardato l'analisi utilizzando un anticorpo primario, capace di mappare l'analita nell'epitopo compreso tra il residuo amino acidico 189 e quello 195 (clone 39E10, monoclonale). Questo recettore è stato immobilizzato attraverso un processo di amino-coupling che ha richiesto l'ottimizzazione del pH di immobilizzazione attraverso un saggio di pre-concentrazione. In particolare l'utilizzo di tampone acetato (10 mM) a pH 4.0 ha dato i migliori risultati, permettendo l'immobilizzazione sulla superficie destranica di un chip CM5 commerciale. Grazie a questo saggio, la sensibilità ottenuta sia in buffer che in liquido cerebro spinale simulato (aCSF) è stata nell'ordine del nM. Migliori sono state le performances in aCSF in termini di ripetibilità e estensione del range lineare del saggio sviluppato (Figura 6).

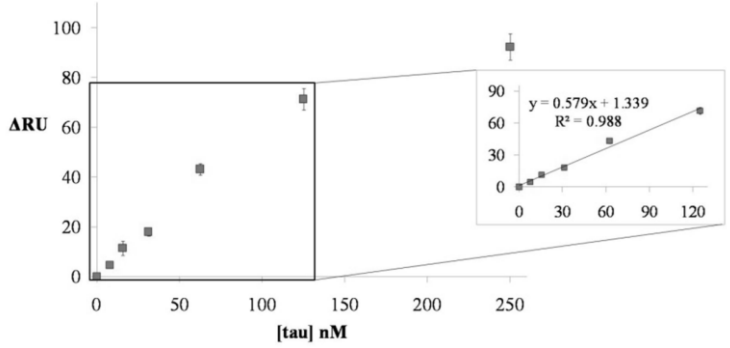

Figura 6: Saggio diretto con il solo anticorpo primario, in matrice simulata.

Questo risultato, a confronto con altri metodi SPR presenti in letteratura, è decisamente buono, in quanto ottenuto utilizzando un singolo anticorpo per la quantificazione. Ciò è dovuto probabilmente all'utilizzo di una superficie che, grazie alle sue proprietà, riesce ad incrementare la cattura di analita. L'analisi in aCSF ben si sposa con le necessità di applicazione

nella pratica clinica, supportando la necessità dello sviluppo di un materiale di riferimento certificato (CRM) per migliorare le performances dei metodi di routine nella diagnosi precoce della malattia di Alzheimer.
Il sandwich è stato sviluppato a partire dal saggio diretto, utilizzando un anticorpo secondario (clone tau12) che lega la proteina tau nella sua parte N-terminale. Le performances del metodo in aCSF sono state migliorate, fino a raggiungere un limite di rivelabilità due volte migliore rispetto a quello precedentemente osservato (Figura 7B).

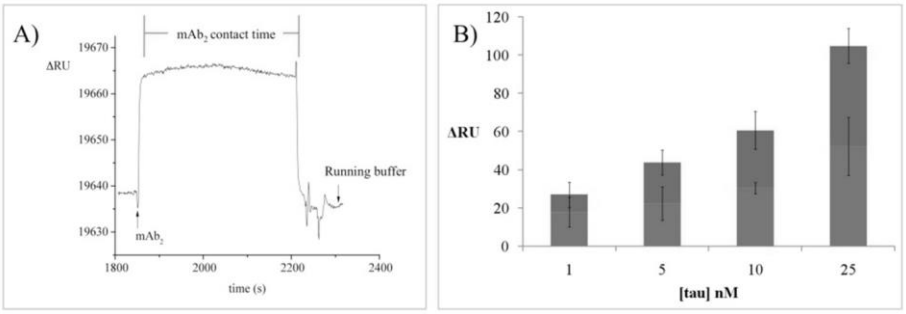

Figura 7: A) Assenza di interazione non specifica dell'anticorpo secondario sulla superficie del sensore, in assenza di analita. B) contributo del segnale ottenuto per una concentrazione di tau tra 1 e 25 nM grazie al saggio diretto e al sandwich.

In assenza di analita non è stata osservata interazione non specifica tra gli anticorpi (Figura 7B) o in presenza di albumina sierica umana (HSA).
Per poter raggiungere livelli dell'ordine del pM, il gruppo del professor Cicchi ha realizzato l'ossidazione e la seguente attivazione dei nanotubi, in modo da consentire sia la loro dispersione nei mezzi acquosi, sia di fornire i siti attivi per la formazione del legame covalente con gli anticorpi. Tale processo viene facilitato grazie alla presenza di gruppi O-succinimmide, la cui presenza è stata verificata attraverso spettroscopia IR e analisi elementare. Dopodiché tre parametri sono stati presi in considerazione per rendere più efficace la bioconiugazione. In particolare il tempo di sonicazione (in assenza di anticorpo), il tampone di

coniugazione e la concentrazione totale di anticorpo (Figura 8) si sono rivelati fondamentali nel mantenimento di una dispersione stabile.

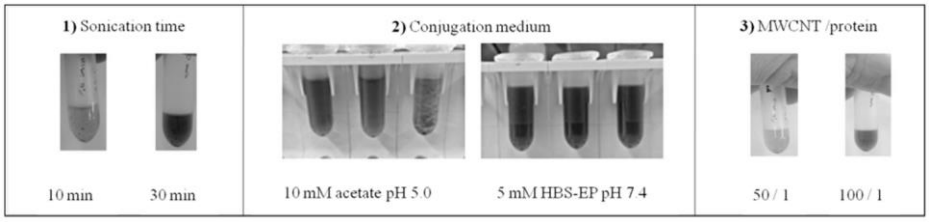

Figura 8: Ottimizzazione della coniugazione dell'anticorpo secondario. 1) tempo di sonicazione; 2) mezzo di immobilizzazione; 3) rapporto tra la concentrazione di CNT e quella di anticorpo.

In queste condizioni una prima prova di concetto in buffer è stata effettuata, dando luogo ad una amplificazione di segnale significativa (Figura 9A). Tuttavia la rigenerazione del sensore, che nella configurazione precedente era effettuata grazie a brevi iniezioni di NaOH (10 mM - 50 mM; 24 – 48''), risultava impossibile.

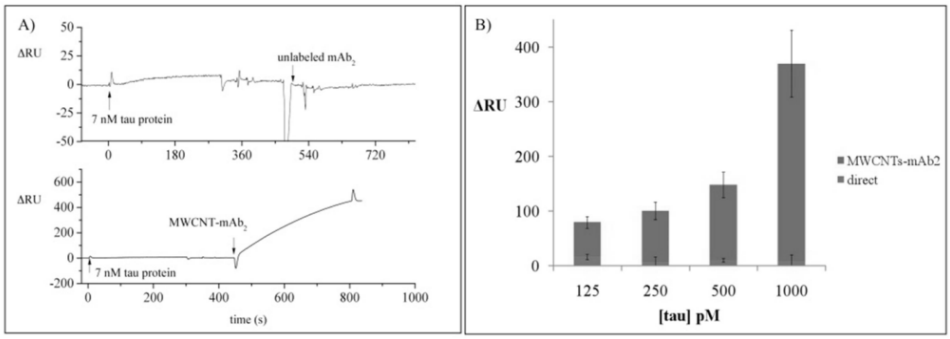

Figura 9: A) amplificazione segnale in buffer grazie all'utilizzo di nanotubi in cella confrontata con il segnale ottenuto utilizzando una concentrazione equivalente di anticorpo secondario; B) investigazione dell'amplificazione ottenuta con nanotubi "selezionati", a concentrazioni pM di proteina tau.

Tra le varie ragioni che possono spiegare quanto osservato, l'ampia dispersione delle lunghezze dei nanotubi sembra la più probabile. È infatti noto che i CNTs sono composti da frazioni la cui lunghezza varia notevolmente, fino a raggiungere valori nell'ordine dei µm. Questi ultimi

hanno una tendenza elevata ad interagire fra loro, e possono dunque produrre uno spesso strato di CNTs, inaccessibile alla soluzione rigenerante. Riducendo questa frazione attraverso un passaggio di centrifugazione blando (3000 rcf, 3'), la capacità rigenerante è stata ripristinata con successo, permettendo l'investigazione del range pM (Figura 9B) con un solo sensore. In questo range di concentrazione si è osservata una chiara correlazione della dose di analita con il segnale ottenuto a seguito dell'iniezione del coniugato anticorpo-nanotubi. Tale curva era descritta da una funzione esponenziale che deviava dalla linearità a concentrazioni di analita che si approssimano al nM, probabilmente a causa di un maggior impaccamento dei CNT dovuto alla loro maggiore concentrazione locale in cella.

Conclusioni-immunosensore

In questa prima parte del lavoro l'utilizzo di CNT per la rilevazione di proteina tau ha mostrato interessanti proprietà, lasciando intravedere possibili applicazioni nella diagnosi dell'Alzheimer. L'amplificazione del segnale, di circa 2 ordini di grandezza rispetto al saggio diretto, ha permesso l'ottenimento di un limite di rivelabilità sperimentale vicino ai valori fisiologici del target e buone prestazioni in termini di ripetibilità sono state ottenute (CV 14%) usando diversi lotti di nanotubi funzionalizzati. Queste prestazioni possono essere ulteriormente migliorate soprattutto grazie ad una selezione più fine delle lunghezze delle nanostrutture coinvolte, ma anche allo stato attuale, i CNT possono essere considerati come una nuova frontiera per l'uso di SPR nella diagnosi dell'Alzheimer.

Selezione di un recettore a base aptamerica per la rivelazione della proteina tau

La seconda parte del lavoro è stata condotta grazie alla collaborazione con l'Università di Grenoble al dipartimento di farmacologica chimica molecolare. Come già accennato, scopo di questa seconda parte era lo sviluppo di un recettore per la proteina tau, con proprietà diverse da quelle degli anticorpi, in particolar modo in termini di stabilità e riproducibilità di sintesi dei vari lotti. I recettori aptamerici sono noti in letteratura per rispondere a queste necessità, e sono stati usati in diversi campi applicativi dal momento del loro sviluppo. Essi vengono selezionati *in vitro* usando di solito la tecnologia SELEX, che prevede diversi passaggi, ma che è essenzialmente basata su un'alternanza di step separativi e di amplificazione tramite PCR. In questo lavoro una strategia di selezione alternativa è stata sviluppata e prende il nome di CE-Non-SELEX, in cui a seguito di vari passaggi separativi, una singola amplificazione PCR viene realizzata. L'aumento di affinità per le sequenze isolate è stato analizzato interpolando la dipendenza tra concentrazione di tau e segnale attraverso due tecniche: Anisotropia di Fluorescenza (FA) e SPR. Grazie all'SPR le sequenze più affini sono state identificate.

Risultati e discussione

In primo luogo la separazione tra target e pool di sequenze a DNA (marcata con fluoresceina modificata, e composta da 77 nucleotidi, 30 dei quali disposti in posizione casuale per la composizione del pool) si è resa necessaria per poter identificare la finestra di collezione da cui poi si sarebbero ricavate le sequenze più affini per la proteina tau. Per ottenere questo obiettivo uno dei primi ostacoli da superare ha riguardato l'identificazione del tempo di migrazione del target, data la molteplicità dei picchi presenti nell'elettroferogramma. Questo è stato fatto misurando la mobilità elettroforetica della proteina (dipendente dal pH del tampone di corsa) a tre diversi pH (3,0; 5,5; 7,4). Identificando il picco la cui mobilità fosse dipendente da questo parametro, si è potuta definire la zona di separazione per entrambe le metodiche (SELEX e Non- SELEX) usate in questo lavoro (Figura 10).

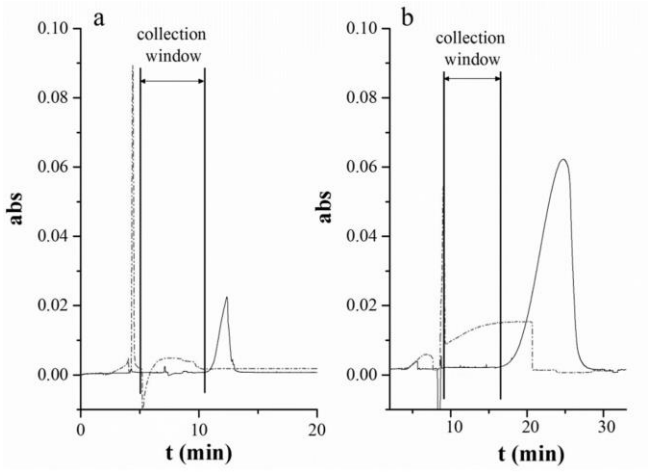

Figura 10: a) finestra di collezione per CE-SELEX e b) CE-Non-SELEX. Il picco della proteina tau (linea tratteggiata) migra più rapidamente rispetto alla library (linea continua), che viene esclusa dalla frazione collezionata. Entrambi gli elettroferogrammi sono ottenuti a 20 kV, 25°C e la rivelazione è effettuata grazie alla misura dell'assorbimento a 200 nm.

Dopodiché, l'analisi con PCR quantitativa (qPCR) ha permesso di mettere a punto la concentrazione iniziale di proteina tau necessaria e il numero di cicli di amplificazione, in modo da evitare amplificazione dei sottoprodotti (dimeri dei primer). Infatti, per quanto riguarda il primo aspetto, la concentrazione di proteina tau da incubare nel primo round di selezione con il DNA deve essere 2 ordini di grandezza inferiore rispetto al valore della costante termodinamica di dissociazione (nel nostro caso 0,750 µM). Tuttavia l'analisi qPCR ha rivelato una debole quantità di complesso di affinità in queste condizioni, che non permettevano perciò l'amplificazione selettiva delle sequenze più affini. Aumentando la concentrazione di tau fino a 7,5 µM, la qPCR ha mostrato la formazione di un complesso di affinità, mettendo in evidenza come il numero di cicli soglia (Ct) della frazione in presenza dell'analita fosse sensibilmente inferiore a quello della frazione in assenza del target. Data la relazione di proporzionalità inversa che lega il numero di ampliconi al numero dei Ct, tale analisi ci ha permesso di identificare le condizioni di partenza per la selezione.

Nonostante ciò la selezione con CE-SELEX non ha portato evoluzione positiva, che invece è stata realizzata attraverso l'utilizzo del CE-Non-SELEX (Figura 11).

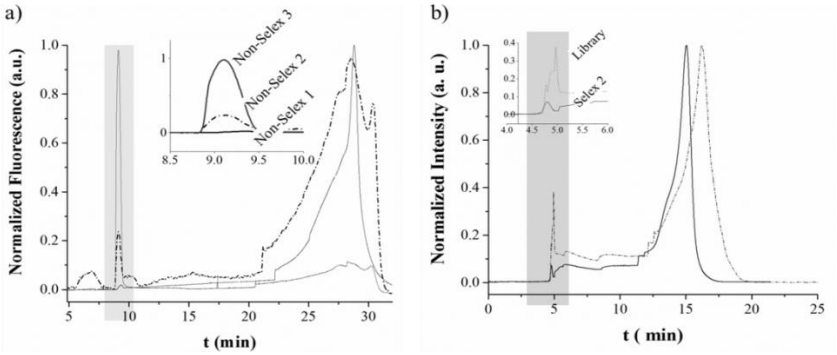

Figura 11: Complesso di affinità derivante dai due processi di selezione. a) CE-Non-SELEX; aumento delle aree rappresentanti il complesso di affinità stabile (osservato ad un tempo di 9,8 minuti) nei tre round di selezione. Vista la sostanziale uguaglianza tra le aree del complesso formatosi in presenza della library e del primo round di selezione, solo quest'ultima è riportata. b) CE-SELEX; aree del complesso in presenza della library (linea tratteggiata) e in presenza del pool selezionato dopo il round 2 (linea continua).

Tra le ragioni che possono aver impedito la selezione attraverso CE-SELEX, c'è sicuramente la formazione di sottoprodotti della PCR, evidenziati attraverso misure della temperatura di denaturazione (T_m) del doppio filamento, associate con una probabile amplificazione di sequenze di acido nucleico più corte rispetto all'originale.

Il campione di sequenze selezionate per via CE-Non-SELEX è stato sottoposto a sequenziamento con un processo diverso da quello convenzionale in cui è previsto l'uso di un plasmide per l'identificazione del motivo consenso. In particolare l'applicazione di un processo di NGS[7] ha permesso l'ottenimento di un numero molto elevato di sequenze (4792), tutte potenzialmente valide per legare selettivamente il target, la cui analisi è stata effettuata attraverso software bioinformatici. Nel primo caso l'uso di PATTERNITY- Seq (software che permette l'analisi della similarità tra le sequenze secondo un parametro detto distanza di

[7] NGS: next generation sequencing

Levenshtein) non ha mostrato un arricchimento del pool in poche famiglie, ma ha invece evidenziato un'elevata variabilità delle sequenze selezionate. L'analisi con MEME[8] ha sostanzialmente mostrato una situazione simile, sebbene, grazie ad esso, una classificazione secondo lunghezza dei motivi presenti ha permesso la scelta di cinque sequenze isolate, la cui affinità è stata caratterizzata sperimentalmente (FA e SPR).

La caratterizzazione con FA è stata scelta poiché permette l'utilizzo di ridotti volumi di campione (10 µL) e allo stesso tempo riflette in maniera fedele le condizioni di selezione (vale a dire omogeneità della soluzione, presenza della sonda fluorescente). Grazie a questa tecnica le sequenze 4133, 3146 e 433 hanno mostrato una affinità doppia rispetto alla library iniziale (Figura 12a e b).

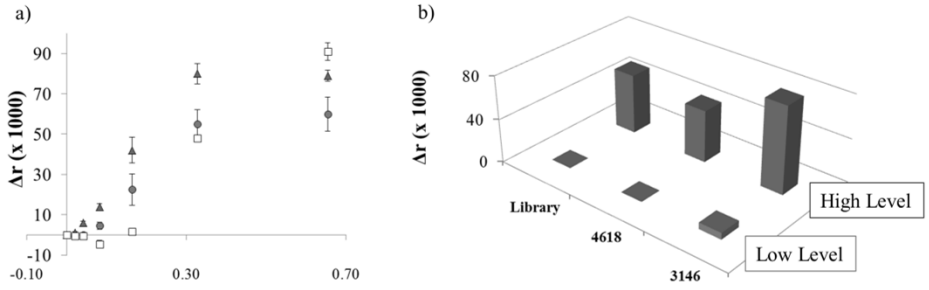

Figura 12: a) Variazioni di anisotropia di fluorescenza per gli aptameri 3146 (triangoli), 4618 (quadrati) e per la library (cerchi) in presenza di concentrazioni di tau comprese tra 0.02 e 0.7 µM. b) valori per le stesse sequenze a bassa concentrazione (0.04 µM) e alta concentrazione (0.3 µM) di analita.

L'immobilizzazione di tau, attraverso amino-coupling su un sensore CM5 commerciale ha permesso l'analisi SPR delle sequenze in condizioni *label-free*, a differenza di quanto accade in FA. Il sensorgramma delle sequenze è riportato in Figura 13.

[8] MEME: Multiple Em for Motifs Elicitation

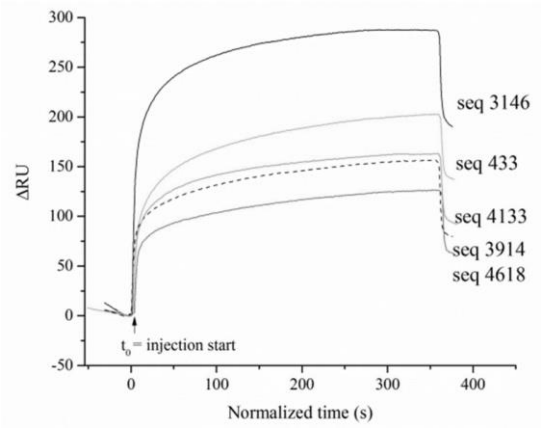

Figura 13: sensorgrammi delle sequenze isolate analizzate attraverso SPR.

Questa seconda analisi ha mostrato risultati simili a quanto riportato per la FA, pur basandosi su un principio fisico completamente diverso e su un diverso assetto strumentale, in cui l'analita era immobilizzato rispetto alle sequenze. In questo approccio la K_d stimata iniettando le sequenze a concentrazione costante sul sensore (1 µM) è risultata nel range del nM per la sequenza 3146, considerando il suo segnale come pari al valore di riflettività massimo (R_{max}), che può essere approssimato a 100 K_d. Il superamento dei problemi strumentali legati alla stabilità della proteina tau immobilizzata sul sensore potrà in futuro permettere una delucidazione più completa di quanto osservato.

Conclusioni

In questo lavoro è stata riportata la selezione rapida di aptameri per proteina tau. Se l'approccio convenzionale si è dimostrato inefficace per ottenere un'evoluzione positiva delle affinità, la CE-Non-SELEX ha permesso di ottenere gli scopi prefissi. Una riduzione sostanziale dei cicli di selezione è stata osservata, in particolar modo in confronto con gli aptameri contro la proteina tau selezionati in letteratura.

Sebbene grazie alla riduzione dei cicli di amplificazione sia stato possibile superare i limiti osservati, una elevata variabilità delle sequenze è stata riscontrata e l'uso di tecniche bioinformatiche per l'analisi dei dati (PATTERNITY-Seq e MEME) si è reso necessario. Pur considerando l'elevata variabilità vantaggiosa in termini di potenzialità ancora inesplorate per la tecnologia degli aptameri, questo ha reso molto complessa l'analisi dei dati. Alcune soluzioni sperimentali possono essere prese in esame per migliorare il processo di selezione, come ad esempio l'uso di diversi tipi di PCR.

Tra gli aptameri selezionati, cinque sequenze sono state analizzate attraverso SPR e anisotropia di fluorescenza, dando risultati confrontabili.

In conclusione la selezione grazie al CE-Non-SELEX ha permesso di ottenere numerosi candidati. Allo stato attuale l'affinità sembra insufficiente per un'applicazione diretta in diagnostica molecolare, tuttavia l'elevato numero di sequenze ottenuto e l'analisi critica dei vari step di selezione suggeriscono ampi margini di miglioramento in questo senso.

Conclusioni Generali

In questa tesi la rivelazione della proteina tau a scopo diagnostico è stata affrontata considerando due diversi aspetti. In primo luogo, il lavoro ha riguardato lo sviluppo di un saggio immunochimico sfruttando anticorpi monoclonali commerciali, mentre secondariamente è stato sviluppato un metodo di selezione per ottenere uno o più potenziali aptameri specifici per la proteina tau.

In entrambi gli approcci l'utilizzo della tecnica SPR è stato fondamentale, grazie alle sue proprietà e versatilità, per quantificare il target (grazie agli anticorpi) o per stimare le potenzialità di legame dei recettori selezionati.

Lo sforzo maggiore durante lo sviluppo dell'immunosensore ha riguardato la concezione di strategie di amplificazione innovative per raggiungere i livelli di proteina tau (pM) generalmente presenti nel liquido cerebrospinale, sia nei soggetti sani che nei soggetti affetti da demenza. Per raggiungere gli scopi prefissi, l'utilizzo di nanostrutture di carbonio (CNTs), come marcatori per un anticorpo secondario, ha permesso lo sviluppo di un saggio sandwich grazie al quale un limite di rivelabilità sperimentale di 125 pM è stato raggiunto. La possibilità di monitorare la formazione di un legame di affinità in tempo reale ha permesso di ipotizzare un processo di impaccamento, dovuto alla formazione di aggregati tra i diversi nanotubi in dispersione, che impediva inizialmente la rigenerazione del sensore. Il processo è stato ripristinato servendoci di un passaggio di centrifugazione che abbattesse le nanostrutture più grandi in soluzione; ulteriori sviluppi, che permettano una selezione più fine dei nanotubi secondo la loro lunghezza, possono essere previsti.

Altrettanto utile può essere l'utilizzo di CNTs direttamente immobilizzati sulla superficie del sensore in modo da sfruttare le residue proprietà

dovute alla loro conducibilità, e di incrementare la capacità totale di cattura della superficie.

La selezione è stata permessa da un processo di CE-Non-SELEX, che ha permesso con un volume estremamente ridotto di analita (circa 187 nL) e in pochi cicli di selezione (3), di arricchire il pool di sequenze, solo in quelle più affini per la proteina tau. Le caratteristiche del processo utilizzato sono sicuramente vantaggiose in termini di tempi necessari e costo globale, ma l'assenza di ripetuti passaggi di amplificazione ha portato ad una popolazione di sequenze altamente differenziata, messa in luce grazie a metodi di sequenziamento NGS.

In questo senso lo sviluppo di aptameri per la proteina tau può ancora permettere enormi sviluppi grazie all'utilizzo di strumenti bioinformatici, attraverso i quali la grande variabilità dei pool ottenuti può essere completamente analizzata, e anche le sequenze più rare (ma più affini) possono essere mantenute. In questo lavoro l'analisi delle sequenze ha riguardato essenzialmente la ricerca di famiglie secondo motivi comuni a più aptameri del pool, ma la presenza di una stretta e univoca relazione tra sequenza e specificità per il target è stata recentemente messa in discussione. Infatti analisi QSAR[9] sembrano dare maggiore importanza alla presenza di strutture secondarie, indipendentemente dalla successione delle basi presenti nella sequenza aptamerica.

In conclusione, se ad oggi l'immunochimica gioca un ruolo predominante anche grazie alla combinazione con le nanotecnologie, la selezione degli aptameri, avendo ancora ampi margini di crescita, può sicuramente giocare un ruolo nell'affiancare e, in certi, casi sostituire l'uso dei recettori anticorpali nella diagnostica clinica.

[9] QSAR: quantitative structure activity relationship

Appendix II. Résumé de thèse – Français

Introduction

La maladie d'Alzheimer (AD) a été découverte par Alois Alzheimer en 1906, grâce à l'observation de plaques amyloïdes et d'agrégats neurofibrillaires localisés dans l'encéphale d'August Deter, une femme de 50 ans qui a été le premier cas avéré de AD. Selon le *World Alzheimer Report*, environ 46 millions d'individus sont atteints de démence et parmi eux on estime une prévalence des cas d'Alzheimer à environ 60-70 % de la totalité. En ce qui concerne les plaques amyloïdes, leur composition révélée en 1985, montre l'existence de fortes concentrations de peptide bêta-amyloïde (Aβ), alors que pour les agrégats neurofibrillaires (1986), leur composition a montré essentiellement la présence de protéine tau. A l'heure actuelle, aucune technique existante ne s'est révélée efficace pour le diagnostic précoce de la maladie d'Alzheimer (ce qui serait fondamental pour intervenir dans les premières phases de la pathologie). Parmi les différentes techniques qui peuvent supporter le diagnostic *in vivo* nous pouvons énumérer les techniques d'imagerie médicale comme l'imagerie par résonnance magnétique (MRI en anglais), la tomographie par émission de positons (PET en anglais) ou les techniques basées sur des tests immunochimiques.

Ces derniers se basent sur la variation des niveaux (reportés dans le Tableau 1) des biomarqueurs de certaines biomolécules dans le liquide céphalo-rachidien (LCR) : le peptide Aβ, la protéine tau (tau totale représentant le total des isoformes) et la protéine tau phosphorylée.

Tableau 3 : Niveaux de concentration et valeurs seuils correspondantes, obtenus à travers test ELISA, pour les biomarqueurs de l'Alzheimer.

Biomarker	Healthy subject (ng L^{-1})	AD subject (ng L^{-1})
$A\beta_{1-42}$	794±20 (184.3 pM)	<500 (<116.3 pM)
Total tau protein (21-50 years)	136±89 (3.0 pM)	n.d.
Total tau protein (51-70 years)	243±127 (5.3 pM)	>450 (>9.8 pM)
Total tau protein (>71 years)	341±171 (7.4 pM)	>600 (>13.1 pM)
Phosphorilated tau protein (181)	23±2 (0.5 pM)	>60 (>1.3 pM)

Ces biomarqueurs dérivent de l'hypothèse de la cascade amyloïde (représentée dans la Figura 1), développée entre les années 1990 et 2002 et selon laquelle l'agrégation de l'Aβ précède celle de la protéine tau.

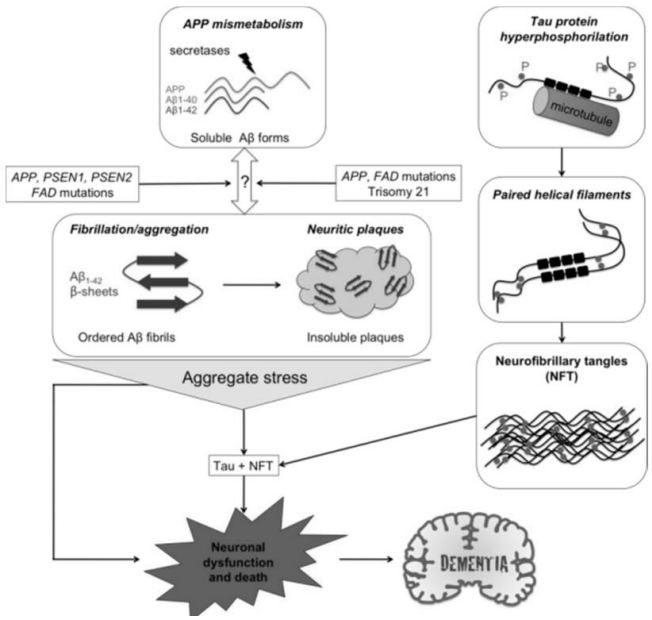

Figure 1 : Schéma représentant les voies suivies par la protéine tau et le peptide amyloïde selon l'hypothèse de la cascade amyloïde.

Des développements dans l'étude des biomarqueurs ont ensuite décrit l'existence d'espèces oligomères qui, étant donné leur plus grande vitesse de diffusion, ont montré une toxicité plus élevée que les agrégats.

Même si de nombreux travaux ont décrit la quantification de celles-ci ou d'autres espèces dans le LCR ou dans d'autres matrices biologiques, les

techniques immunochimiques de routine sont actuellement basées sur le dosage multiple des biomarqueurs telle que Aβ, tau-totale et tau hyperphosphorylée. L'utilisation de cette combinaison de biomarqueurs a démontré une sensibilité et une spécificité élevées, qui permettent de distinguer les sujets sains des sujets malades. De moins bonnes performances ont, en revanche, été rencontrées dans la distinction des différents types de démence.

Dans les paragraphes suivants, nous présenterons les caractéristiques de plusieurs techniques immunochimiques et des approches innovantes basées sur des récepteurs synthétiques.

Techniques immunochimiques adoptées dans les analyses de routine

Suite à l'introduction d'anticorps commerciaux spécifiques pour les biomarqueurs de *core*, le test ELISA (*Enzyme Linked Immunosorbent Assay)* et le test xMAP ont été développés et utilisés dans la recherche biomédicale. Ils sont désormais adoptés dans les pratiques hospitalières (après l'introduction en 2007 des directives publiées par le NINCDS-ADRDA[10]) dans le but de poser le diagnostic d'une probable maladie d'Alzheimer. Malgré les standards très élevés atteints en matière de répétabilité analytique et les excellentes performances en matière de sensibilité, avec des limites de détection (LOD) de l'ordre du picomolaire, ces deux techniques présentent encore plusieurs limites. La variabilité inter-laboratoires peut en effet atteindre des valeurs élevées, exprimées à travers le coefficient de variation (CV en %), qui peuvent atteindre 30%. De plus, l'utilisation de marqueurs fluorescents ou colorimétriques et de

10 NINCDS-ADRDA: National Institute of Neurological and Communicative Disorders and Stroke and the Alzheimer's Disease and Related Disorders Association.

récepteurs comme les anticorps (fiables, mais sujets à une variabilité intrinsèque due au système de production des différents lots) peut fortement limiter l'application de ces tests. Pour cette raison, des techniques alternatives, basées notamment sur l'utilisation de biocapteurs, ont été développées.

Biocapteurs d'affinité à base d'anticorps pour le diagnostic de la maladie d'Alzheimer

Les principaux biocapteurs d'affinité (dont le principe sera explicité dans la section suivante) qui se servent des anticorps mono- ou poly-clonaux, utilisés pour le diagnostic sans marquage de la maladie d'Alzheimer, sont listés dans le Tabella 2.

Tableau 4 : Techniques immunochimiques innovantes pour le diagnostic sans marquage de la maladie d'Alzheimer. Abréviations : SPR : résonance des plasmons de surface ; LSPR : SPR localisée ; SWV : voltamétrie à signaux carrés de potentiel ; EIS : spectrométrie d'impédance ; CNT-FET : transistors à effet de champ utilisant des nanotubes de carbone ; ADDL : oligomères dérivés du peptide amyloïde ; mAb : anticorps monoclonal.

Transduction principle	(1)	Target	LOD	Medium	Detection strategy	Antibody
Optical	SPR	$A\beta_{1-40}$	0.3 fM	buffer	sandwich	Fragmented mAb
		$A\beta_{1-40}$ $A\beta_{1-42}$	20.0 pM	CSF	sandwich	mAb
		tau (captured) $A\beta_{1-42}$ (flowed)	3.0 nM	buffer	sandwich	mAb
		$A\beta_{1-42}$	0.2 µM	buffer	direct	mAb
		ADDLs	280 nM	buffer	sandwich	mAb
		ADDLs	n.d.	buffer	direct	mAb
	LSPR	ADDLs	10.0 pM	human brain cells and CSF	sandwich	mAb
		Tau	0.2 pM	buffer	direct	mAb, protein G oriented
Electrochemical	SWV	$A\beta_{1-40}$ $A\beta_{1-42}$	20.0nM	rat CSF	direct	mAb
	EIS	$A\beta_{1-42}$	0.57 nM	buffer	direct	mAb, protein G oriented
	CNT-FET	$A\beta_{1-42}$	2 nM	serum	direct	mAb

Sans détailler les techniques développées, celles-ci, comme résumé dans le tableau, permettent l'obtention de limites de détection comparables ou supérieures à celles des techniques de routine. L'absence de marquage et la possibilité de détecter l'interaction en temps réel (dans la plupart des techniques présentées) augmentent la versatilité d'utilisation des biocapteurs, en simplifiant également l'interprétation des données obtenues. Par ailleurs, des possibilités élevées de miniaturisation instrumentale sont associées à l'utilisation de techniques telle que la LSPR et les techniques électrochimiques, ce qui les rend hautement adaptables à une utilisation sur le terrain (dans le cas d'analyses sur des matrices dont le prélèvement est moins invasif par rapport au LCR).

La sensibilité des techniques immunochimiques peut être énormément améliorée à travers l'utilisation d'approches de type *sandwich* et grâce à l'utilisation de marqueurs de diverses natures (schématisés dans la Figura 2).

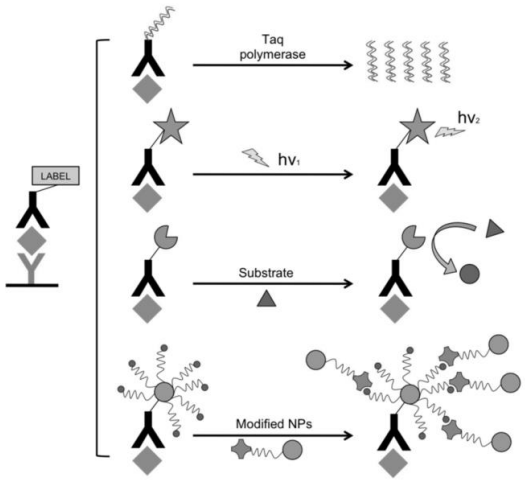

Figure 2 : Représentation des classes de marqueurs utilisés dans les tests bioanalytiques pour l'analyse des marqueurs de l'Alzheimer (les dessins ne sont pas à l'échelle). De haut en bas : ADN, fluorophores, enzymes catalytiques et nanoparticules métalliques.

Les oligonucléotides ont trouvé une application dans les méthodes qui permettent leur amplification à travers la réaction en chaîne par

polymérase (PCR en anglais) ou qui exploitent la possibilité de prédire les appariements entre les bases. La première propriété est exploitée par l'immuno-PCR, dans laquelle un des anticorps utilisés pour la reconnaissance de l'analyte est marqué avec un brin d'ADN et amplifié pour augmenter la sensibilité du test. Un exemple de l'association entre l'utilisation des oligonucléotides et des nanoparticules métalliques est décrit dans le test appelé *biobarcode*. Grâce à cette technique, la sensibilité de l'analyse a été abaissée jusqu'à des niveaux de l'ordre de attomolaire pour les oligomères issus du peptide amyloïde.

Dans d'autres exemples, les nanoparticules métalliques ont trouvé une application dans l'amplification de phénomènes comme la diffusion Raman ou dans d'autres méthodes optiques, tandis que les fluorophores organiques ont été exploités pour l'étude de résonance Foster (FRET) ou pour des mesures traditionnelles de l'intensité de fluorescence.

Les marqueurs enzymatiques, comme la phosphatase alcaline, sont adaptés pour une utilisation par des méthodes électrochimiques (électrodes conventionnelles ou *SPE*[11]), dont on trouve par contre un nombre inférieur d'exemples dans la littérature scientifique.

En conclusion, les techniques émergentes semblent avoir besoin d'une attention particulière pour leur utilisation dans une matrice biologique. L'utilisation d'un marqueur ou tout du moins le développement de tests de type *sandwich* semble être favorisé et, à l'heure actuelle, la plupart des méthodes sont orientées vers l'analyse d'espèces dérivées de l'APP[12] (oligomères de l'amyloïde, Aβ). On s'aperçoit également qu'il est nécessaire d'accroitre le nombre de techniques pour le dosage de la protéine tau.

[11] SPE: Screen Printed Electrode
[12] APP: amyloid precursor protein

Récepteurs biomimétiques

On énumère dans la famille des récepteurs biomimétiques tous ceux qui ont des propriétés similaires (voire meilleures dans certains cas) aux récepteurs de type biologique (anticorps, enzymes, etc.) et qui proviennent d'un processus de synthèse chimique effectué *in vitro* grâce auquel on obtient une production contrôlée et reproductible. De manière générale, les aptamères, les peptides, les peptoïdes (oligo-glycines substituées à l'atome d'azote) et les MIP *(polymères à empreinte moléculaire)* sont assurément les récepteurs synthétiques les plus utilisés en bioanalyse.

Dans le secteur des techniques bio-moléculaires appliquées au dépistage de la maladie d'Alzheimer (AD), on trouve de nombreux exemples de peptides. En particulier on exploite leur plus grande capacité de repliement sur eux-mêmes, par rapport à des récepteurs protéiques comme les anticorps. Cette caractéristique a été utilisée dans les méthodes d'impédance qui exploitent le processus de transfert de charge du peptide (marqué avec un médiateur chimique, tel que le ferrocène) à la surface d'une électrode. Au moment de l'interaction analyte-récepteur la conformation du peptide change, et une variation du signal analytique est détectée.

En ce qui concerne les aptamères et les *MIP*, à ce jour on ne relève pas d'applications diagnostiques pour ces éléments de reconnaissance, même si un développement croissant a été enregistré à partir de 2002. Les deux classes de récepteurs sont connues pour garantir une reconnaissance sélective des cibles, à travers les structures tridimensionnelles qu'ils assument lors de l'interaction avec la molécule d'intérêt. Certains auteurs ont décrit, pour les aptamères, un manque substantiel de sélectivité envers les espèces dont l'agrégation se fait par la formation de feuillets bêtas

(protéines prioniques, alpha-synucléine, amyloïde, tau, etc.). Toutefois, comme nous le décrirons dans les prochaines sections de la thèse, les résultats obtenus au cours de ce travail montrent une amélioration significative de l'affinité des séquences isolées par rapport à celle mesurée pour le *pool* de séquences initiales, suggérant ainsi un mécanisme de sélection vis-à-vis de la cible.

D'autres récepteurs synthétiques ont été développés et utilisés dans des capteurs de type électrochimique en garantissant les limites de détection de l'ordre du picomolaire.

En conclusion, même si l'aboutissement vers un diagnostic précoce de l'AD reste encore un défi difficile à relever, l'état de l'art indique que les développements dans le domaine des biocapteurs et de la bioanalyse, peuvent apporter une évolution positive dans cette direction. Toutefois, un rôle important est aussi à envisager pour tout ce qui concerne l'amélioration des techniques d'échantillonnage, le développement de matrices de référence, l'adoption de protocoles standardisés et enfin l'augmentation de la connaissance des aspects biochimiques associés au développement de la pathologie. Toutes ces améliorations pourraient aboutir à un diagnostic et un traitement plus précoce de la maladie d'Alzheimer.

Principes de Résonance des Plasmons de Surface et sélection des aptamères

La Résonance des Plasmons de Surface (SPR en anglais) et l'Electrophorèse Capillaire (EC) ont été utilisées pendant le travail de thèse.

Dans cette partie, les principes des techniques sont détaillés, notamment en portant une attention particulière aux principes physiques qui permettent la détection par SPR et aux différentes méthodes de sélection où l'EC est utilisée pour l'obtention des aptamères.

Principes de base de SPR

Parmi les phénomènes physiques exploités pour le développement d'un biocapteur, la SPR est sans doute un de ceux qui rencontre le plus de succès. Bien que de nombreux exemples de capteurs en milieu homogène aient été décrits dans la littérature scientifique, un biocapteur est composé d'un élément biologique, tel qu'un anticorps, un brin d'ADN etc., en contact direct avec un transducteur du signal. Ce dernier sert à transformer l'interaction biologique en un signal détectable analytiquement (Figure 3).

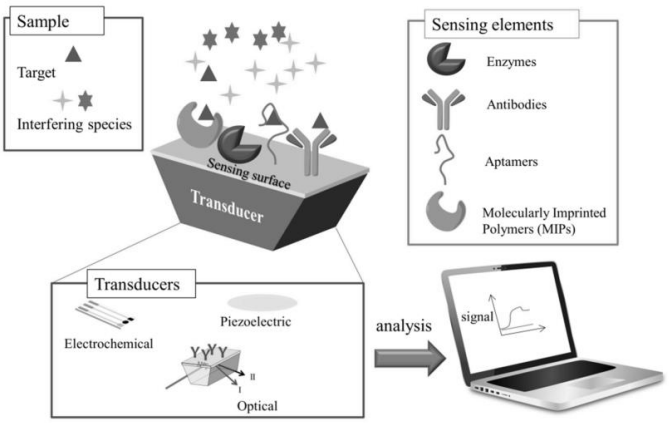

Figure 3 : Principes et principaux composants d'un biocapteur.

En SPR la transduction est assurée à travers le couplage entre les polaritons de surface, présents à l'interface métal-matériel diélectrique, et une radiation à longueur d'onde adaptée pour pouvoir résonner avec les électrons du métal.

Dans des conditions favorables, qui varient en fonction des matériaux utilisés (type de film métallique, prisme) et de la configuration des éléments optiques dans l'espace (angle d'incidence de la source de radiation), les électrons sont excités collectivement et produisent, en conséquent, une onde évanescente qui traverse le film métallique jusqu'à ce qu'elle rejoigne la face en contact avec le matériel diélectrique. Pour cette raison, les phénomènes qui apportent des variations à l'indice de réfraction du diélectrique et qui sont produits dans les 500 nm de distance de la surface métallique (dans le cas des films d'or), sont immédiatement détectés de manière très sensible.

Si l'on compare cette technique avec celle la plus couramment utilisée (la technique ELISA) nous pouvons mettre en avant plusieurs avantages : a) la révélation se fait en temps réel et sans avoir besoin du marquage avec des chromophores, ce qui facilite grandement l'analyse des paramètres cinétiques de l'interaction, b) la surface de la puce peut être utilisée pendant plusieurs cycles, grâce à des protocoles de régénération, c) l'amplification du signal peut-être réalisée à travers différentes stratégies, dont une des plus communes concerne l'utilisation de nanostructures d'or ou d'autres matériaux.

Dans les paragraphes suivants les propriétés de la SPR qui ont été exploitées au cours du travail sont reportées de manière plus détaillée, en mettant en évidence la versatilité de la technique dans plusieurs domaines de la bioanalyse.

SELEX et Non-SELEX : avantages et inconvénients des techniques pour la sélection des aptamères

Au cours de l'année 1990, deux groupes de recherches ont développé une technique appelée aujourd'hui SELEX[13]. Cette technique est divisée principalement en quatre passages fondamentaux : a) la banque d'ADN ou d'ARN est synthétisée et ensuite incubée avec la cible pour laquelle on veut obtenir l'aptamère (b). La séparation des séquences d'ADN affines de celles à faible affinité est effectuée à travers différentes méthodes, et elle est suivie par une amplification PCR (c) qui enrichit l'échantillon seulement avec les brins souhaités. A la fin de la méthode, le brin complémentaire qui est produit est éliminé (d) et le processus peut être répété jusqu'à 15 fois (comme résumé dans la Figure 4).

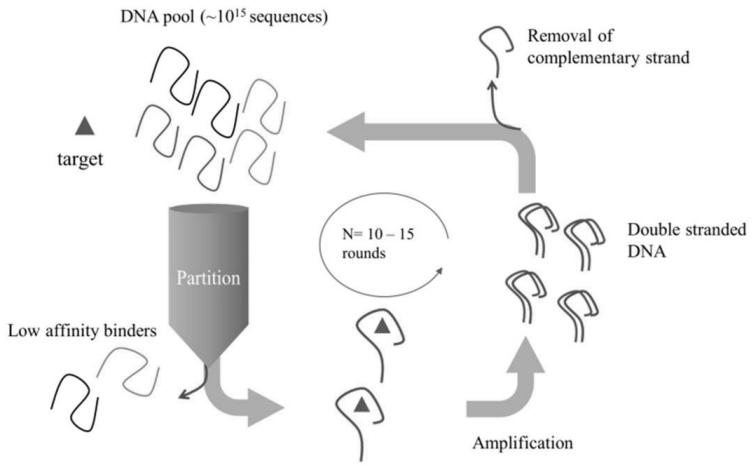

Figure 4: Technique de sélection SELEX pour l'identification des aptamères.

La forte résolution de l'EC a été exploitée durant ce travail. Cette technique est très avantageuse par rapport à d'autres méthodes de

[13] SELEX: Systematic Evolution of Ligands by EXponential enrichment

séparation habituellement employées. Par exemple, la cible n'a pas besoin d'être immobilisée sur un support adapté à la sélection, donc elle reste complètement accessible à la banque d'ADN en exposant tous les sites qui garantissent le mécanisme de reconnaissance moléculaire. De plus, avec l'EC il est également possible d'estimer l'affinité de la banque envers la cible, en calculant la surface du pic des espèces présentes dans le capillaire.

Comme pour l'EC-SELEX, l'EC-Non-SELEX possède les mêmes propriétés. La différence principale entre les deux méthodes demeure au niveau de l'amplification PCR, qui, pour l'EC-Non-SELEX, n'est pas effectuée entre chaque cycle, mais uniquement lors du dernier cycle de séparation. Ceci permet une réduction très importante du temps de sélection, avec un processus réalisé en quelques heures. Cependant, elle fournit un échantillon très peu enrichi et qui est donc plus difficile à interpréter à cause de la présence d'une population très variée de brins d'ADN.

Objectif de la thèse

Cette thèse a comme objectif principal le développement d'une méthode d'analyse de la protéine tau par voie SPR. Au début, des anticorps commerciaux ont été utilisés pour leur capacité à interagir sélectivement avec la cible, et les informations récoltées durant la mise au point de la méthode directe ont servi au développement de l'essai *sandwich*, avec ou sans nanostructures de carbone. Ces structures permettent une grande amplification du signal analytique dont le principe est décrit dans le paragraphe suivant.

Afin d'étudier la faisabilité d'un processus de sélection des aptamères pour la protéine tau, deux techniques ont été comparées (EC-SELEX et EC-Non-SELEX) et les raisons qui ont permis le choix de l'une ou de l'autre sont analysées. Ici la SPR a été utilisée pour évaluer l'affinité des séquences obtenues, en montrant sa versatilité dans plusieurs domaines de la bioanalyse.

Immunocapteur pour la protéine tau

Le développement de l'immunocapteur pour quantifier la protéine tau a été réalisé grâce à une étroite collaboration avec le groupe de recherche du professeur Cicchi, qui a fourni les nanotubes de carbone (CNTs) utilisés pour l'amplification du signal analytique (Figure 5)

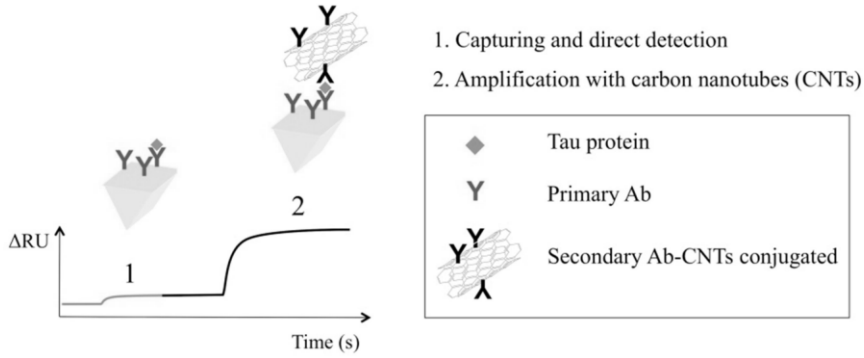

Figure 5 : Représentation de l'essai immunochimique. 1) Essai direct ; l'anticorps primaire est immobilisé sur la surface du capteur et sert pour assurer la reconnaissance moléculaire afin de capturer la cible. 2) Amplification du signal avec un anticorps secondaire marqué avec des nanotubes de carbone (CNTs).

Les CNTs ont déjà été utilisés pour l'analyse de l'EPO[14] par voie SPR et ils ont montré de fortes capacités d'amplification du signal analytique. Ces structures peuvent donc être proposées comme alternatives aux structures métalliques, qui sont plus souvent utilisées en SPR. Parmi les caractéristiques qui permettent l'amélioration du signal, on peut indiquer leur géométrie, à travers laquelle l'indice de réfraction du milieu est modifié de manière significative, et l'augmentation de la surface dont l'anticorps et l'analyte disposent pour établir leurs interactions.

[14] EPO : érythropoïétine, protéine du corps humain de 34 KDa.

Résultats et discussion

Durant l'analyse, la protéine tau a été injectée à une concentration comprise entre 125 pM et 250 nM, et détectée tout d'abord avec un anticorps monoclonal qui reconnait la cible dans la région comprise entre l'acide aminé 189 et 195 (clone de l'anticorps 39E10). L'immobilisation du récepteur a été réalisée grâce à la technique du couplage-amino, qui permet l'exploitation des groupes aminés présents à la surface des protéines pour assurer la formation d'une liaison covalente entre la surface du capteur et la protéine elle-même. La valeur du pH (4,0, maintenu avec tampon acétate 10 mM) pour l'immobilisation a été définie grâce à un test de pré-concentration qui a permis d'identifier les conditions les plus adaptée à la fonctionnalisation de la surface à base de dextrane d'une puce d'or (CM5, commercialisé par GE Healthcare). La méthode ainsi développée permettait de quantifier la protéine tau dans un tampon et dans une solution conçue pour avoir une composition similaire à celle du liquide céphalorachidien (LCR). Dans ces conditions la sensibilité analytique reportée était de l'ordre du nM pour les deux milieux considérés, mais de meilleures performances en matière de répétabilité et d'amplitude du range linéaire ont été observées dans le LCR artificielle (aLCR) (Figure 6).

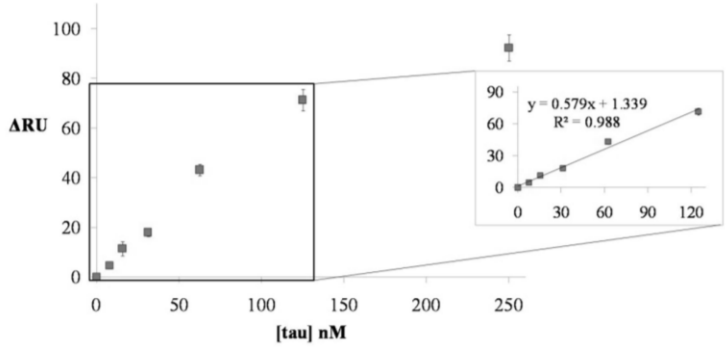

Figure 6: test direct avec l'anticorps primaire en aLCR

Le résultat obtenu est prometteur si on le compare à ceux obtenus par d'autres auteurs en littérature, car une limite de détection de l'ordre du nM avait été précédemment reportée avec l'utilisation d'un essai *sandwich*, tandis qu'ici nous n'avons utilisé qu'un seul anticorps. Les raisons qui ont permis d'obtenir ces performances doivent être probablement recherchées dans la capacité de la surface de dextrane à capturer une quantité de cible plus importante grâce à sa conformation 3D. De plus, l'analyse du aLCR est sans doute plus adaptée pour le dépistage précoce de la maladie d'Alzheimer. Elle révèle aussi la nécessité de développer une matrice certifiée (CRM), qui pourrait améliorer les performances des méthodes conventionnelles.

Le test *sandwich*, conçu en utilisant comme base l'essai direct décrit auparavant, a utilisé un anticorps secondaire (clone tau12, monoclonal) qui reconnait la protéine tau dans sa partie N-terminale. Comme pour le premier essai, la méthode développée a permis la détection en aLCR en améliorant les performances de l'essai jusqu'à une limite de 2,5 nM (Figure 7B), deux fois inférieure à la précédente, mais loin des niveaux atteints par l'analyte dans la matrice.

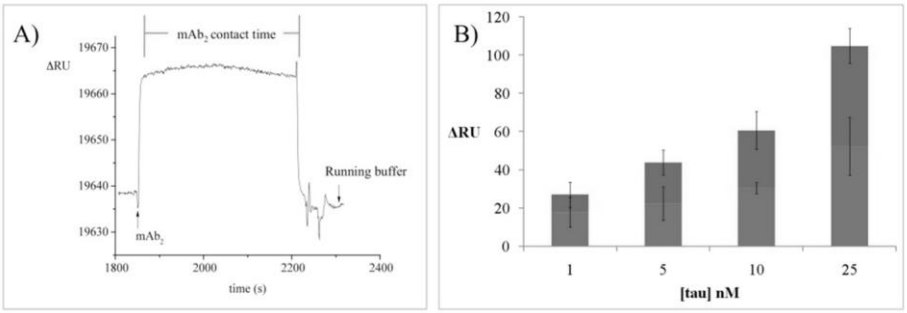

Figure 7 : A) absence d'interaction entre l'anticorps secondaire et la surface fonctionnalisée avec l'anticorps primaire ; B) signaux enregistrés pour des concentrations de protéine tau de 1 à 25 nM.

L'injection du récepteur secondaire en absence d'analyte n'a pas montré de contributions aux signaux venants de l'interaction entre les deux anticorps (Figure 7A), et le même comportement a été observé pour l'interaction avec l'albumine humaine (HSA).

Afin d'atteindre le niveau pM nécessaire, le groupe du professeur Cicchi a réalisé l'oxydation et l'activation des nanotubes, pour permettre à la fois leur dispersion dans une solution aqueuse et l'ancrage covalent de l'anticorps. Cette deuxième étape a été facilitée par l'activation des nanostructures avec des groupes fonctionnels O-succinimide, dont nous avons vérifié la présence par spectrométrie infrarouge (IR) et par analyse élémentaire. Ensuite, trois paramètres ont été considérés pour rendre plus efficace la conjugaison de l'anticorps et permettre également l'obtention d'une dispersion stable. En particulier, le temps de sonication sur les nanostructures sans l'anticorps, le milieu où nous avons effectué la réaction et la concentration globale de l'anticorps ont montré leur importance dans le processus de conjugaison (Figure 8).

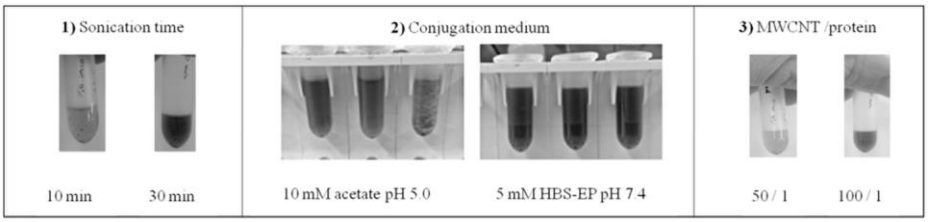

Figure 8 : Optimisation des paramètres pour la conjugaison de l'anticorps secondaire. 1) temps de sonication ; 2) milieu de conjugaison ; 3) rapport entre les concentrations de CNTs et la cible.

Une amélioration significative du signal a été obtenue dans l'essai réalisé dans le tampon (Figure 9A). Toutefois, la régénération de la puce, avec des injections de courte durée de NaOH (24-48'', 10 mM – 50 mM), n'a pas été possible, à cause de la présence des CNTs.

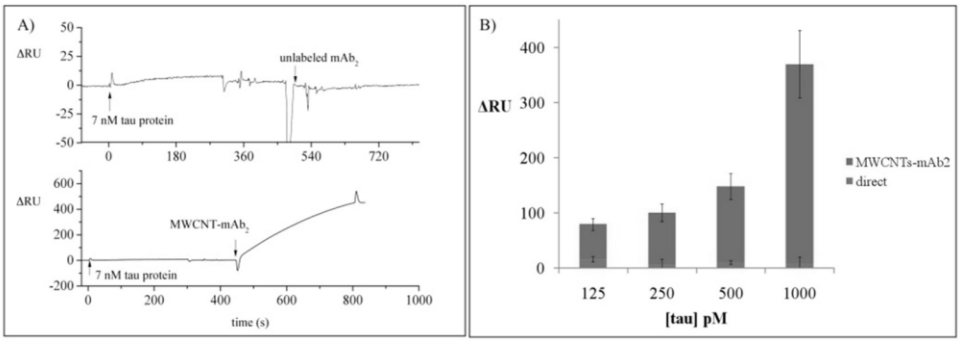

Figure 9 : A) amplification du signal en tampon à travers l'utilisation des CNTs, comparé avec le signal d'une concentration équivalente de l'anticorps secondaire non marqué ; B) utilisations de CNTs centrifugés pour l'amplification des signaux de la protéine tau dans la région du pM.

Parmi toutes les causes pouvant affecter le processus de régénération du capteur, la dispersion des nanotubes en structure de différentes longueurs semble la plus probable. Il est connu que les CNTs sont composés de fractions dont la taille varie beaucoup, pouvant arriver à des valeurs de l'ordre du µm. Ces derniers ont une tendance à interagir de manière très forte avec eux-mêmes et peuvent donc produire une couche très épaisse de CNTs qui n'est pas accessible à la solution de régénération. En

introduisant une étape de centrifugation, réalisée à faible régime de rotation (3000 rcf) et pour une courte durée (3 minutes), nous avons réduit la présence en dispersion de la fraction de plus grande taille. De cette manière, la régénération de la puce a été rétablie. Grâce à cela, le comportement de l'interaction cible-anticorps à des concentrations de l'ordre du pM a pu être étudié (Figure 9B). Une relation linéaire entre la concentration de l'analyte et le signal a été observée, jusqu'à des valeurs de tau de l'ordre du nM. Au-delà, une déviation de la linéarité vers une relation de type exponentielle a été montrée, probablement due à une augmentation des interactions entre les différents nanotubes.

Conclusions – immunocapteur

Dans cette partie du travail, l'utilisation des CNTs pour augmenter le signal d'un immunocapteur SPR conçu pour la détection de la protéine tau a été décrite. Grâce à l'utilisation de ces nanostructures, le signal analytique a été amplifié de deux ordres de grandeur par rapport à la méthode directe. Ceci permet ainsi d'atteindre une limite de détection pour la protéine tau de l'ordre du pM avec une répétabilité acceptable (CV 14%) entre les différents lots de nanotubes testés.

Une optimisation de la méthode pourrait porter sur une sélection plus fine des longueurs des nanotubes afin d'obtenir un processus globalement plus contrôlable, mais, même en l'état actuel des choses, ces nanostructures peuvent être considérées comme une nouvelle étape pour le dépistage de la maladie d'Alzheimer.

Sélection d'un élément de reconnaissance de type aptamère pour la détection de la protéine tau

La deuxième partie des travaux de recherche a été réalisée au sein du département de pharmaco-chimie moléculaire (DPM) de l'Université Grenoble Alpes. Le but de cette partie du travail était de se consacrer au développement d'un élément de reconnaissance moléculaire de type aptamère, capable, par ses propriétés de stabilité et de reproductibilité dans la production des différents lots, de rivaliser avec les anticorps comme outil d'analyse de la protéine tau. La technique qui permet l'obtention des aptamères prend le nom de SELEX et se base sur l'enchaînement d'étapes de séparations, suivies par des étapes d'amplification, qui sont répétées en boucle jusqu'à environ 15 fois afin d'atteindre des résultats optimaux. Dans la méthode, moins répandue, appelée EC-Non-SELEX, les étapes séparatives sont effectuées consécutivement, avant de procéder à une seule amplification par PCR.

Après avoir comparé les résultats de ces deux méthodes, nous avons isolé un nombre limité de séquences d'ADN pour déterminer leur affinité envers la cible via Anisotropie de Fluorescence (FA) et par SPR.

Résultats et discussion

Avant de pouvoir commencer la sélection, des étapes préliminaires ont été réalisées pour assurer la séparation entre la banque d'ADN (marquée avec de la fluorescéine et constituée de 77 nucléotides, dont 30-mer sont obtenues d'une manière aléatoire afin d'obtenir un nombre de séquences différentes de l'ordre de 10^{15}) et la protéine tau. Cette étape a permis d'identifier la zone de l'électrophérogramme où les séquences d'intérêt ont été récoltées. A cause de la présence de plusieurs pics dans le tracé électrophorétique, nous avons mesuré la mobilité des espèces présentes à

des valeurs de pH différentes (3,0 ; 5,5 ; 7,4). Seule la mobilité de la protéine tau était sensible à ces types de variations : cela nous a permis d'identifier la fenêtre de collection (Figure 10).

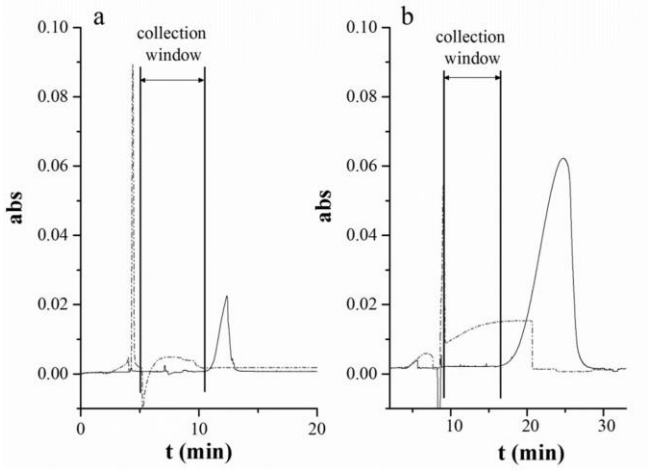

Figure 10 : a) fenêtre de collection pour le CE-SELEX et b) pour le CE-Non-SELEX. Le pic de la protéine tau (trait en pointillé) migre plus rapidement par rapport aux molécules d'ADN libre (trait continu), qui sont éliminées. Les tracés électrophorétiques sont obtenus dans les mêmes conditions à 20 kV, 25°C et les molécules sont détectées par UV, en mesurant l'absorbance à 200 nm.

Ensuite, une analyse de PCR quantitative (qPCR) a permis de mettre au point la concentration initiale de cible à incuber avec la banque et les nombres de cycles PCR optimaux pour ne pas amplifier des « primer dimer ». En effet, en ce qui concerne le premier aspect, la concentration de cible doit être de deux ordres de grandeur plus faibles que la constante de dissociation K_d estimée lors de l'interaction avec la banque. Nous avons estimé une concentration de tau initiale de 0,750 µM en se basant sur les tracés électrophrétiques ; cependant, l'analyse en qPCR a révélé la formation d'une quantité de complexe très faible, qui ne permettait pas une amplification sélective des brins les plus affins. En augmentant la concentration de protéine en contact avec la banque à 7,5 µM, la qPCR a permis de montrer qu'il y avait formation de complexe entre la banque et

la protéine tau. En effet, la fraction collectée en présence de la cible a montré des C_t (nombre de cycles seuils à partir desquels il y a amplification) inférieurs à la fraction collectée sans cible (C_t= 6 contre C_t= 18 ; plus la valeur est faible, plus le nombre d'amplicons est important). Bien que la sélection par voie SELEX ait été réalisée dans ces conditions, l'évaluation du complexe par EC, n'a pas montré une évolution vers des brins plus affins pour la tau. Par contre, l'utilisation de la méthode EC-Non-SELEX, a montré une évolution après seulement trois cycles de sélection

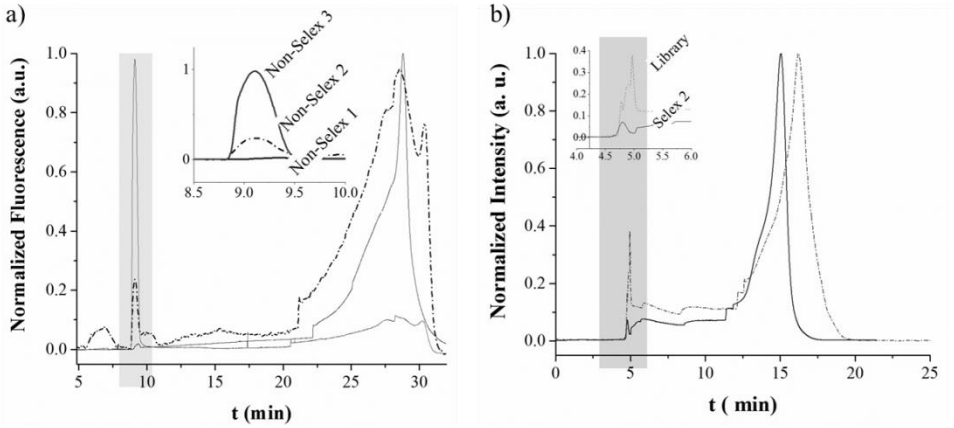

Figure 11 : Complexe d'affinité issu de deux processus de sélection. a) EC-Non-SELEX ; augmentation des aires qui représentent le complexe d'affinité stable (observé avec un temps de migration de 9,8 minutes) durant les trois rounds. Les aires dérivées de la formation du complexe avec la banque et celle en présence du premier round ont montré les mêmes valeurs, donc seulement la deuxième est représentée dans la figure. b) EC-SELEX ; aire du complexe d'affinité avec la banque (trait pointillé) et avec le *pool* sélectionné après le round 2 (trait continu).

La formation de sous-produits, dérivants de l'amplification PCR, est peut-être une des causes qui ont empêché au SELEX conventionnel de produire l'augmentation d'affinité recherchée. En effet, lors de la formation du double brin, une température de fusion (T_m de l'anglais *Temperature of Melting*) plus faible par rapport à celle de la banque a été observée pour

l'EC-SELEX, indiquant, probablement, l'amplification prépondérante de brins plus courts que la banque (que l'on peut associer à des sous-produits).

L'échantillon collecté du processus d'EC-Non-SELEX a été retenu et envoyé à l'étape de séquençage, qui a été réalisé par NGS[15]. Dans cette méthode, l'absence de l'étape de clonage et l'utilisation d'un système d'analyse très rapide ont permis d'avoir des milliers de séquences (4792), toutes potentiellement capables d'interagir avec la protéine tau avec la même affinité. Une analyse des séquences par logiciels bio-informatiques (PATTERNITY – Seq et MEME[16]) a montré une très grande diversité de la population des séquences récoltées. Cependant, cinq brins ont été isolés afin d'effectuer une caractérisation de l'affinité par voie expérimentale (FA et SPR). L'anisotropie de fluorescence permet, en utilisant des volumes d'échantillon très petits (10 µL), d'estimer l'affinité dans des conditions qui reflètent celles de la sélection. Suite aux résultats obtenus en FA, les séquences 4133, 3146 et 433 ont montré une meilleure affinité par rapport à celle de deux autres testées et de la banque (Figure 12a et b).

[15] NGS: Next Generation Sequencing
[16] MEME: Multiple Em for Motifs Elicitation

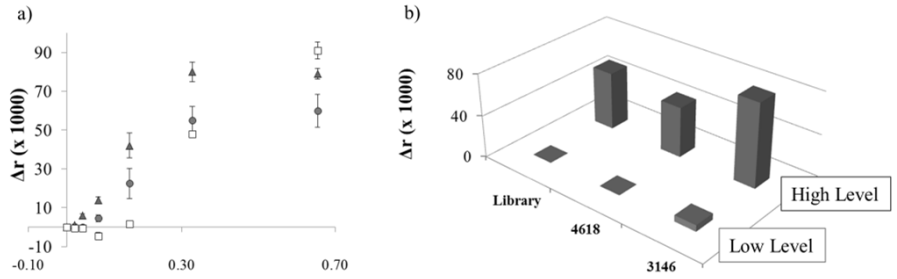

Figure 12 : a) Variations d'anisotropie de fluorescence des aptamères 3146 (triangles), 4618 (carrés) et pour la banque (cercles) à des concentrations de tau croissantes (entre 0.02 et 0.7 µM). b) valeurs de mêmes séquences à faible (0.04 µM) et haute concentration (0.3 µM) de cible.

L'analyse par SPR se fait en immobilisant la protéine tau sur la surface à base de dextrane d'un capteur CM5 commercial, en exploitant les groupes carboxyles présents sur le capteur pour le couplage-amino. Les courbes dérivantes de l'interaction sont reportées en Figure 13.

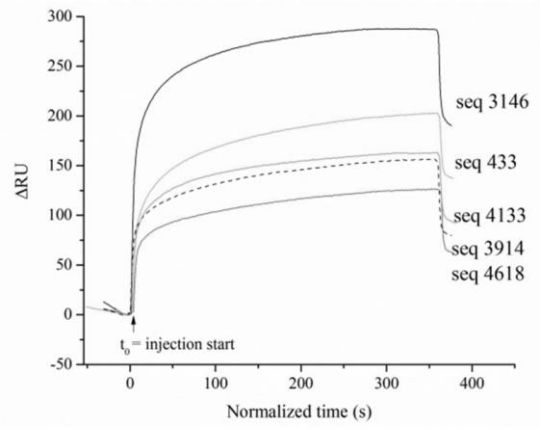

Figure 13 : Signaux des séquences isolées mesurées par SPR.

Les résultats obtenus lors de l'analyse par SPR concordent avec les mesures d'anisotropie, ce qui est très encourageant si l'on considère la diversité des deux techniques. Les séquences ont été injectées à la même concentration (1 µM) sur la puce fonctionnalisée avec la tau. L'aptamère

3146 possède une meilleure affinité par rapport aux autres. Si l'on considère le signal obtenu du brin 3146 comme R_{max}, il est possible d'estimer une K_d autour du nM, bien que cette valeur ait besoin d'analyses supplémentaires afin de la confirmer.

Conclusions

La sélection rapide d'aptamères pour la protéine tau a été décrite dans ce travail de recherche, où la méthode conventionnelle de l'EC-SELEX a montré des limites par rapport à l'EC-Non-SELEX, qui a en revanche permis d'accomplir la tâche que nous nous étions fixée.

Même si les rounds de sélection ont été fortement réduits par rapport à d'autres techniques reportées dans la littérature scientifique, une grande variabilité des séquences a été observée.

Des techniques issues de la bio-informatique ont été appliquées pour surmonter cette difficulté, en s'appuyant sur l'utilisation de logiciels préalablement développés (PATTERNITY-Seq et MEME). Ces derniers n'ont pas réussi à identifier des séquences consensus aptes à limiter la variabilité observée ; il reste donc un grand potentiel inexploré, qui pourrait conduire à des aptamères plus fortement affins pour la tau, par rapport à ceux qui ont été décrits dans cette thèse.

Conclusions Générales

Dans cette thèse, la détection de la protéine tau dans un but de diagnostic a été évaluée en considérant deux aspects différents. En premier lieu, le travail a concerné le développement d'un test immunochimique avec des anticorps monoclonaux commerciaux. Dans un deuxième temps, nous avons développé une méthode de sélection pour obtenir un ou plusieurs aptamères spécifiques de la protéine tau.

Dans ces deux approches, l'utilisation de la technique SPR, grâce à ses propriétés et à sa versatilité, a permis de quantifier la cible (immunodosage) ou d'estimer les affinités des récepteurs sélectionnés.

Pendant le développement de l'immunocapteur, une partie importante du temps a été consacrée à la conception de stratégies d'amplification innovantes pour atteindre une sensibilité suffisante et adaptée aux niveaux de la protéine tau (pM) généralement présents dans le CSF, aussi bien chez les sujets sains que chez les sujets atteints de démence.

Pour atteindre ces objectifs, l'utilisation de nanostructures de carbone (CNTs), comme marqueurs d'anticorps secondaire, a permis le développement d'un test *sandwich* grâce auquel nous avons obtenu une limite de détection expérimentale de 125 pM. La formation d'agrégats entre les différents nanotubes en dispersion a initialement empêché la régénération du capteur. Le système a pu être à nouveau régénéré en utilisant une étape de centrifugation qui a abattu les nanostructures de plus grande taille. Ainsi, des développements ultérieurs, qui permettraient une sélection plus fine des nanotubes selon leur longueur, pourraient être envisagés. L'utilisation de CNTs directement immobilisés à la surface du capteur pourrait être tout autant utile, car elle permettrait d'exploiter les propriétés résiduelles de conductivité des nanostructures et aussi d'augmenter la capacité totale de capture du récepteur immobilisé.

La sélection d'aptamères anti-tau a été effectuée grâce au processus d'EC-Non-SELEX qui a permis, en peu de rounds de sélection (3), de sélectionner un *pool* de séquences affines vis-à-vis de la protéine tau. Si les caractéristiques du processus utilisé sont assurément avantageuses en matière de temps nécessaire et de coût global, l'absence de passages répétés d'amplification, combinée au nombre limité de séquences qu'il est possible d'introduire dans le capillaire pour la séparation électrophorétique, a conduit à l'obtention d'une population de séquences très diversifiée. Cet aspect a été mis en évidence grâce au séquençage NGS qui a abouti à un séquençage très rapide de la population entière.

Dans ce travail, l'analyse des séquences a concerné essentiellement la recherche de familles sur la base de motifs communs à plusieurs aptamères du pool, mais la présence d'une relation étroite et univoque entre séquence et spécificité pour la cible a été récemment mise en discussion. En effet, des analyses QSAR[17] semblent attribuer plus d'importance à la présence de structures secondaires, indépendamment de la succession des bases présentes dans la séquence aptamérique. Des tests bioinformatiques plus poussés, ou sur des critères différents de ceux utilisés, pourraient être envisagés.

En conclusion, si à ce jour, grâce également aux techniques qui permettent le couplage des anticorps avec les nanotechnologies, l'immunochimie joue un rôle prédominant, la sélection des aptamères, ayant encore une grande marge de croissance, peut assurément permettre à ce type de récepteurs d'être utilisés voire même de remplacer les récepteurs conventionnels dans le domaine du diagnostic clinique.

[17] QSAR: quantitative structure activity relationship