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NEUROPROTECTIVE PEPTIDES IN ALZHEIMER'S DISEASE: STRUCTURAL CHARACTERIZATION AND AFFINITY OF B-AMYLOID AND HUMAN CYSTATIN C PEPTIDES INTERACTIONS

Ana Almeida

MASTER IN HUMAN BIOLOGY AND ENVIRONMENT

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For my wonderful parents Maria and Julio Almeida.

“Do not follow where the path may lead. Go, instead, where there is no path and leave a trial.”
Anonymous

“Learn from yesterday, live for today, hope for tomorrow. The important thing is to not stop questioning. Curiosity has its own reason for existing.”
Albert Einstein

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All my friends in Konstanz and Lisbon.

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1. INTRODUCTION

1.1 Alzheimer's disease: Pathophysiological characteristics and potential therapeutical approaches

Age-related impairments in cognition and memory have been known since ancient times, but the clinical-pathological features of the syndrome, now termed Alzheimer's disease (AD), were not documented in the literature until the first decade of last century (1906), when the german neurologist Alois Azheimer reported the case of a middle-aged woman who developed memory deficits and progressive loss of cognition. AD is characterized by cognitive symptoms like memory loss, disorientation and confusion, problems with reasoning and thinking and by behavioral symptoms that include agitation, anxiety, depression, hallucination, insomnia and wandering.

It is established that AD is a progressive, degenerative disorder of the brain and the most common form of dementia among the elderly, affecting more than 20 million people worldwide. It is recognized as a major public health problem in developed nations and the third more expensive disease to treat in the U.S., costing society close to $100 billion annually. There is currently no cure for AD and the exact causes of the disease are still unknown, but current research is beginning to allow a greater understanding of how this disease develops and potential therapeutic approaches to treat or prevent AD under investigation.

1.1.1 Biochemistry of Alzheimer's disease

Microscopic examination of the AD brain reveals a number of characteristic histological lesions comprising intracellular neurofibrillary tangles (NFTs), extracellular amyloid plaques, congophilic angiophaty (CA) of the vessels, neuropil treads that lead to extensive neuronal loss, predominantly of the cholinergic neurons. Since the innervate

the hippocampus and cortex and are believed to be involved in information processing and storage, their loss is responsible for severe memory loss and behavioural and intellectual deterioration seen in AD.

Neurofibrillary tangles are neuronal cytoplasmatic accumulations of paired helical filaments consisting of hyperphosphorilated *tau* protein, a neuron-specific phosphoprotein that is the major constituent of neuronal microtubules. The microtubules help transport nutrients and other important substances from one part of the nerve cell to another. In AD the *tau* protein is abnormal and the microtubule structures collapse. Intracellular protein accumulation is not specific for Alzheimer's disease alone, but has been shown in other degenerative disease.

The other neuropathological feature of AD is the accumulation and invariant deposition of extracellular beta-amyloid plaques (senile plaques) in the brain. Two major types of senile plaques have been identified: the classic (neuritic) and the diffuse senile plaques. The major component of either plaque type is the amyloid beta peptide (Aβ), a 39-43 amino acid peptide derived from its precursor amyloid protein (βAPP) by proteolytic processing. Whereas classic senile plaques consist of a central core of amyloid, made up of Aβ fibrils and surrounded by a halo of degenerating and dystrophic neuritis, the diffuse senile plaque contains non-fibrillar Aβ and contains neither an amyloid core nor neuritic changes (they are more abundant in AD brains than the classic senile plaques, but are also found in the brains of non-demented eldery).

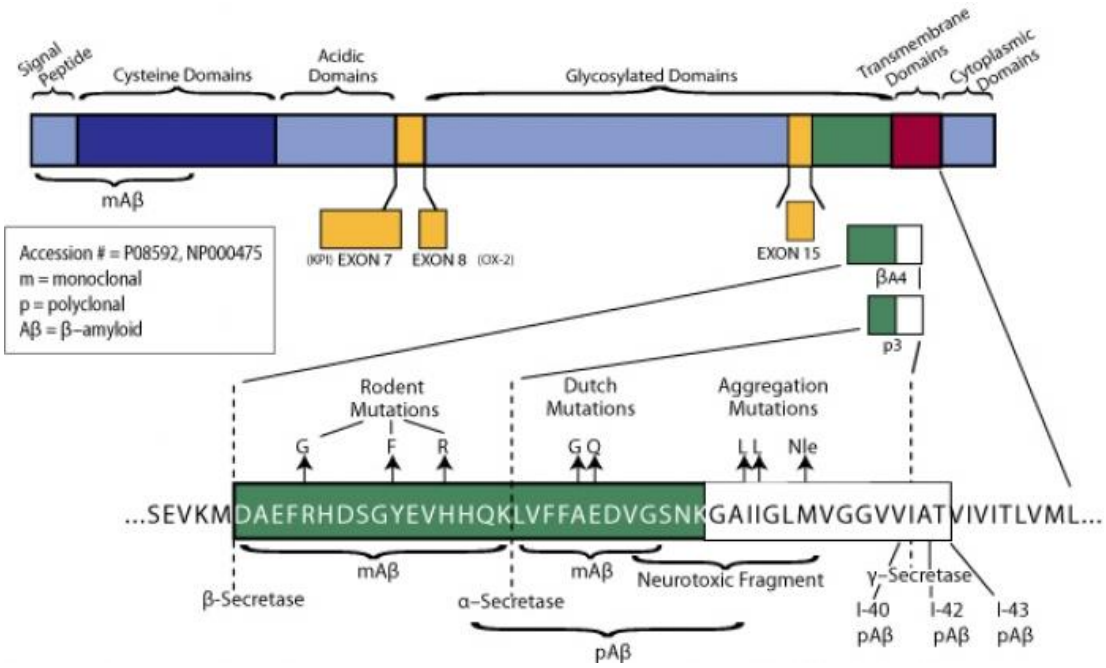


Figure 1: APP and Aβ protein schematic.

βAPP is a transmembrane glycoprotein with a single membrane spanning region, a large extracytoplasmatic domain (ectodomain) and a small C-terminal intracytoplasmatic part. It is expressed in the heart, kidneys, lungs, spleen and intestines, as well as in the brain. The most abundant isoforms in the brain are APP695, APP751 and APP770. APP695 is the shortest of the three isoforms and is produced mainly in neurons. APP 751, which contains Kunitz-protease inhibitor domain (KPI), and APP770, which contains both the KPI and an Ox^{-2} antigen domain, are found mostly in non-neuronal glial cells. All three isoforms share the same Aβ transmembrane and intracellular domains and are thus all potentially amyloidogenic.

The initial step of the metabolic pathway of APP involves the alfa-secretase enzyme that cleaves out APP within the Aβ sequence, allowing for the release of its transmembrane fragment. This metabolic pathway is not amyloidogenic because it precludes the formation of Aβ and leads to the release of APPα wich appears to exert neuroprotective activity. Several reports suggested that mainly adamalysin proteinases such as ADAM 10 (a disintegrin and metalloprotease) or ADAM 17 (also know as

TACE, tumor necrosis factor converting enzyme) are involved in the constitutive and regulated alpha-secretory pathway of APP.

The alternative pathway of APP secretion results in the cleavage of APP at β-and γ-cleavage sites, liberating secreted APP (sAPPβ) and Aβ peptide. Recently, proteases that cleave APP at the β-site have been cloned and identified. They are namedBACE 1 and 2 for beta-site cleaving enzyme and belong to the family of aspartyl-proteases.

γ-Secretase is a hetero-oligomer containing at least four protein components, presenilins (PS-1/PS-2), nicastrin, APH-1 and PEN-2, in a high molecular mass complex of unknown stoichiometry.

Aβ and Aβ-like peptides have been found to be produced with strong N- and C- terminal heterogeneity. Under normal conditions, the most abundant species in the brain is the Aβ40; however much of the fibrillar Aβ is composed of the longer, more fibrillogenic Aβ42. Aβ40 comprises 90-95% of the secreted Aβ and it is the predominant species in cerebrospinal fluid (CSF). In contrast, less than 10% of secreted Aβis Aβ42. Originally, Aβ42 peptide was assumed to be released by a pathogenic event; it is now well established that Aβ42 is released from cells during normal cellular metabolism of the Alzheimer amyloid precursor protein. Aβ42 is the predominant species found in the plaques and is deposited initially to form insoluble amyloidogenic aggregates more rapidly tha Aβ40.

1.1.2 Amyloid cascade hypothesis of Alzheimer's disease

Several lines of evidence suggest that Aβ plays a key role in the pathogenesis of AD. It was found that all patients with AD accumulate deposits of first Aβ42 and then also Aβ40 in regions of the brain important for memory, cognition and behavioural stability. Aβ42 diffuse plaques occur increasingly with age in neurologically normal individuals, strongly suggesting that Aβ42 accumulation precedes all other pathological feature of AD.

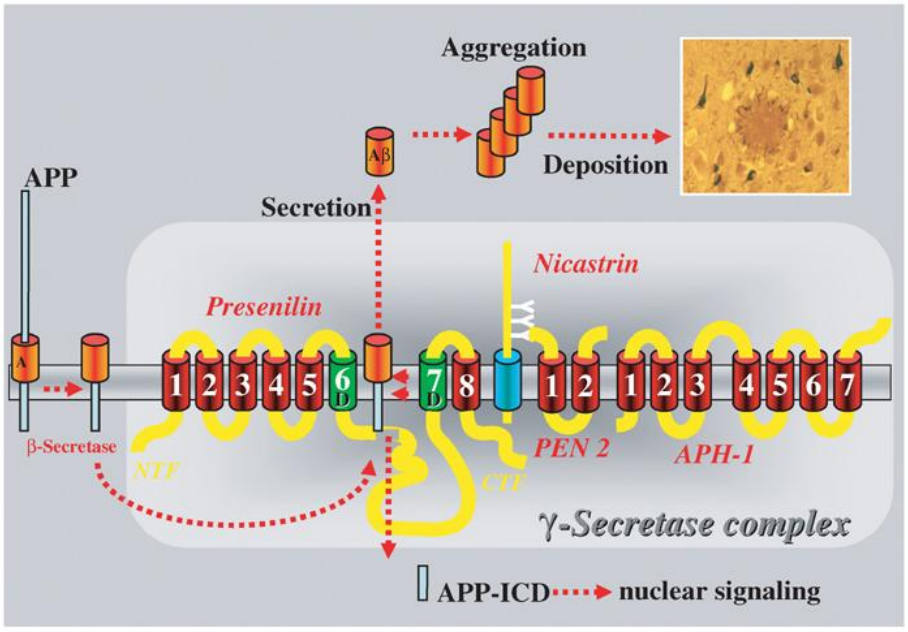


Figure 2: Generation of Aβ from APP via proteolytic processing by β- and γ-secretase (for details, see text). Aβ aggregates and finally precipitates in amyloid plaques. This event initiates the amyloid cascade resulting in additional intracellular aggregations of the tau protein, which then form tangles (the black structures surrounding the amyloid plaque).

1.2 Human cystatin c peptides in Alzheimer's disease

Alzheimer's disease (AD) and AD-related neurodegenerative disorders have become the predominant form of progressive cognitive failure in elderly humans, a development presently accelerating due to the significant increase in life expectancy in the last decades. Consequently, uncovering details of AD pathology has become of paramount importance. Major neuropathological features in AD brain are cortical atrophy, neuronal loss, regionspecific amyloid deposition, neuritic plaques and neurofibrillary tangles.[1,2] A major constituent of amyloid fibrils in brain of patients with AD and AD-related diseases, as well as in aged individuals without any neurological disorder is the β-amyloid polypeptide (Aβ). Aβ arises from a large precursor, the amyloid precursor protein (APP); [3,4] it is produced by normal cells and detected as a circulating peptide in plasma and cerebrospinal fluid (CSF) of healthy humans.[5,6] Although the physiological role of APP is not well understood, specific missense mutations confer autosomal dominant inheritance of AD (FAD) and have pointed out pathogenic,

proteolytic processing mechanism(s).6 The accumulation of Aβ, a 39-42 amino acid proteolytic fragment of APP, in neuritic AD plaques is thought to be causative for disease progression.5,6 The N-terminal sequence of Aβ(1-42) is part of the extracellular region of APP, while the major C-terminal Aβ sequence is contained within the transmembrane domain.

Despite the lack of details on degradation pathways and mechanism(s) of formation of Aβ-derived plaques, recent studies towards the development of immunisation methods for AD based on therapeutically active antibodies have yielded initial success in generating antibodies capable of disaggregating Aβ-plaques and reversing the memory impairments in transgenic AD mice. Both active immunisation with pre-aggregated Aβ(1-42) and administration of antibodies against Aβ significantly attenuated plaque deposition and neuritic dystrophy.[7,8,9] Thus, active or passive immunization of AD patients may emerge as a therapeutic approach targeting the production, clearance, and aggregation of Aβ.[10,11] The molecular antigen recognition of antibodies produced by immunisation with Aβ has been identified using selective proteolytic excision (epitope-excision) of antigen-antibody complexes in combination with high resolution mass spectrometry [12] , providing a specific N-terminal epitope, Aβ(4-10); the same epitope of plaque-specific antibodies has been identified in AD plaques, extracts from Aβ-protofibrils, and synthetic Aβ(1-42).[12,13] Recently, natural anti-Aβ- antibodies (Aβ-autoantibodies) have been identified in both blood and CSF of nonimmunized humans,[14] which specifically bind to human Aβ(1-40) as well as to Aβ in brain of transgenic mice and have been shown to reduce Aβ-fibrillation and neurotoxicity.[15-17] Aβ-autoantibodies have been also found in intravenous IgG preparations (IVIgG), and treatment of AD patients with IVIgG caused a reduction of Aβ concentrations in CSF15 and neuroprotective effect in inhibiting Aβ-plaque deposition.[16-18] A specific carboxy-terminal Aβ-epitope has been identified to be targeted by Aβ-autoantibodies using epitope excision mass spectrometry (M. Przybylski et al., *Abstr. 7th Austral. Pept. Sympos.* **2007**; Cairns, Oct. 21-25; 32), in contrast to Aβ-antibodies produced by active immunisation.[17,18]

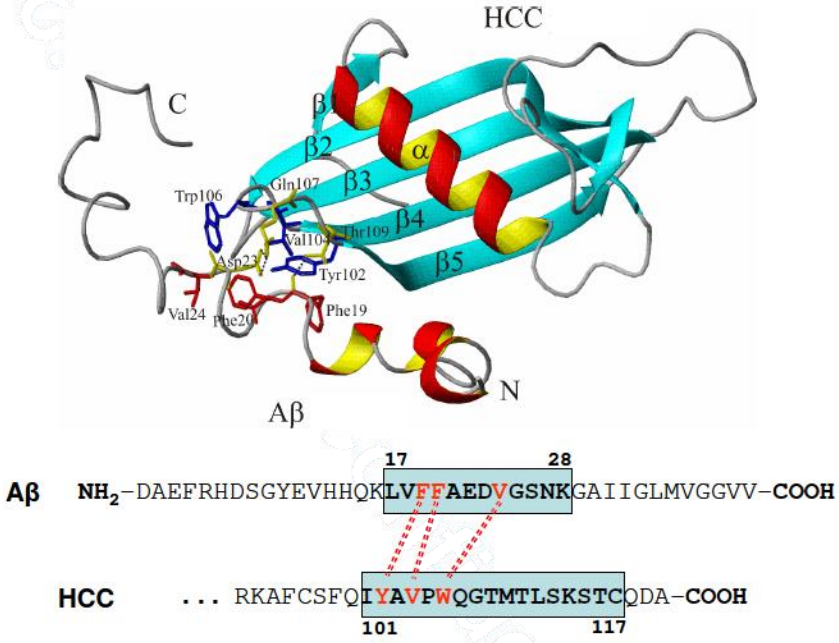


Figure 3: Interaction structure of the HCC-Aβ complex revealed by molecular dynamics simulation. Hydrophobic interactions between HCC and Aß, Tyr-102, Val-104 and Trp-106 residues of HCC are colored in blue, Phe-19, Phe-20, Val-24 residues of Aß are colored in red. Hydrogen bonds between Gln-107 and Thr-109 residues of HCC and Asp-23 and Phe-19 of Aβ are indicated by black dashed lines, respectively; amino acids and carbonyl groups (Phe-19) forming hydrogen bonds are colored in yellow.

Although amyloid plaques in brain of AD patients contain predominantly Aβ-aggregates, immunohistochemical studies have shown the co-deposition of several other proteins, such as the protease inhibitor cystatin C, apolipoprotein E, clusterin, transthyretin and gelsolin.[19-22] In particular, the presence of human cystatin C (HCC) in amyloid deposits has found much interest,[23,24] and a wide spectrum of activities has been associated with HCC such as modulation of neuropeptide activation and neurite proliferation.[25,26] The 13 kDa protein HCC is the main cysteine protease inhibitor in mammalian body fluids27,28 and has been found with high concentrations in CSF. While wild type cystatin C has no aggregation tendency, the naturally occurring mutant L68Q shows a high tendency to form amyloid fibrils, causing hereditary cerebral hemorrhage of the amyloidosis-Icelandic type.[20-23] The presence of HCC in Aβ-plaques has been suggested to result from its binding to APP, or alternatively, HCC may bind to Aβ prior to the secretion or following the deposition in brain.[23] Sastre et al. found that the association of HCC with Aβ causes an inhibition of fibril formation, and

suggested an N-terminal Aβ-sequence to be responsible for the interaction, with formation of a stoichiometric HCC-Aß complex.[25]

The recent results suggesting an important role of HCC in the processing and/or aggregation of Aβ prompted our interest in the molecular characterization of the Aβ-HCC interaction. Here we report the identification of the interacting epitopes of HCC and Aβ using selective proteolytic excision of the HCC-Aβ complex (epitope-excision) and mass spectrometry (MS).[18] The general analytical scheme of the epitope excision-MS approach is shown in Figure 1. Briefly, the immobilised ligand-binder (antigen-antibody) complex is subjected to specific, limited protease digestion followed by mass spectrometric analysis of the eluted affinity-bound epitope fragments. In the proteolytic step the ligand epitope is protected from digestion due to the shielding of the ligand-binder interaction, enabling subsequent specific dissociation and MS analysis of the bound epitope(s).18 In a variation of this approach (“epitope-extraction”) the ligand is first subjected to proteolytic digestion and the mixture of peptide fragments presented to the immobilised binder. Using both MS approaches the Aβ-epitope interacting with HCC was identified in a central, C-terminal Aβ sequence interfering with the Aβ-aggregation; an analogous proteolytic-MS approach with immobilised Aß provided the identification of a specific Aβ-binding epitope at the C-terminus of HCC, HCC(101-117). Structures and affinities of both the Aβ and HCC epitopes were characterised by ELISA, surface plasmon resonance (SPR), direct mass spectrometric analysis of HCC-Aβ-epitope peptide complexes, and a structure model of the HCC-Aβ complex obtained by molecular docking simulation. Furthermore, we show the functional activity of the identified synthetic HCC-epitope by analysis of its Aβ- fibril inhibitory effect *in vitro.*

1.3 Methods and instrumentation of biopolymer

Mass spectrometry (MS) is today one of the major analytical methods. The information gained from the analyses of the intact or fragmented molecular ions can be used for the determination of structure and composition of pure substances or mixtures.

The general build-up of mass spectrometers suitable for biopolymer analyses can be summarized in a simple scheme of their basic components: ion source, mass analyzer and detector. The ion source is the place of ion formation from sample. The mass analyzer is responsible for the separation of ions according to their m/z. The ion signals from the detector, combined with other information from the ion source or the analyzer are transferred for processing to the computer that delivers the mass spectra.

The mass spectrometry became widespsread in the biopolymer analyses only afer the introduction of soft ionization methods, two most used today being matrix-assisted laser desorption ionization (MALDI) and electrospray ionization (ESI). Their importance has been also recognized by the awarding of the 2002 Nobel Prize for Chemistry to John B. Fenn [1] and Koichi Tanaka. [2]

Matrix-assisted laser desorption ionization (MALDI) [3] is a pulsed method in which the sample under vacuum or at atmospheric pressure [10] is irradiatedwith a laser (usually nitrogen laser, wavelength 337 nm, pulse duration 3-4 ns). The analyte is embebbed in a so called matrix, which consists of crystals of small organic molecules that absorb strongly in the UV domain, lose to the wavelength of the laser. Their role is to protect the analyte molecules from the high energy of the laser beam and allow them to remain intact during the ionization. They must also be acidic, in order to provide a proton source for the sample ionization. A typical used matrix is α-cyano-4-hidroxycinnamic acid.

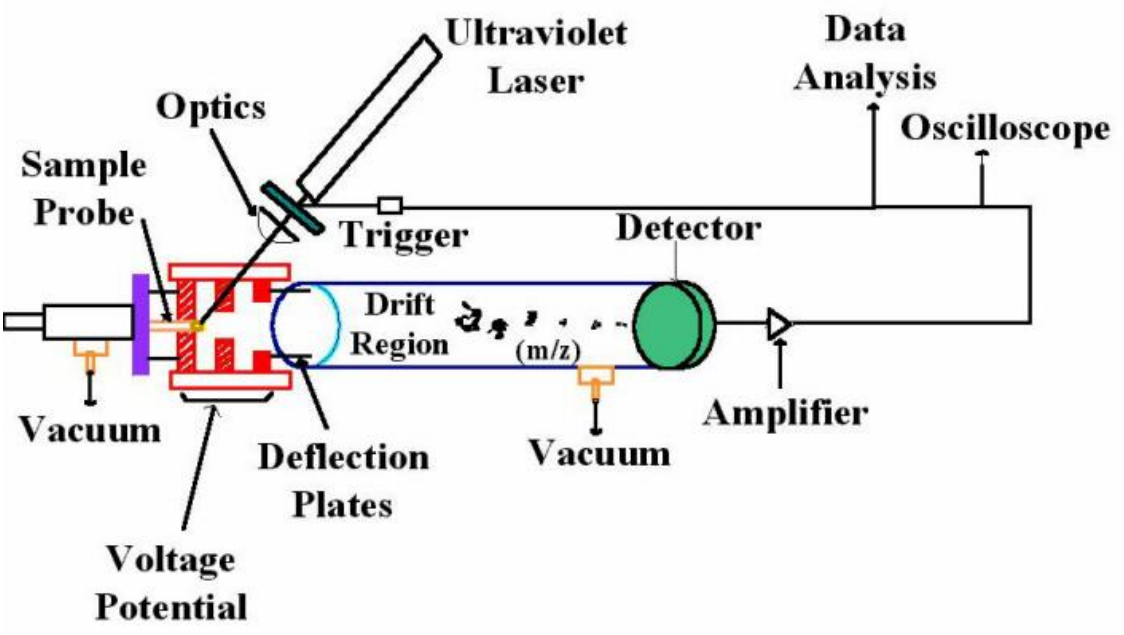


Figure 4: Matrix-assisted laser desorption ionization (MALDI).

Protonation and deprotonation is the main source of charging for biologically relevant ions in ESI. In fact, ions of proteins, peptides, oligonucleotides and other molecules with acid/base functionality are often found with several sites of protonation or deprotonation. The multi-charge ions are a typical characteristic for ESI, bein alsoa major advantage. The multiple peaks from the ESI mass spectrum make the measurements more accurate. The large number of charges on the ions allows the mass spectrometers with limited m/z ranges to analyze high molecular weight molecules. Aditionally to the ionization of very large molecules, ESI enables alo the study of non-covalent bio-macromolecular complexes [14]. But the most important feature of ESI is the ease with each it can be coupled with other instruments. Particularly the association with HPLC systems is a very successful practice. Generally, ESI may be coupled with any kind of other instrument that delivers a continuous flow of solution to be analysed. However, ESI has also two notable deficiencies: i) the continuous sparaying leads inevitable to wasdte of sample, as no mass spectrometer ca continuously analize ions; ii) the presence of salts in samples has a very negative impact on ESI formation. The second drawback can be overcome by first desalting the samples, this intermediate step being

rendered by different methods.

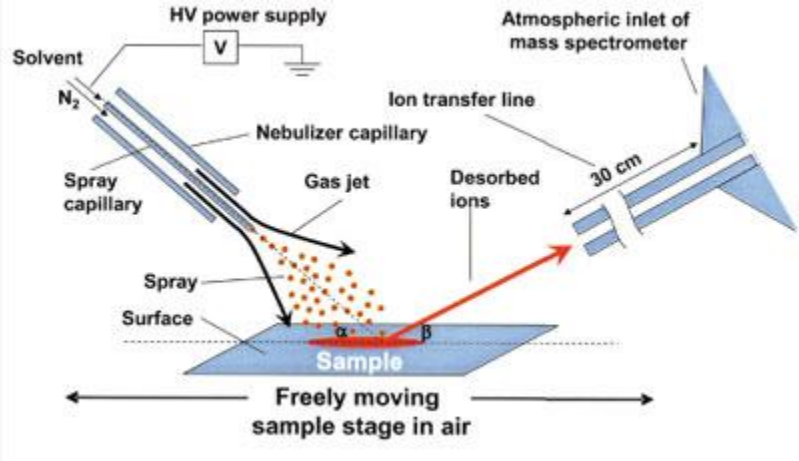


Figure 5: Electrospray ionization – principle of functioning: high positive potencial is applies to the capillary (anode), causing positive ions in solution to drift towards the exit, where the liquid surface is distorted, forming a cone ("Taylor" cone); from the tip of the cone is emitted a spary of droplets with an excess of positive charge; gas phase ions are formed from charged droplets in a series of solvent evaporation steps.

1.4 Introduction to affinity-mass spectrometry: Analytical methods for identification of ligand-binder interactions

Affinity binding refers in general to those binding interactions between complex molecules of biological nature that are non-covalent and originating in a multitude of different physical interactions, like dipole interaction, hydrogen bond or hydrophobic interaction. Although every single physical interaction involved is weak, the affinity binding is their specificity. This is explained through a highly specific structure with a certain special location of different sites of interaction, required in order that all the types of non-covalent bindings are formed. This simplistic description is a lock-and key model [22,23] of the affinity interaction.

Affinity bindings are of crucial importance in most of the biochemical interactions involved in a living organism. Therefore their study has been always a source of precious information for understanding the mechanisms of different biological

processes. Particularly the investigation of the different diseases at molecular levels, in search of a new drug, has stimulated the study of affinity bindings. A major class of biomolecules, the proteins, owe their biological role to the capability to affinity bind other molecules like other proteins, peptides, carbohydrates, lipids, metal ions, antibody to its antigen is very interesting through the potential of the antibodies to become highly specific drugs against different types of targets. The antigen region of binding to the antibody is called antigenic determinant or epitope. The analogue region from antibody that binds to the epitope is called paratope. In the case that the antigen is a peptide or a protein, the epitope consists of one or more short sequences of amino acids. When there is only a single sequence, the epitope is said to be linear (continuous). In case of more sequences, the epitope is discontinuous.

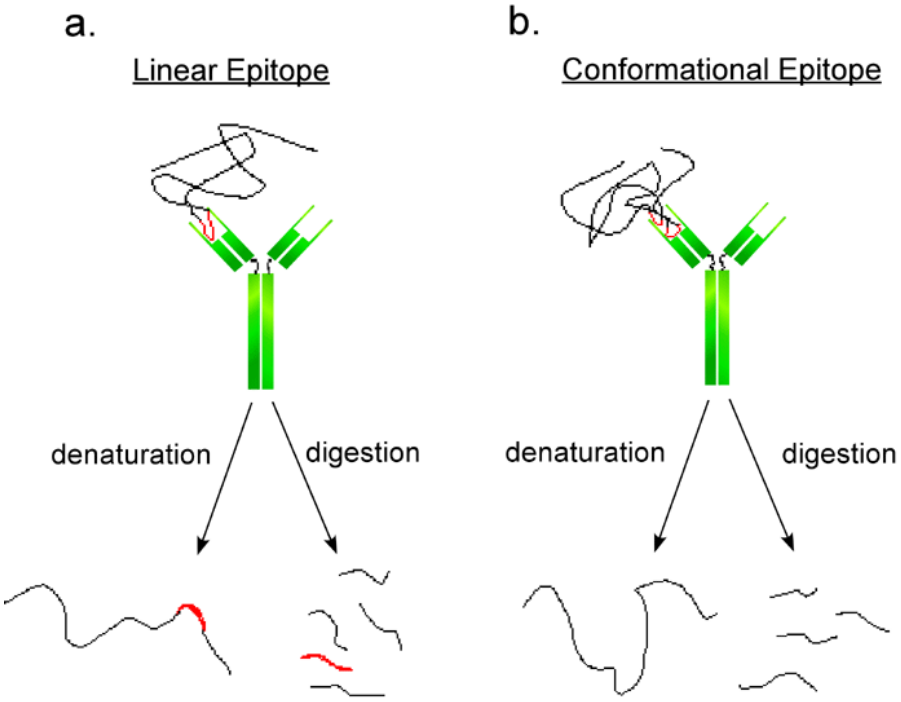


Figure 6: Schematic representation of the two types of epitopes found in proteins.

The identification and analysis of antigenic determinant is of crucial importance for the understanding of the binding between an antibody and an antigen, providing a starting point for the design of diagnostic tools or for the development of new vaccines [24,32]. There are several methods for the epitopoe identification, such alanine-sacanning

mutagenesis [25] or X-ray christallography [26].

A molecular approach for identification of epitopes from peptide and protein antigens is the mass spectrometric epitope excision or extraction [27, 28]. Beside the capacity of investigation at the molecular level, this method offers many other advantages, such as time consume, sample quantity and purity, and employment of solution sample in the work flow.

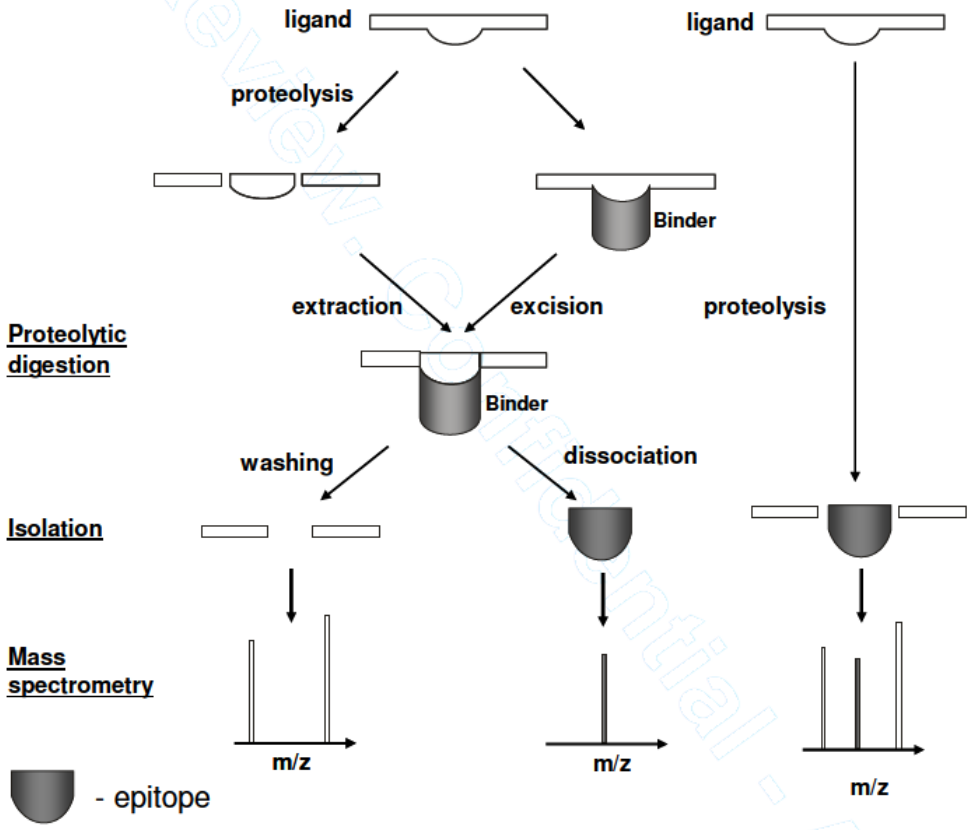


Figure 7: Schematic representation of mass spectrometric epitope excision and extraction procedures.

The principle of mass spectrometric epitope identification has been developed by our laboratory, and is based on the finding that the contact sides of the antigen are sterically protected by the antibody during the proteolyses [29]. In practice the antibody is covalently immobilized on a sepharose matrix and the antigen is allowed to bind to it. A specific protease digestion is then performed and the resulted fragments are eluted.

Bound to antibody remain only the epitope fragments, which are eluted under acidic conditions and then analyzed by mass spectrometry, for sequence identification (epitope excision). Alternatively, the digestion can be performed first and the mixture is added to the antibody micro-column. The epitope peptides can be separated through binding to the antibody, and then analyzed by mass spectrometry (epitope extraction). The discontinuous epitopes are more difficult to identify, their identification requires frequently a differential chemical modification [30, 62-65].

1.5 Affinity interaction analysis using a surface acoustic wave biosensor system

In the last three decades, the biosensors have been developed as a new and efficient tool of analytical chemistry. After Thévenot [33], “biosensors are chemical sensors in which the recognition system utilizes a biochemical mechanism”. Furthermore, he defines a chemical sensor as a combination of two elements: “a chemical (molecular) recognition system (receptor) and a psycho-chemical transducer”.

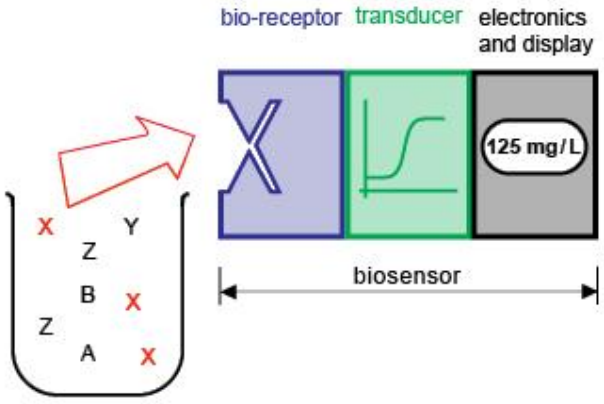


Figure 8: Schematic representation of the basic components of a biosensor: the biochemical sensitive element (bio-receptor) and the physical element responsible for signal generation (transducer).

Therefore, a biosensor has two major components: the biochemical sensitive element

and the physical element of signal generation. For the signal processing and results display a computer is usually used. Both major components are of various types and an exhaustive classification is not the purpose of this work. However, the physical elements of the biosensors are generally optical, electrochemical or electro-mechanical. Further, most of the electro-mechanical biosensors are based on the piezoelectric effect. The piezoelectric crystals can be made to vibrate at a specific frequency with the application of an electrical signal of a specific frequency. These oscillations are actually mechanical waves that travel through the bulk matter. Their frequency is dependent on the electrical frequency apllied to the crystal as well as the crystal's mass. Therefore, when the mass increases due to binding of chemicals, the oscillation frequency changes and the resulting change can be measured electrically and be used to determine the additional mass of the crystal. This is the function principle of a quartz crystal microbalance (QCM) [34]. If the oscillation is confined in a thin layer on the surface of the crystal, one speaks of surface acoustic waves (SAW) [35, 36]. Different types of acoustic waves can be employed in a SAW device, but Love waves [35] offer particularly high sensitivities due to the confinement of the acoustic energy to the sensing surface (Figure 8). The Love wave are in fact shear horizontally polarized guided waves [36], being especially suited for sensing liquids [37]. The recently introduced K% biosensor system uses surface acoustic wave of Love type [38].

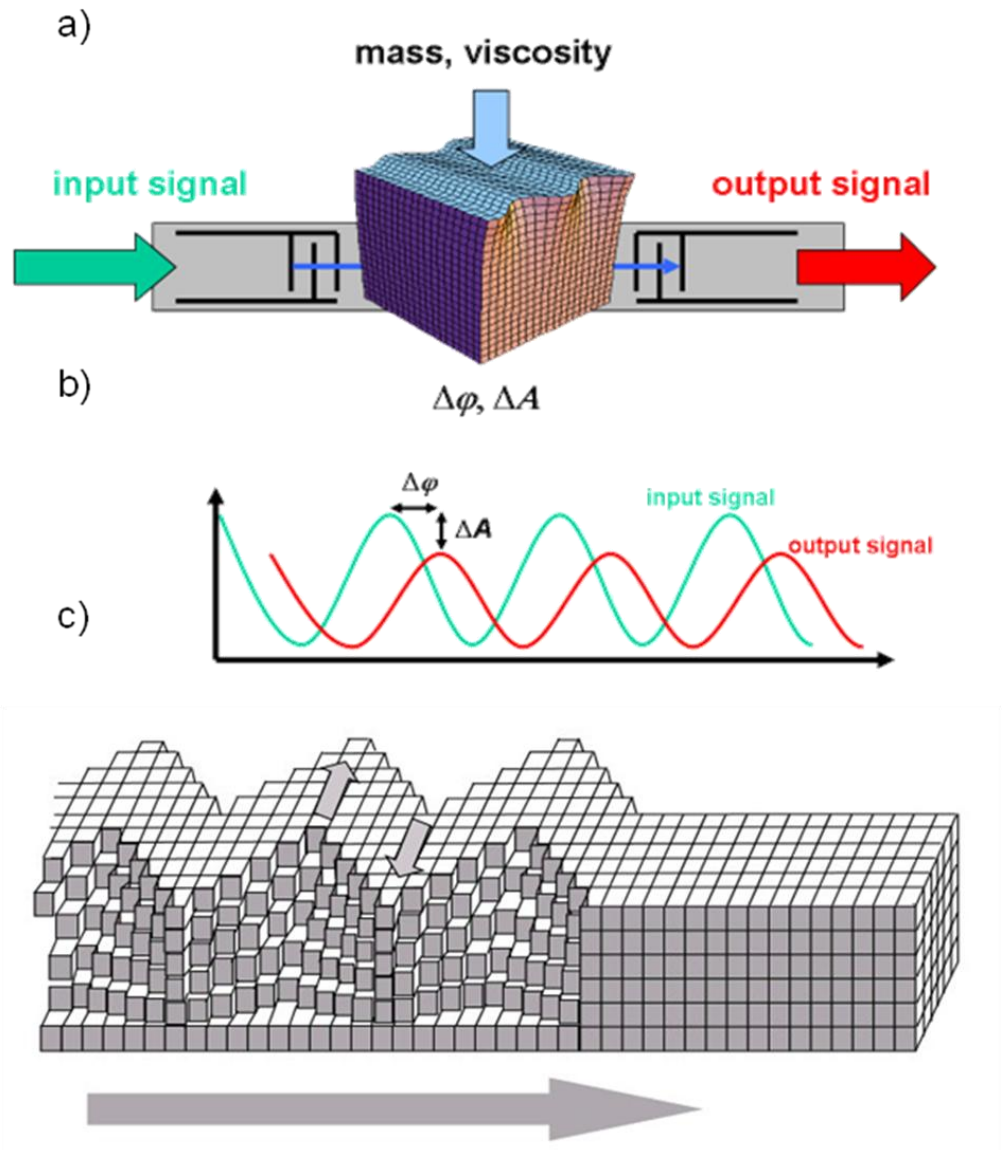


Figure 8: a) Functioning principle of the SAW sensor: an electrical signal is transformed in mechanical wave through piezoelectricity; the wave changes its amplitude and phase due to surface mass loading and liquid viscosity changes; the wave is then transformed back into electrical signal for processing; b) The input and output signals differ in phase and amplitude; mass loadings render phase shifts, while viscosity changes induce modification in both phase and shift; c) Love waves are horizontally polarized transversal waves.

The essential part of the instrument is the microstructured sensor-chip. This is a thin plate of quartz (the substrate) on which are deposited, by standard thin film deposition

and optical lithography processes, the contact pads for electronics and the interdigital transducers (IDTs) [38, 39].The split finger design is used in order to reduce distortions from reflections. Over the IDTs there is deposited another layer of amorphous SiO_2, with a 4.5 mm thickness. This is the “guiding layer” for the Love waves. On top of it the sensitive surface is covered with a thin (150 nm) film of Cr/Au alloy. On one single chip there are five sensor elements, which can be operated independently (Figure 9).

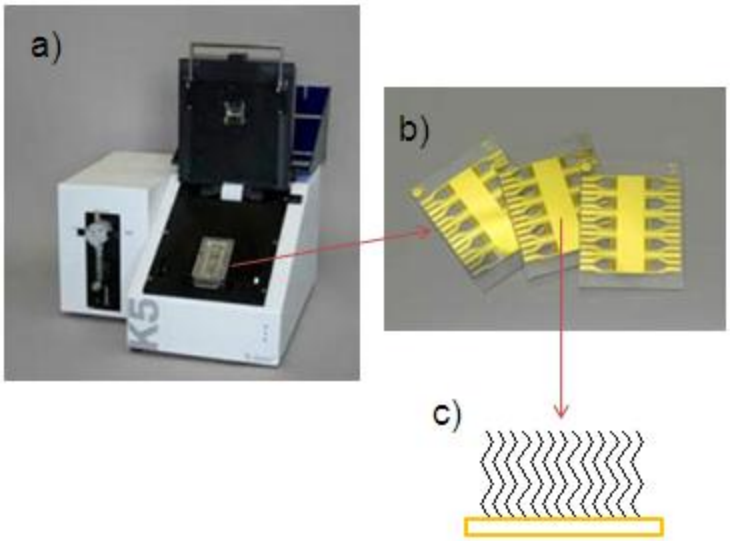


Figure 9: a) The K5 biosensor instrument, containing the electronics, the pump and the flow cell; b) the SiO_2 chip, covered with a layer of gold. There are visible the sensing surfaces of the five channels, the contacts for electronics and the regions of interdigital transducers (IDTs). c) self assembled monolayer (SAM) formation on he gold sensitive surface, used as a linker for the covalent immobilization of different other molecules.

Further, the chemical components used in analysis are physically absorbed on the gold surface, or are fixed through a covalent bound (or a linker) to the gold layer. The most convenient method is the covalent binding to gold surface. This can be easily achieved with organic molecules containing free thiol groups employing the well known chemical affinity of sulphur for gold. With bifunctional molecules like 16-mercaptohexadecanoic acid different other molecules, capable of forming bonds with the carboxyl group (amines, alcohols, etc.), can be attached to the gold-surface. When employing 16-mercaptohexadecanoic acid, or other similar molecules, as a linker, an important

process occurs on the gold surface: the saturated carbon chains align themselves parallel one to another due to the hydrophobic interactions. This is the so called self assembled monolayer (SAM) [40-43]. The carboxyl groups of the 16-mercaptohexadecanoic acid can be activated using 1-ethyl-3-(3-(dimethylamino)propyl) carbodiimide hydrochloride (EDC) and N-hydroxy-succinimide (NHS) [44].

1.6 Aims of the thesis

The aims of the present thesis are summarized as follows:

1. *Synthesis and purification* of amyloid β-peptides including Aβ (1-40) and Aβ (17-28), as well as of hCC-peptides, more specifically hCC (101-117) and hCC (93-120).

2. *Characterization of affinity binding between Aβ-peptides and hCC-peptides by Surface Acoustic Wave (SAW) and ELISA* to compare the resultes obtained from both methods.

3. *Determination of the dissociaton constant* (kd) of Aβ peptides (using SAW biosensor).

4. Study of the inhibition effect on the fibrillization process of Aβ observed in presence of hCC.

2. RESULTS AND DISCUSSION

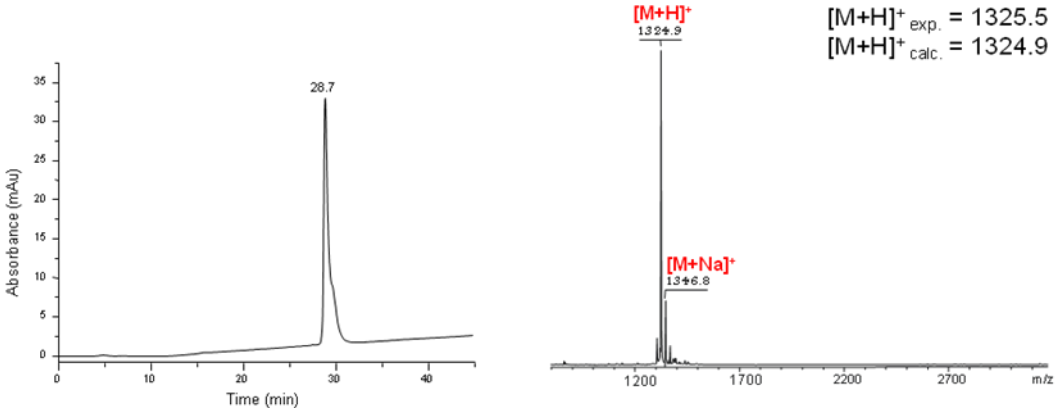
2.1 Synthesis and structural characterization of beta-amyloid and HCC peptides

The synthesis of the peptides employed in this study was performed on a NovaSyn TGR resin according to fluorenylmethyloxycarbonyl (Fmoc)/tert-Butyl (tBu) chemistry. Double coupling was used to reach an almost complete formation of amide bond for each amino acid. The protocol of the synthesis consisted of removal of Fmoc group with 20% piperidine in dimethylformamide (DMF) for 8 minutes, coupling of PyBOP/NMM-activated amino acid for 30 minutes and washing. DMF was employed as a solvent for all the reactions and it was also used for all washing steps. After the desired sequence was completed, the cleavage of the peptide from the resin was carried out with TFA, triethylslane and water for 3 hours at room temperature. The crude product was precipitated with t-butylmethylether, filtered, re-dissolved in 5% acetic acid and lyophilized. The crude product was purified by reversed phase high performance liquid chromatography (RP-HPLC) and analyzed by ESI ion trap MS. The amino acid sequences, the HPLC chromatograms and the mass spectra are shown in Table 1 and Figure 10.

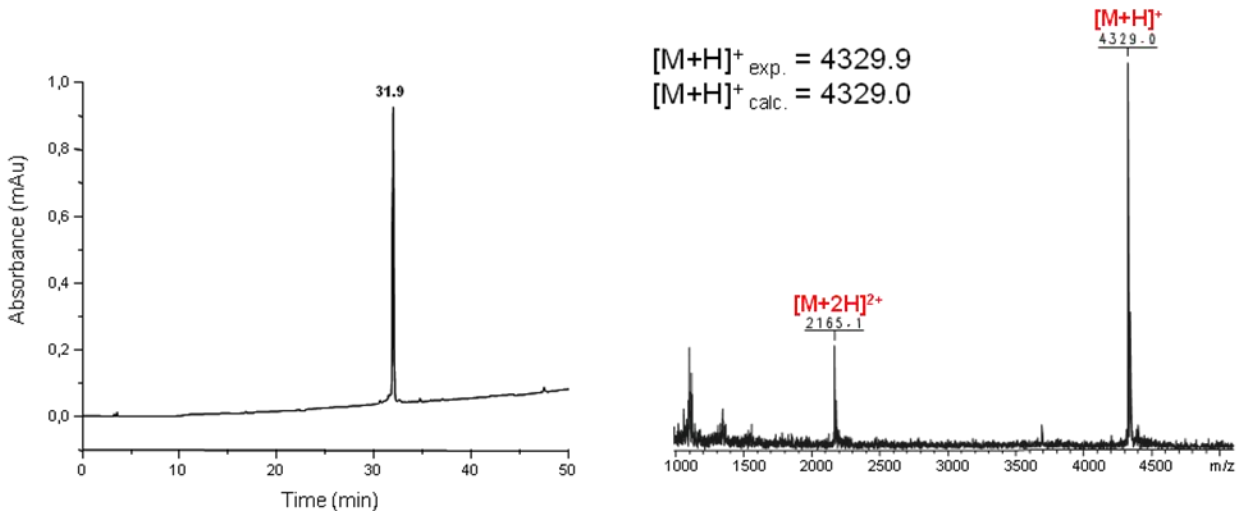
Table 1: Chemical characteristics of Aβ-peptides.

Peptide	Sequence	R_t (min) [a]	Purity (%) [b]	$[M+H]^+$ Theoh/Exp. [c]
Aß(1-40)	NH_2-DAEFRHDSGYEVHHQKLVFFAE DVGSNKGAIILMVGGVV-$CONH_2$	31.9	94	4329.9 / 4329.0
Aß(17-28)	NH_2-LVFFAEDVGSNK-$CONH_2$	28.7	90	1325.5 / 1324.9
HCC (101-117)	NH_2-IYAVPWQGTMTLSKSTC-$CONH_2$	25.3	96	1886.2 / 1884.7
HCC (93-120)	NH_2-RKAFCSFQIYAVPWQGTMTLSKS TCQDA-$CONH_2$	31.9	95	3168.7 / 3168.2

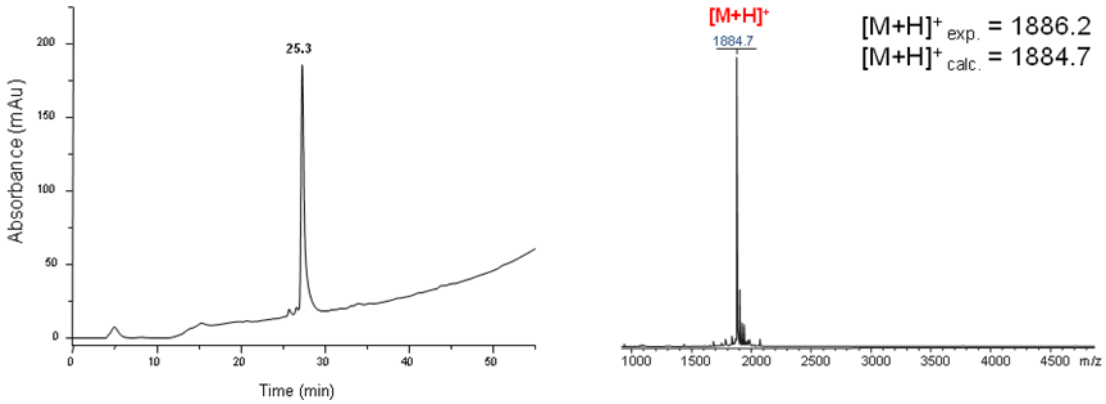
a.


28.7
Absorbance (mAu)
Time (min)
[M+H]+
1324.9
[M+H]+ exp. = 1325.5
[M+H]+ calc. = 1324.9
[M+Na]+
1346.8
m/z

b.


31.9
Absorbance (mAu)
Time (min)
[M+H]+ exp. = 4329.9
[M+H]+ calc. = 4329.0
[M+H]+
4329.0
[M+2H]2+
2165.1
m/z

c.


25.3
Absorbance (mAu)
Time (min)
[M+H]+
1884.7
[M+H]+ exp. = 1886.2
[M+H]+ calc. = 1884.7
m/z

d.

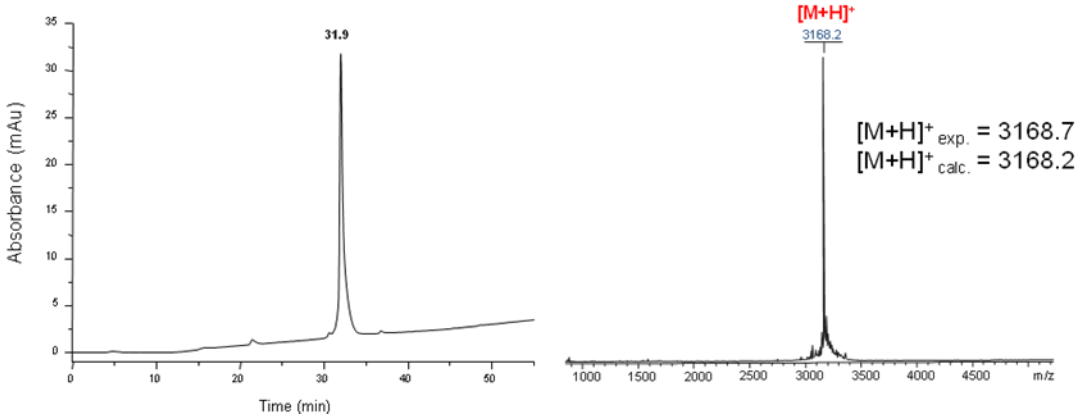


Figure 10: HPLC profile and the ESI ion trap mass spectrum of: ***a.*** *Aβ (17-28),* ***b.*** *Aβ (1-40),* ***c.*** *hCC (101-117),* ***d.*** *hCC (93-120)*

The investigation of the brain tissue from patients with Alzheimer's disease revealed the accumulation of Aβ peptides.

The protease inhibitor human cystatin C (HCC) co-associates with ß-amyloid (Aß) in Aß- fibrils in brain of Alzheimer's disease patients by a specific interaction, the molecular basis of which has been elucidated in the present study. The binding epitopes and interaction structure of HCC and Aß in the stoichiometric Aß-HCC complex have been identified using a combination of selective proteolytic excision and high resolution mass spectrometry; they encompass a central sequence of Aß that inhibits fibril formation, and is consistent with the structure of the Aß-HCC complex obtained by molecular docking simulation. The molecular characterisation of the Aß-HCC interaction provides a basis to derive new neuroprotective AD and HCC amyloidosis therapeutic lead structures and AD diagnostics.

To proceed to the interaction studies, the synthesis of both peptides was necessary. Aß (17-28) comprehends the epitope of the larger peptide (Aß(1-40)) and theoretically an higher affinity, but the last one is one of the most common in the AD patients brains. In this way. In hCC case, the epitope (hcc(101-117)) and the larger peptide are of interested to see which one will interact more with the Aβ peptides.

2.2 Mass spectrometric characterization of the HCC-Aß epitope peptide complex.

Further evidence for the specific interaction of the HCC- and Aβ- epitopes was obtained by direct high resolution ESI- mass spectrometry of the complexes of HCC- and Aβ-peptides. The nano ESI-FTICR mass spectrum of the peptide complex between the HCC(101-117) and the Aβ(17-28) epitopes is shown in Figure 11. The specific formation of a stoichiometric complex between the two minimal epitope peptides is ascertained by the triply and quadruply charged molecular ions (M+3H)3+ (m/z 1070,1408) and (M+4H)4+ of the complex which were determined with a relative mass accuracy of approximately 5 ppm. Likewise, the complex between HCC(93-120) and Aβ(17-28) yielded molecular ions (M+3H)3+ (m/z 1497,6012) and (M+4H)4+ (m/z 1123,4403) (_m 6.5 ppm; not shown). Thus, the ESI- MS analysis of the peptide complex ascertained the specific interaction of the C-terminal HCC epitope with the Aβ epitope located in the middle- to C- terminal domain.

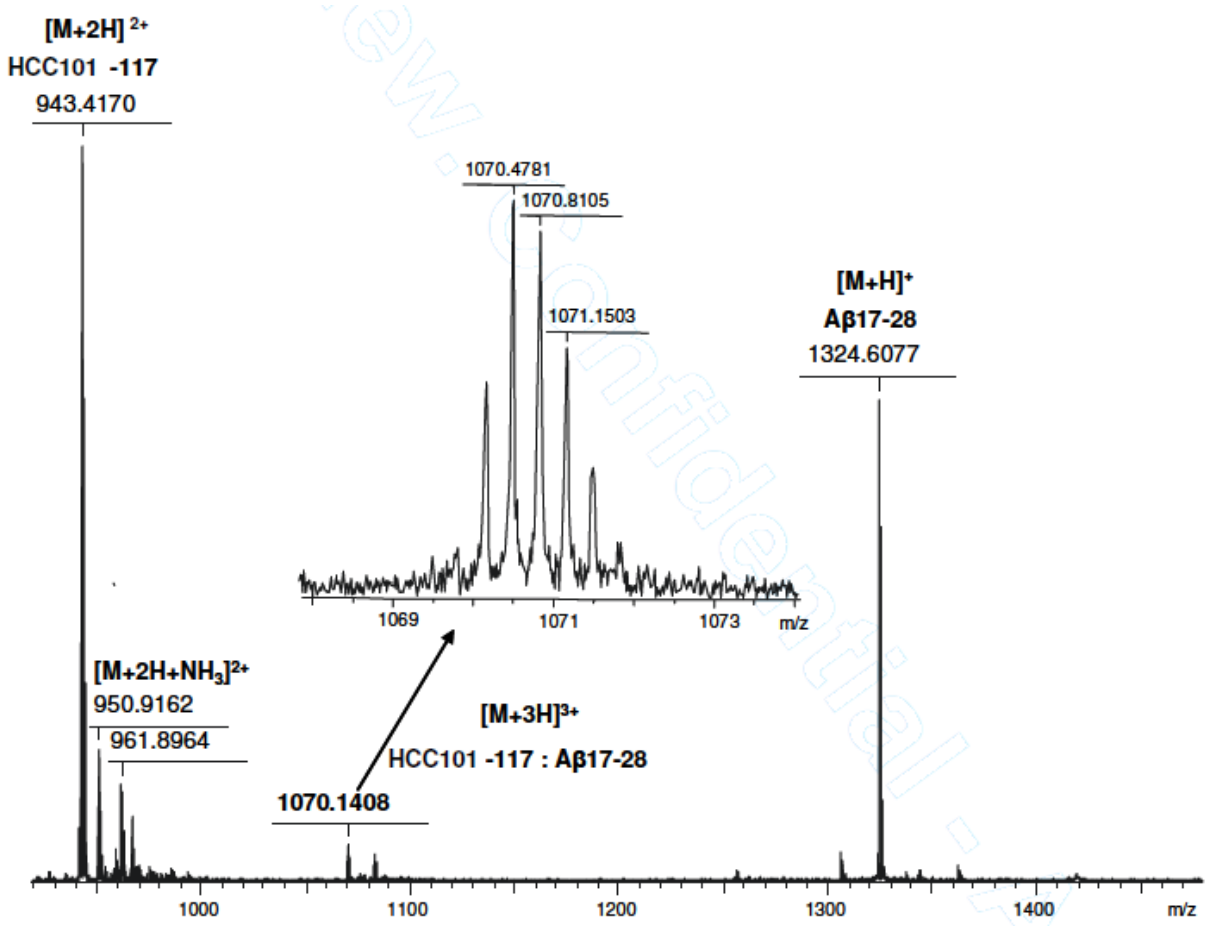


Figure 11: Nano ESI-FTICR mass spectrum of the peptide complex between the HCC(101-117) and the Aβ(17-28) epitopes.

2.3 Affinity interaction hCC and Aβ-peptides system by ELISA

The affinities interactions between the hCC-peptides and Aβ-peptides were investigated by ELISA.

The Enzyme-Linked ImmunoSorbent Assay (ELISA) experiment was performed by coating the 96-well microtiter plate with 12 serial dilutions of peptides. The unspecific binding sites were blocked with bovine serum albumin (BSA) before adding the specific antibody with a dilution 1:1000. The systems investigated were …. For the detection was used anti-mouse antibody conjugated with horseradish peroxydase (HRP), diluted

1:5000.

In Figure 12 the optical density (OD) at λ 450 nm is plotted versus antigen concentration.

The specificities and affinities of the epitope interactions were analysed by ELISA of the HCC and Aß peptides in an analogous manner as employed for antibody binding to an antigen-coated plate. A suitable ELISA approach for the Aβ-HCC complex was developed to determine the affinities of Aβ peptides to HCC, by absorption of HCC on the ELISA plate and binding the N-terminally biotinylated Aß- peptides via a spacer peptide (Biotin-(Gly)5- Aβ peptides), using of an antibiotin detection antibody. No anti-HCC or anti-Aß antibody was used to exclude a possible interference of the antibody epitope with the binding sites of the HCC-Aβ complex. Biotinylated peptide derivatives of full length Aβ(1-40), Aβ(12-40), Aβ(17-28) and Aβ(1-16) were tested in a comparative ELISA experiment (Figure 12a). All Aβ peptides comprising the HCC-binding epitope showed binding affinity to HCC, with Aβ(17-28) having the highest affinity, while the N-terminal peptide Aβ(1-16) did not show any binding. Thus, the ELISA results were in complete agreement with the mass spectrometric epitope identification.

Further comparative ELISA studies with the Aβ(17-28) epitope were performed using the C-terminal HCC peptides identified by epitope excision-mass spectrometry, by coating the ELISA plate with intact HCC and the HCC(93-120), HCC(101-117), and HCC(101-114) fragments. The results confirmed the C-terminal HCC epitope, with the carboxy-terminal sequence being essential for binding affinity to Aβ (Figure 12b).

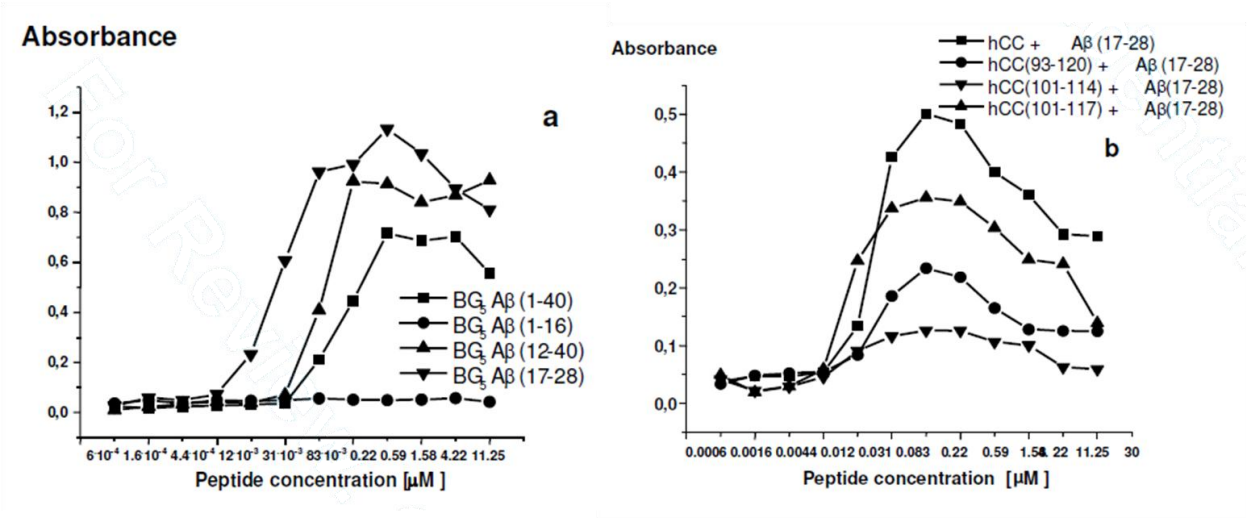


Figure 12: Antigen-antibody affinity binding for Aβ peptides.

2.4 SAW bioaffinity studies of β-amyloid and hCC peptides

The affinity interaction of the peptides was investigated using the SAW biosensor. The antibodies were immobilized on the active gold surface of the active gold surface of the biosensor chip and their interaction with peptides in dissolved in the liquid phase was observed. The studies show that the antibodies recognize β-amyloid peptides in concentration ranges from 1.22 nM to 2.5 μM (table 2). These concentrations represent the minimum and the maximum limits of detection. The purpose was to demonstrate that the affinity between the antibody and peptides is detectable with the SAW biosensor, having the first peptide covalently immobilized and the second peptide free in the aqueous phase, but also the determination of the dissociation constant of the peptides involved. These limits of detection are needed to be determined in order to be possible the determination of the dissociation constant. For this determination is also necessary the existence of a minimum of 8 different concentration, being used in the present work 12 different concentrations between 1.22 nM and 2.5 μM.

For the affinity component of the study, the mass loading was the value found.

Table 2: Mass loading after affinity injection (Aβ peptides)

	hCC (101-117)			hCC (93-120)		
C [M]	Aβ [1-40] [µg cm^{-2}]	Aβ [12-40] [µg cm^{-2}]	Aβ [17-28] [µg cm^{-2}]	Aβ [1-40] [µg cm^{-2}]	Aβ [12-40] [µg cm^{-2}]	Aβ [17-28] [µg cm^{-2}]
1.22 10^{-9}	-	0.0002751	-	0.0000005	0.0001254	0.0000075
2.44 10^{-9}	-	0.0003112	-	0.0000636	0.0001517	0.0000251
4.88 10^{-9}	-	0.0003224	-	0.0001009	0.0001942	0.0001690
9.7 10^{-9}	-	0.0003368	-	0.0002598	0.000206	0.0001691
19.5 10^{-9}	-	0.0003665	0.0000059	0.0002601	0.000207	0.0001863
39 10^{-9}	0.00002636	0.0004354	0.0000486	0.0002599	0.0002607	0.0004583
78 10^{-9}	0.00009711	0.0004809	0.0000832	0.0002598	0.0002923	0.0004584
150 10^{-9}	0.00015905	0.0005246	0.0000911	0.000413	0.0003183	0.0005295
300 10^{-9}	0.00028039	0.0005526	0.0001082	0.0005139	0.0003459	0.0006444
625 10^{-9}	0.00032153	0.0005850	0.0001310	0.0005824	0.0003652	0.0007659
1.25 10^{-6}	0.00044227	0.0006069	0.0001809	0.0005821	0.0003826	0.0009231
2.5 10^{-6}	0.00050948	0.0007108	0.0003891	0.0008564	0.0006333	0.0009622

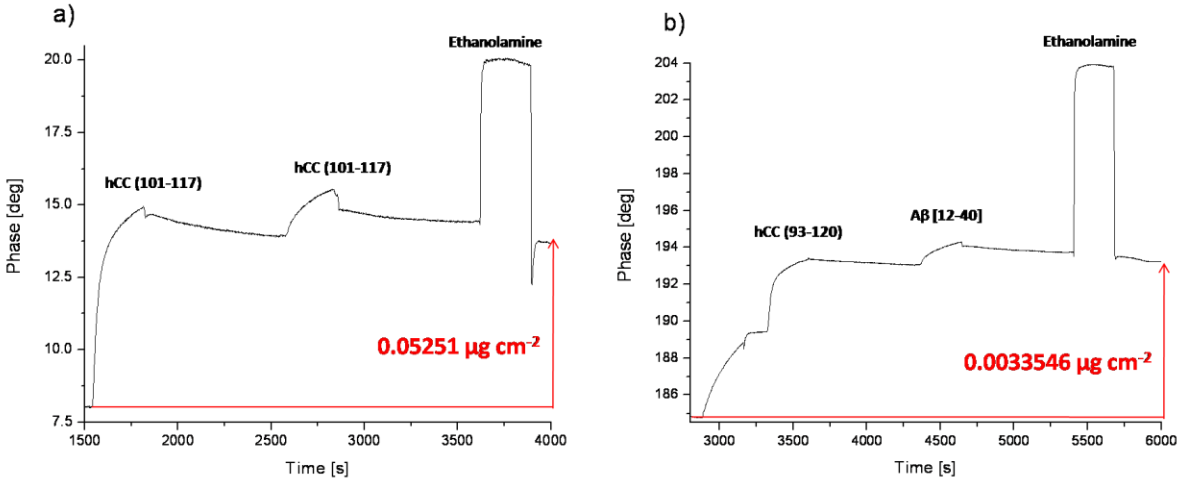


Figure 17: SAW binding curves referring to the immobilization of hCC a) (101-117) and b) (93-120) on the surface of the chip.

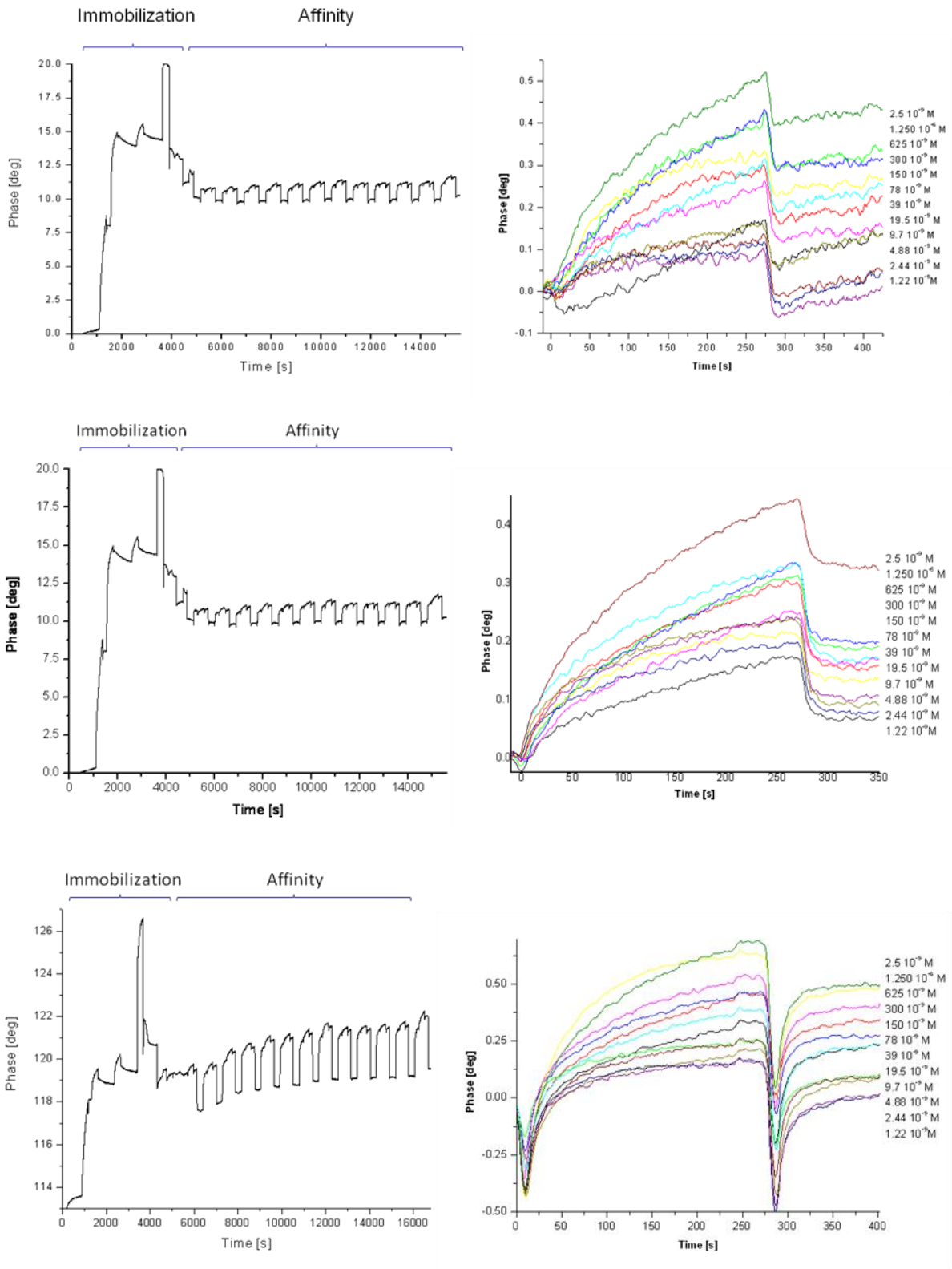
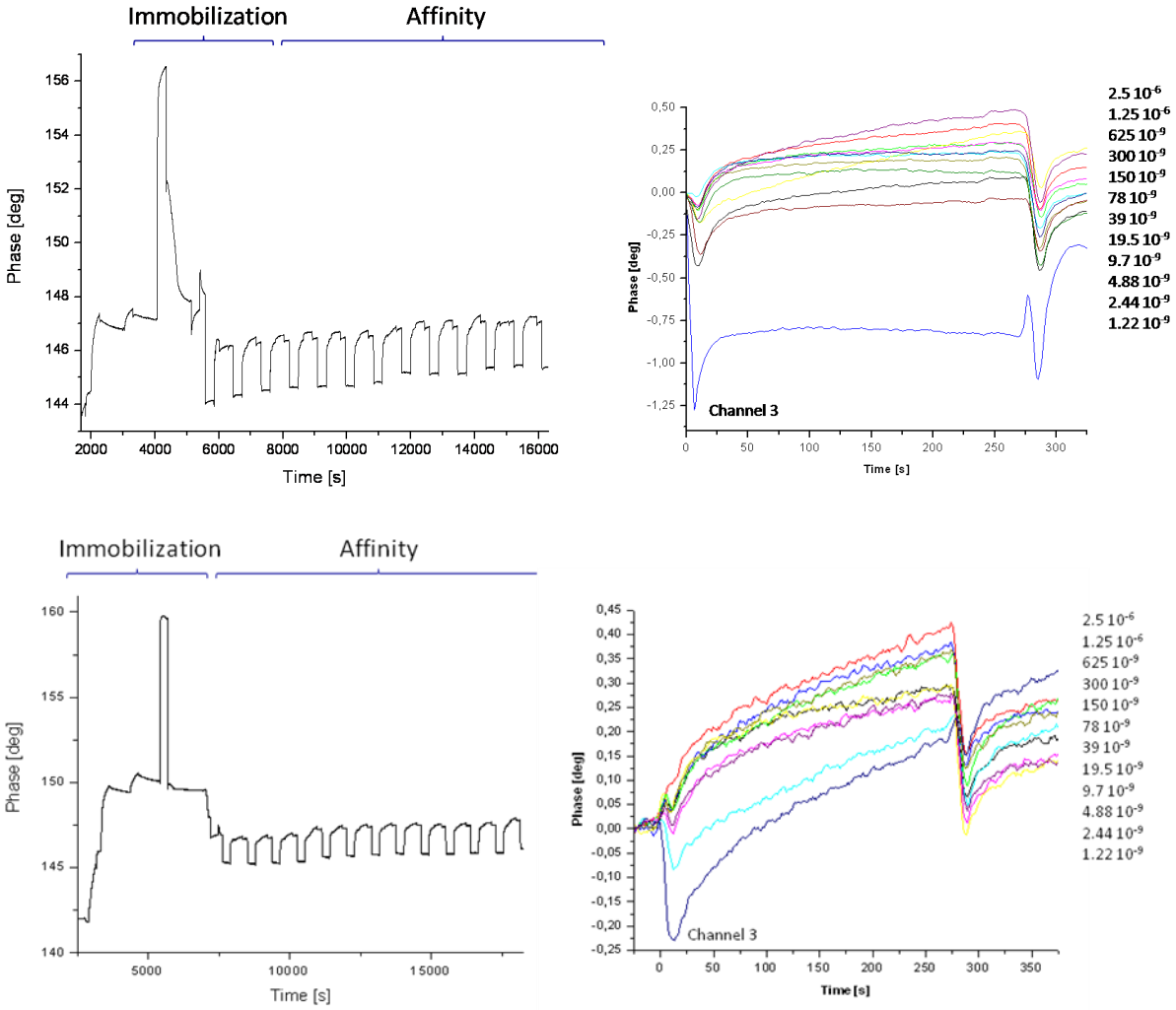


Figure 18: SAW binding curves referring to the: on the left the complete spectra and on the right the

affinity component with all the injections overlapped. The immobilization was performed with HCC (101-117) and then, in this order, the affinity with Aβ (1-40), Aβ (12-40) and Aβ (17-28).



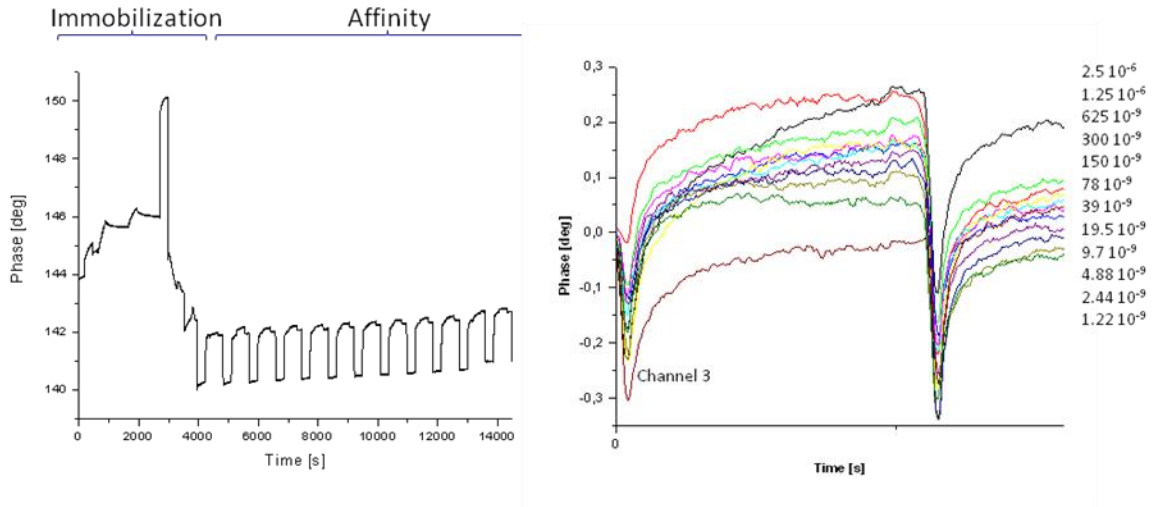


Figure 19: SAW binding curves referring to the: on the left the complete spectra and on the right the affinity component with all the injections overlapped. The immobilization was performed with HCC (93-120) and then, in this order, the affinity with Aβ (1-40), Aβ (12-40) and Aβ (17-28).

2.5 Determination of dissociation constant of Aβ and hCC peptides by SAW

A dissociation constant (Kd) is a specific type of equilibrium constant that measures the propensity of a larger object to separate (dissociate) reversibly into smaller components, as when a complex falls apart into its component molecules, or when a saltsplits up into its component ions. The dissociation constant is the inverse of the association constant.

For a general reaction:

$$\mathrm{A}_x\mathrm{B}_y \rightleftharpoons x\mathrm{A} + y\mathrm{B}$$

in which a complex A*x*B*y* breaks down into *x* A subunits and *y* B subunits, the dissociation constant is defined

$$K_d = \frac{[A]^x \times [B]^y}{[A_x B_y]}$$

where [A], [B], and [AxBy] are the concentrations of A, B, and the complex AxBy, respectively.

The dissociation constant is commonly used to describe the affinity between a ligand (L) (such as a drug) and a protein (P) i.e. how tightly a ligand binds to a particular protein. Ligand-protein affinities are influenced by non-covalent intermolecular interactions between the two molecules such ashydrogen bonding, electrostatic interactions , hydrophobic and Van der Waals forces. They can also be affected by high concentrations of other macromolecules, which causes macromolecular crowding.[1][2]

The formation of a ligand-protein complex (C) can be described by a two-state process

$$\mathrm{C} \rightleftharpoons \mathrm{P} + \mathrm{L}$$

the corresponding dissociation constant is defined

$$K_d = \frac{[\mathrm{P}]\,[\mathrm{L}]}{[\mathrm{C}]}$$

where [P], [L] and [C] represent the concentrations of the protein, ligand and complex, respectively.

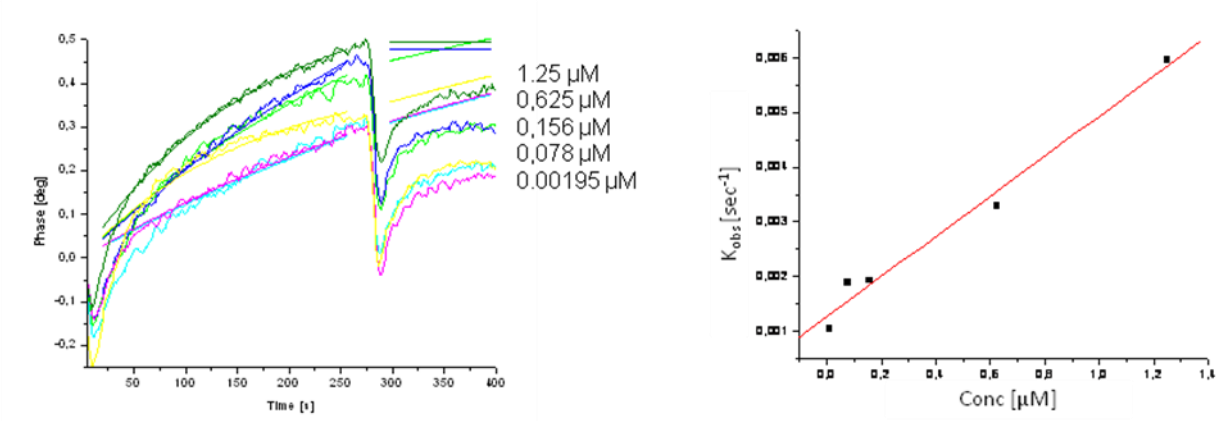
The dissociation constant has molar units (M), which correspond to the concentration of ligand [L] at which the binding site on a particular protein is half occupied, i.e. the concentration of ligand, at which the concentration of protein with ligand bound [C], equals the concentration of protein with no ligand bound [P]. The smaller the dissociation constant, the more tightly bound the ligand is, or the higher the affinity between ligand and protein. For example, a ligand with a nanomolar (nM) dissociation constant binds more tightly to a particular protein than a ligand with a micromolar (μM) dissociation constant.

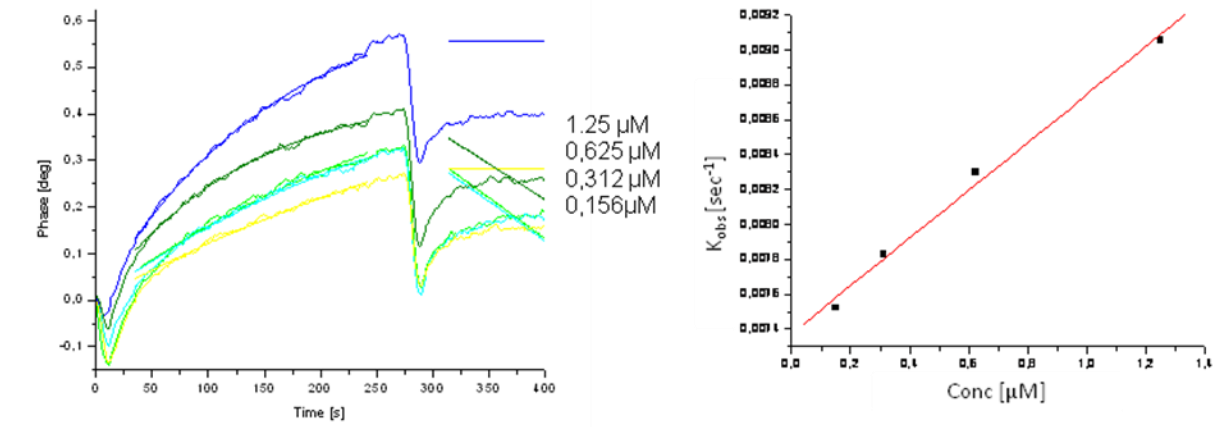
The dissociation constant for a particular ligand-protein interaction can change significantly with solution conditions (e.g. temperature, pH and salt concentration). The effect of different solution conditions is to effectively modify the strength of any intermolecular interactions holding a particular ligand-protein complex together.

Knowing this, it is possible to determine the dissociation constants by using all the data resulting from the 12 different concentrations and the 5 channels that constitute the chip. The values presented result from the average of these 5 channels.

Table 3: Dissociation constants determined using SAW Biosensor.

	$KD_{(Ab\ 1\text{-}40)}$ [µM]	$KD_{(Ab\ 12\text{-}40)}$ [µM]	$KD_{(17\text{-}28)}$ [µM]
hCC [101-117]	1,366	1,045	0,228
hCC [93-120]	0,0652	2,04	1,77





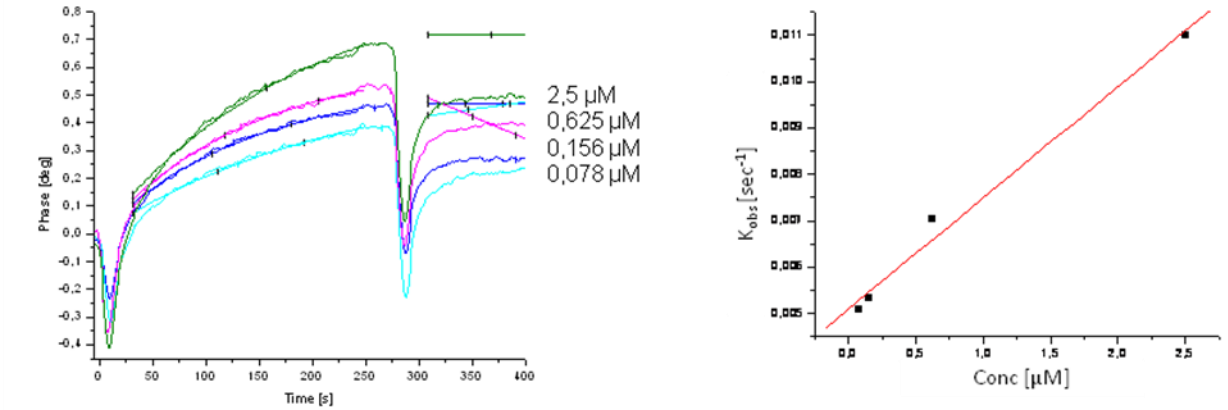
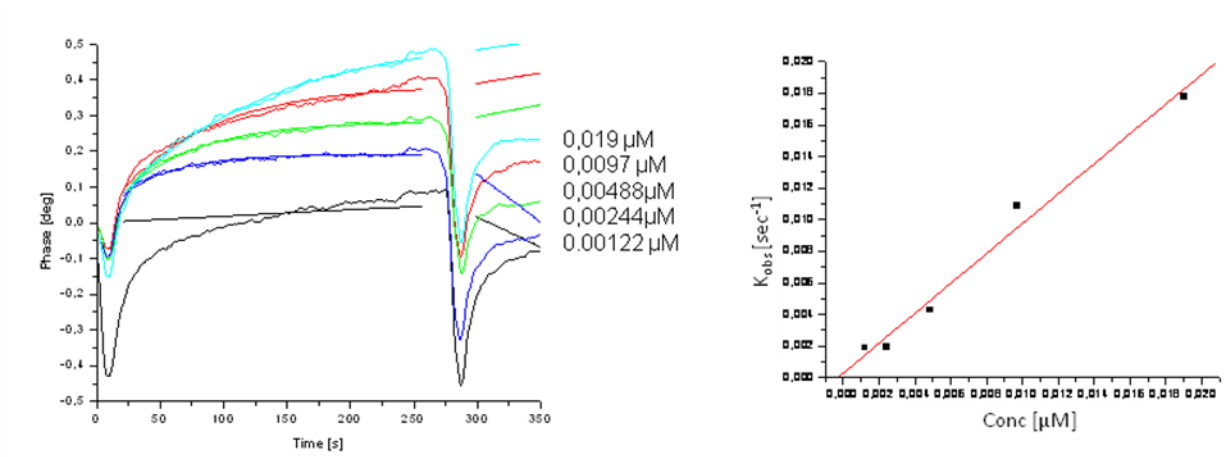


Figure 20: SAW binding curves referring to the: on the left the affinity spectra with the fiting parameters of the best values found among the different 1 concentrations used in the beginning, and on the right the linear regression that give the Kobs value which is used to determine the dissociation constant using the equations previously given. In this order, hCC (101-117) with Aβ (1-40), Aβ (12-40) and Aβ (17-28).



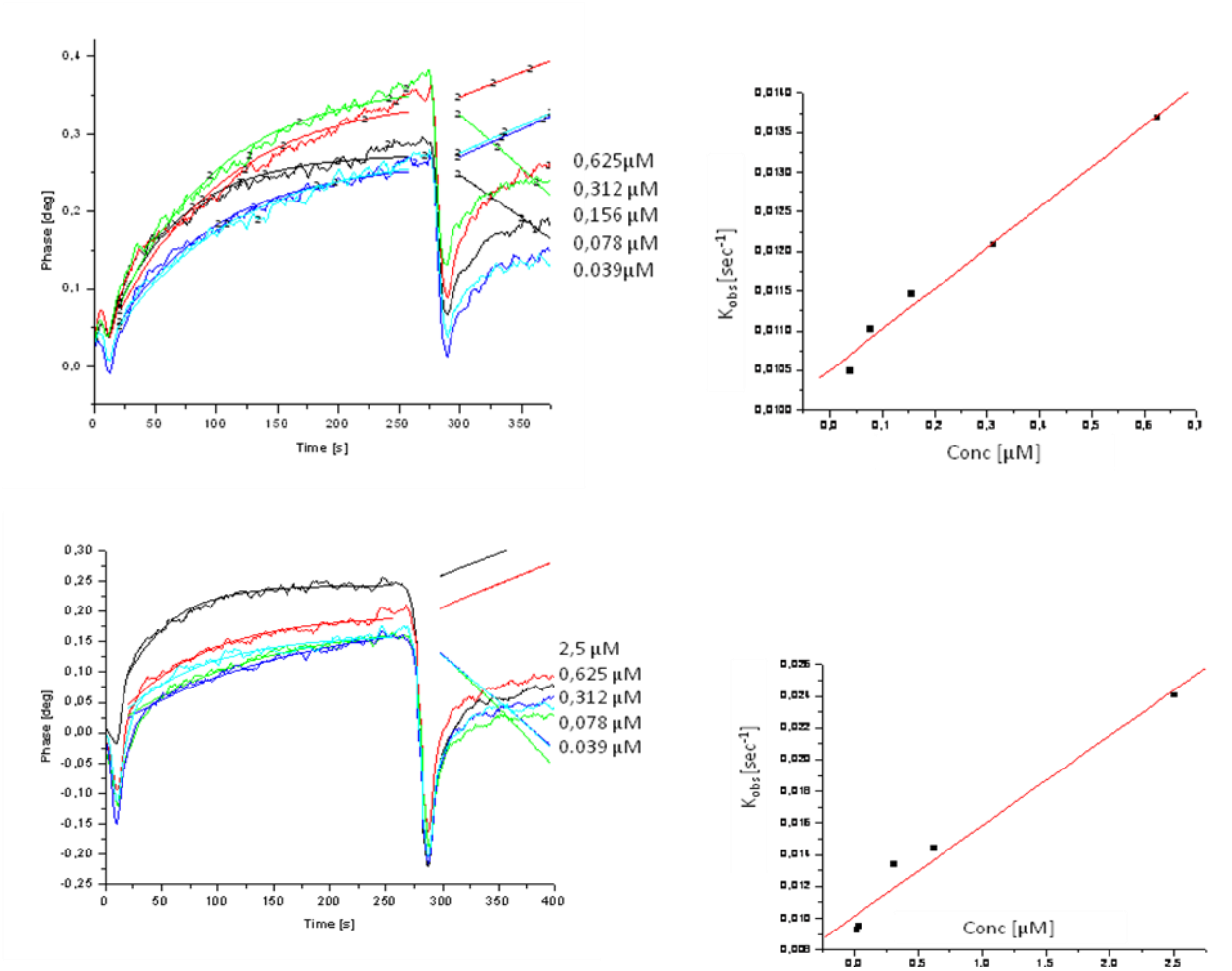


Figure 21: SAW binding curves referring to the: on the left the affinity spectra with the fiting parameters of the best values found among the different 1 concentrations used in the beginning, and on the right the linear regression that give the Kobs value which is used to determine the dissociation constant using the equations previously given. In this order, hCC (93-120) with Aβ (1-40), Aβ (12-40) and Aβ (17-28).

The values of kd found are in agreement with the results obtained with the ELISA procedure and with some data already available in the literature. These data was obtained using a Biacore T100 instrument (Biacore SA, Uppsala, Sweden) with a KD of 3.9 µM for hCC (93-120) in affinity to Biotin-G5-Aβ(1-40), which is a positive reference point.

2.6 Inhibition of Aß-fibril formation by C-terminal HCC epitopes.

Inhibition studies of Aβ-oligomerisation and fibril formation were performed with intact HCC in comparison with the C-terminal HCC epitope as shown in Table 2, using an *in vitro* assay of Aβ- oligomerisation. [25] For formation of Aβ-oligomers, Aβ(1-40) was first dissolved in 100 % TFE to ensure re-formation of monomers, and the sample then lyophilized and redissolved in DMSO. The samples were then diluted to a final peptide concentration of 100 *µ*M in 50 mM phosphate buffer, pH 7.4, and a 10 % final DMSO, and Aβ(1-40) with and without HCCepitope peptides incubated at 37 °C for up to 76 hours. Prior to analysis by SDS-PAGE, the samples were centrifuged at 13,000 x g for 5 min and then lyophilized. These comparative analyses clearly showed a time-and concentration-dependant, inhibitory effect of the HCC epitope peptides on the formation of Aβ-oligomer aggregates, with highest relative effect obtained for the HCC (101-117)-peptide (Table 4). Thioflavine T (ThT) is a dye common used to visualize the amyloid fibrils. For the free dye excitation and emission occur at 385nm and 445 nm; for the bounded – excitation and emission occur at 450nm and 482nm (Figure 22).

Table 4: Relative inhibition (inhibition of oligomer bands) of Aß- fibril formation by HCC-epitope peptides after 72 hrs.

HCC-Peptide / Aβ peptide %	HCC-epitope (µM)	% Fibril inhibition
HCC(93-120) / Aβ(1-40)	10	10
HCC(93-120)/ Aβ(1-40)		
HCC(101-117)/ Aβ(17-28)	10	12
HCC(101-117)/ Aβ(17-28)	100	51

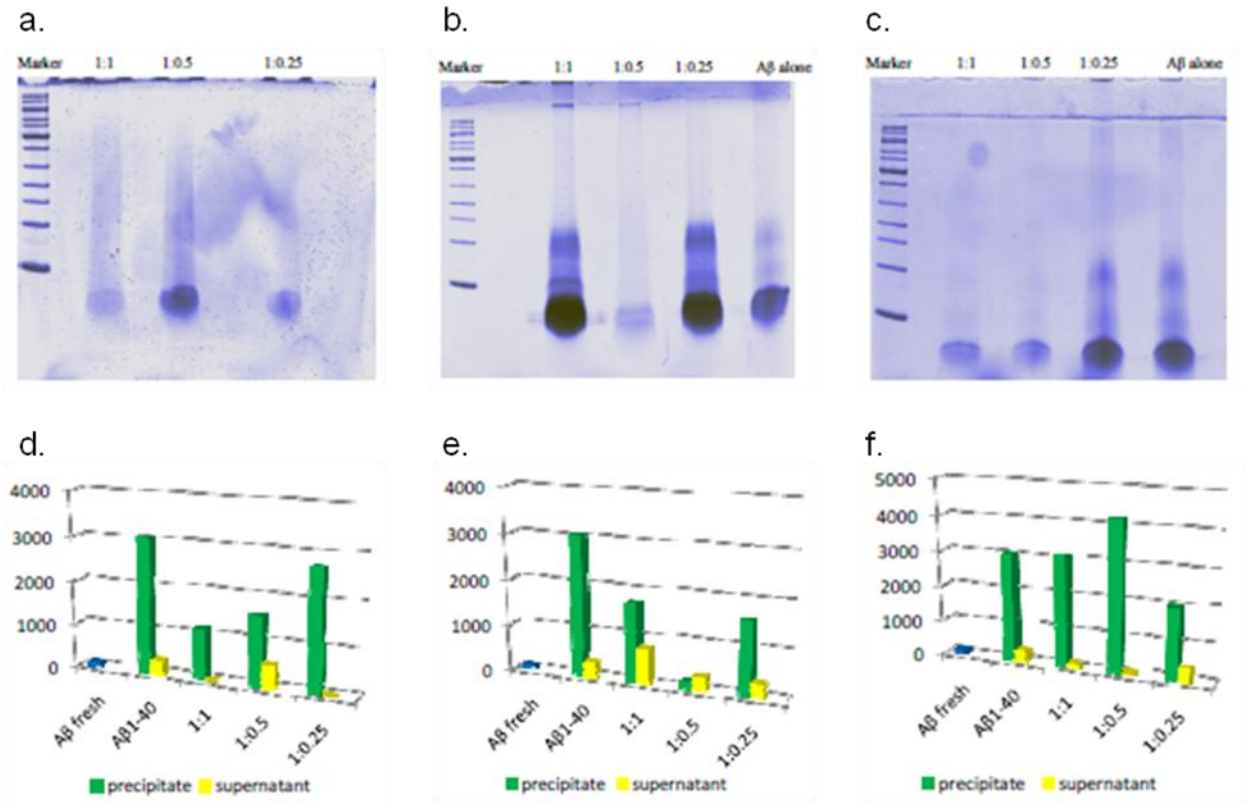


Figure 22: Relative inhibition (inhibition of oligomer bands) of Aß- fibril formation by HCC-epitope peptides after 72 hrs, with the respective information from the thioflavine assay. a. and d. as the combination of Aβ (1-40) with hCC (93-120), b. and e. as the combination of Aβ (1-40) with hCC (101-117); and c. and f. as the combination of Aβ (17-28) with hCC (101-117).

The better experimental results are observed for Thiflavin T test compering to gel electrophoresis. Aβ (1-40) with hCC (93-120) in 1:1, 1:0.5 and 1:0.25 molar ratios shows clearly an inhibition process and it is dependent of the hCC concentration – the best inhibition process was observed in the molar ratio 1:1. Also Aβ (1-40) with hCC (101-117) in 1:1, 1:0.5 and 1:0.25 molar ratio shows inhibitory effect but we cannot see the dependence on the hCC concentration. For Aβ (17-28) with hCC (101-117) in 1:1, 1:0.5 and 1:0.25 molar ratio the Tris-PAGE shows clearly the inhibition dependence on the molar ratio.

2.7 Mass spectrometry.

MALDI-TOF MS analyses were carried out on a Bruker (Bruker Daltonics, Germany) Biflex ITM linear TOF mass spectrometer equipped with a nitrogen UV laser (λ = 337 nm) and a dual channel plate detector, at conditions as previously described.17 Samples were dissolved in 0.1 % trifluoroacetic acid and desalted using the Zip-Tip procedure. High resolution FTICR- mass spectrometry was performed with a Bruker (Bruker Daltonics, Bremen, Germany) APEX II FTICR instrument equipped with an actively shielded 7T superconducting magnet, a cylindrical infinity ICR analyzer cell, and an external Bruker Apollo-II nano-electrospray ion source.40 Sample preparation of HCC- Aβ-epitope complexes was carried out with 10 µL aliquots of freshly prepared solutions of Aβ(17-28) (100 µM; Mr 1323.68) in deionised water, which were added to 10 µL of an equimolar solution of HCC(101-117) or HCC(93-120) in 0.5 mM ammonium acetate, pH 6. After incubation of the HCC-Aβ- peptide complex for three hrs at 25 0C, the solution was infused in the Apollo-II electrospray ionisation source at a flow rate of 4 µL/min. FTICR Mass spectra were obtained by accumulation of 32 single scans, with the capillary exit voltage set to 50 V for ion desolvation external accumulation of ions in a radio frequency-only hexapole for 0.05 s before transfer into the ICR cell. The capillary voltage was adjusted between 960 and 1120 V until a stable spray was obtained. Other experimental conditions were: Setting of skimmer 1, 15; setting of skimmer 2,7; RF Amplitude, 600; ionization pulse time, 2 ms; mass resolution was approximately 150000. Acquisition of spectra was carried out with the Bruker Daltonics Software XMASS and corresponding programmes for mass calculation, data calibration, and processing.

Abnormal accumulation of β-amyloid peptide (Aβ) into extracellular toxic plaques in the brain is responsible for the neurodegeneration and resulting dementia in Alzheimer's disease (AD). Therefore, Aβ represents an important molecular target for intervention in AD, and agents that can prevent its formation and accumulation or stimulate its clearance might be of therapeutic benefit. The immunological approach to the treatment of AD involves either stimulating the host immune system to recognize and attack Aβ or providing passively pre-formed antibodies. It has been shown that active immunization with Aβ (1-42) in transgenic mouse models of AD reduces both the accumulation of Aβ plaques in brain and associated cognitive impairment. However, a therapeutic trial of immunization with Aβ (1-42) in humans was discontinued due to the significant toxic side effects such as meningo-encephalitic cellular inflammatory reactions.

Human cystatin C (HCC) is an ubitquitous protease inhibitor in human body fluids which has a propensity to form β-amyloid (Aβ)-like fibrils and to coassociate with amyloidogenic proteins. A high (ca. 90%) proportion of patients with Alzheimer's disease (AD) has been diagnosed to also suffer from HCCçmyloidosis, and a specific interaction between HCC and Aβ has been found. The molecular characterization of the Aβ-HCC epiope interaction structure provides lead structures of new neuroprotective inhibitors for AD and cystatin C amyloidosis therapy, and mew tools for AD diagnostics.

Although the co-localisation of cystatin C and Aβ has been demonstrated in cerebral arteries of CAA patients [24] cerebral vasculature and brain parenchyma of AD patients[28] and in muscle cells of patients with age-dependent inclusion body myositis[25] the biological role of the HCC- Aβ interaction and mechanism of the HCC-Aβ co-aggregation has been hitherto unknown. In this study we investigated the binding epitopes between HCC and Aβ using a molecular affinity- mass spectrometry approach,[18] and present the first identification of the epitope sequences and interaction structure of the Aβ-HCC complex. The results of proteolytic excision- mass spectrometry show that the HCC binding site is located in the central region of Aβ within residues (17-28) which is critically important for the Aβ structure and aggregation. The Aβ-epitope identified comprises a part of the hydrophobic core at residues (17-22)

(LVFFA) and the β-turn for fibril formation located within residues (25-28).[30] Tycko et al. reported the residues (12-24) and (30-40) to be involved in the formation of the parallel β-sheet structure in fibrils.[31,32] The residues (25-35) are part of the highly hydrophobic C-terminal region of Aβ which is crucial for oligomerization and fibrilogenesis,[33] while residues (17-21) have been proposed to be involved in side-chain interactions and are pivotal in the dimerisation of Aβ.[30] Furthermore, it been suggested that the sequence KLVFFAE (16-22) has an inhibitory effect on fibril formation, indicating the importance of these residues for Aβ assembly.[32,34] This region is very sensitive to single site mutations which cause significant changes of structure, aggregation and toxicity of Aβ.[34-36] The HCC binding results confirm that HCC efficiently binds to this region, and by blocking the residues (17-28) can influence the Aβ oligomerization, decreasing neurotoxicity and plaque formation. The HCC binding site in Aβ identified here is different from that proposed by Sastre et al.,[25] as our results ascertain binding at the central domain of Aβ, not at an N-terminal sequence as previously suggested.

The observed blocking of HCC binding to Aβ by an anti-Aβ(1-17) antibody may be well explained by steric hindrance of HCC access to the binding site starting at Leu-17. Selenica et al. showed that HCC reduced the *in vitro* formation of soluble oligomers and protofibrils of Aβ(1-42); however, HCC did not dissolve preformed Aβ- oligomers.[23] Our results suggest that HCC is interacting only with monomeric Aβ, and binding of the 120 aa protein to the central domain of Aβ may effectively suppress aggregation by blocking the access to the hydrophobic C-terminal part of Aβ, thus inhibiting interaction with a second Aβ molecule. In preformed oligomers the hydrophobic core is involved in Aβ-Aβ interactions thus blocking the access of HCC. This result is in full agreement with the hypothesis that the presence of HCC in Aβ solution can decrease oligomerisation25 and slow down the aggregation process. The HCC binding site in Aβ identified here is localised in a similar position to the Aßepitope recognised by physiological Aβ-autoantibodies in human serum which recognise a specific C-terminal domain of Aβ and seem to play a neuroprotective role in the oligomerisation of Aβ.[16] A similar, neuroprotective effect may be suggested for the HCC binding to Aβ, in contrast to the plaque-specific effect of Aß-antibodies obtained by active immunisation, that target an

N-terminal Aβ-epitope.12 The Aβ- binding site identified in HCC, residues (101-117) is located in the Cterminal part within the L2 loop and β5 strand of HCC which comprise the external part of the protein and are exposed to the environment.[27] The C-terminal binding epitope enables interaction of the Aβ peptide with the L2-β5 part without any restriction. The identification of the binding site in HCC may be of high importance for oligomerisation and fibrillisation studies of HCC and its amyloidogenic mutant L68Q which, due to its structural similarity to HCC [20,25] can be assumed to have a similar binding epitope for Aβ. The knowledge of the binding epitope may be used for future *in vitro* studies of HCC fibril formation, since fibrils can be easily formed with the L68Q HCC mutant by 3D domain swapping, but not by native HCC. In a model postulated by Jaskolski [29] the Aβ binding site is located in the center of the fibrils which would provide the opportunity to disturb the interaction between cystatin molecules by binding of Aβ to the C-terminal part of monomeric HCC. Here, the HCCepitope identified could be used as a new template for designing efficient inhibitors for amyloid angiopathy related to cystatin C oligomerisation.

The interaction of HCC with Aβ may be an important neuroprotective mechanism in brain, and may attenuate the oligomerisation of Aβ and play a regulating role in Aβ amyloidogenesis. The identification of the binding epitope of HCC in the central domain of Aβ confirmed the importance of this protease inhibitor for the aggregation process and amyloidogenesis, since blocking the hydrophobic core may inhibit Aβ oligomerisation and regulate fibril production. On the other hand, the identification of the binding site in HCC should be of importance for oligomerisation studies of cystatin C, and new oligomerisation inhibitors may be designed based on the HCC-epitope.[37,38] It seems somewhat paradox that interaction of two potentially amyloidogenic molecules might give a lead to control or inhibit neuropathological changes in amyloidogenic diseases.

3. EXPERIMENTAL PART

3.1 Materials and reagents

The following commercial available reagents were used in the present work:
Antibodies: anti-Aβ (1-16) antibody (clone 6E10) and anti-Aβ (17-24) antibody (clone 4G8): Chemicon (Millipore); labeled goat anti-mouse IgC: Jackson ImmunoResearch; Coomassie Brilliant Blue G250, Tween-20 (Polyoxyethylensorbitanmonolaureat): Sigma; deionisate water MilliQ (MQ): Millipore; T-buthylmethyleter (99%), diethylether (99%), dimethylformamide (DMF, 99.8%), N-Methylmorpholine (NMM 99.5% p.a.), piperidine (99%), trifluoroacetic acid (TFA, 99 % p.a.), triisopropylsilane (97%): Fluka

The amino acids (N-α Fmoc protected), TGR resin and the activator Benzotriazol-1-yl-N-oxy-tris-pyrrolidino-phosphonium-hexafluoro-phosphat (PyBOP) used for peptide synthesis: NovaBiochem.

Hydrochloric acid (37% p.a.), sulfuric acid (98%), soium hydroxide (99%), acetic acid (100%) ethanolamine (99%), Trizma base (2-amino-2-hidroxymethyl-1,3-propandiol, 99,9%) sodium dihydrogen phosphate monohydrate (NaH_2PO_4, 99.5%), sodium acetate trihydrate (99.5%), hydrogen peroxide (30%), glycine (>99%), N-Hydroxysuccinimid (NHS): Merck; ethanol (99.8% p.a.), disodium hydrogen phosphate-2-hydrate (Na_2HPO_4, 99.5%), Riedel-de Haen; acetonitrile (CAN, 100%, p.a.): Roth; diethanolamine (min. 98%), 1-Ethyl-3-[3-dimethylaminopropyl]carbodiimide Hydrochloride (EDC): Sigma.

3.2 Solid phase peptide synthesis

Solid phase peptide synthesis is a time saving approach to obtain high-yelds of high-purity peptides. It was first introduced in 1963 by Merrifield [59] and since then has

become a widely used method for the small scale peptides synthesis. The method consists of a cyclic repetition of simple chemical reactions which leads to the successive addition of α amino acids into a chain anchored to an insoluble resin support. The main advantage in using the insoluble resin support is the fast and complete separation of the reaction products from the reaction mixture. Further, this allows the use of great excess of reactants, which increases the yield. In present work SPPS was employed for the synthesis of Aβ and HCC peptides, using the Fmoc strategy [60, 61]. This implies the use of 9-fluorenylmethoxycarbonyl as amino-protecting group and other compatible protecting groups for the reactive side chains. Allpeptides were synthesized on a NovaSyn TGR resin (Figure 29). Before starting the synthesis, the resin was washed with 50 mL of DMF and allowed to “swell”.

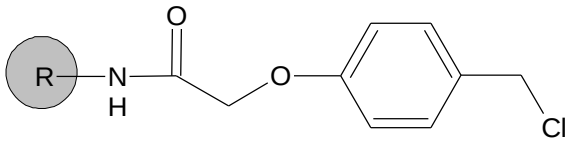


Figure 29: NovaSyn® TGR-resin from Novabiochem. PEG is the plyetthyleneglycol spacer between the resin and the polystyrene matrix.

The synthesis were performed on a semiautomated peptide synthesizer EPS 221 from Abimed,Germany. The following Nα-Fmoc protected amino acids were used in five-fold molar excess: Nα-Fmoc-L-Alanine (Fmoc-Ala-OH), Nα-Fmoc-S-trityl-L-Cystein (Fmoc-Cys(Trt)-OH), Nα-Fmoc-L-Aspartic-acid-β-t-butylester (Fmoc-Asp(OtBu)-OH), Nα-Fmoc-LGlutamicacid-γ-t-butylester (Fmoc-Glu(OtBu)-OH), Nα-Fmoc-L-Phenilalanine (Fmoc-Phe-OH), Nα-Fmoc-Glycine (Fmoc-Gly-OH), Nα-Fmoc-N-im-trityl-L-Histidine (Fmoc-His(Trt)-OH), Nα-Fmoc-L-Iso-Leucine (Fmoc-Ile-OH), Nα-Fmoc-Nε-t-Boc-L-Lysine (Fmoc-Lys(Boc)-OH), Nα-Fmoc-Nβ-trityl-L-Asparagine, (Fmoc-Asn(Trt)-OH), Nα-Fmoc-L-Proline (Fmoc-Pro-OH), Nα-Fmoc-Nγ-trityl-L-Glutamine (Fmoc-Gln(Trt)-OH), Nα-Fmoc-NG-2,2,4,6,7-pentamethyl-dihydrobenzofuran-6-sulfonyl-L-Arginine (Fmoc-Arg(Pbf)-OH), α-Fmoc-O-t-butyl-L-Seine (Fmoc-Ser(tBu)-OH), Nα-Fmoc-O-t-butyl-L-Threonine (Fmoc-Thr(tBu)-OH), Nα-Fmoc-L-Valine (Fmoc-Val-OH), Nα-Fmoc-N-in-t-Boc-L-Tryptophan (Fmoc-Trp(Boc)-OH).

The main steps followed during a synthesis cycle are: a) the deprotection of the α-amino group with 2% DBU and %% piperidine in DMF for 5 minutes; the side products dibenzofulvene is scavenged by the piperidine and removed in the subsequent washingstep; b) washing with DMF; c)activation of the amino acid with 0.9 M PyBOP and 1.3 M NMM in DMF; d) washing with DMF; e) coupling of the activated amino acid on the resin for 30 minutes; the coupling and the subsequent washing step are repeated for every amino acid of the sequence (Figure 30). In the end the last amino acid is also deprotected and the peptide is then cleaved from the resin with 95% TFA as cleavage reagent, 2.5% trietylsilan and 2.5% deionized water for 3 hours at room temperature- Then it was precipitated with 40 mL cold tert-butyl-methylether, filtered, dissolved in 5% acetic acid and lyophilized.

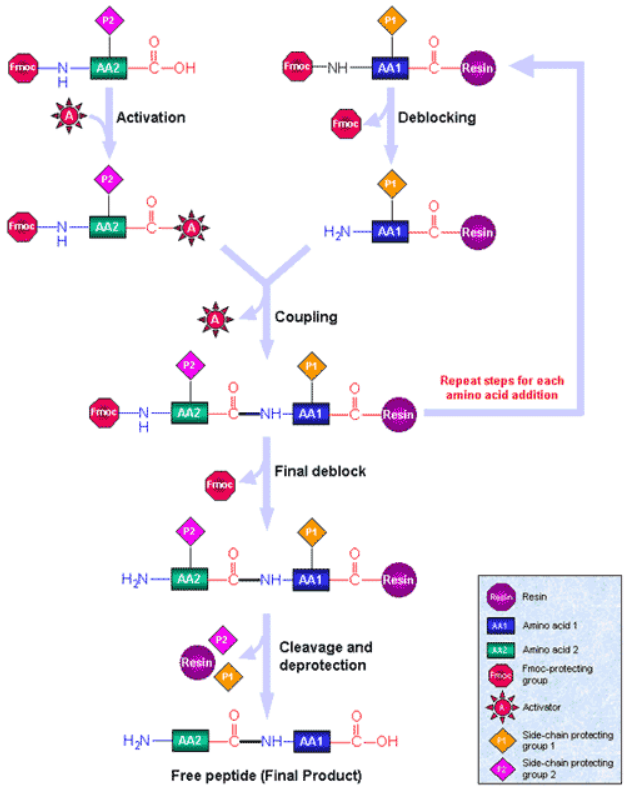


Figure 30: General scheme of solid phase peptide synthesis employing Fmoc_/tBu strategy.

3.3 High performance liquid chromatography

High performance liquid chromatography (HPLC) is a separation method based on

sample partitioning between a coated silica solid phase and a mobile liquid phase. The term high-performance (also known as “high pressure”) refers to the speed and superior separation compared to agarose gel particules that were used before in column-chromatography. For peptide and protein separation the main type of HPLC used today is reversed-phase HPLC (RP-HPLC). This separation mode is based on acid nonpolar adsorption of peptides onto the hydrophobic stationary phase within the column. The peptides are then differentially released from the stationary phase as a function of increasing organic component in the liquid phase. The two major adjustments of the method that made it highly popular in the peptide and protein separation: the pore size of silica particles, which, increased from ~100 to ~300Å, had a dramatic effect on inproving the separation of peptide, and the replacement of phosphoric with trifluoroacetic acid (TFA) as the ion-pairing agent, which was volatile and improved also the peptide separation. The sample is introduced into the HPLC column via a manual injector. The hydrophobic coating of the silica solid phase consists of saturated alkyl chains that interact with the hydrophobic moieties of the analyte.The elution is made with aqueous solvents containing TFA as ionic modifier to adjust the pH and CAN as an organic modifier is applied by using a two-phase mobile system:

Solvent A: 0.1% (v/v) TFA in MilliQ

Solvent B: 0.1% (v/v) TFA, 80% (v/v) acetonitrile in MilliQ

The solutions were thoroughly deaerated prior to use by sonication at low pressure (a vaccum was employed). The sample was dissolved in solvent A. To prevent column damage, the sample was centrifuged before injection. Analytical RP_HPLC was performed on a UltiMate 3000 system (Dionex, Germering, Germany), equipped with LPG-3400A pumps, using a Vydac C4 column (250×4.6 mm I.D.) with 5 µm silica (300 Å pore size) (Hesperia CA). The chromatograms were recorded by UV detection at 220 nm, employing the VWD-3400 variable wavelength detector of the UltiMate system, with a flow cell of PEEKJ, 0.4 mm long and an internal volume of 0.7 µL. The fractions were collected with the FOXY Jr® automated fraction collector.

3.4 Gel Electrophoresis

3.4.1 SDS-PAGE

All peptide samples were analyzed by one-dimensional electrophoresis, to establish their constituency and purity. The electrophoresis was performed on polyacrylamide-gels, using also the SDS detergent in sample preparation. Proteins are biopolymers folded into compact structures held together bya variety of non-covalent interactions like salt bridges, hydrophobic interactions and hydrogen bonds. These interactions can be disrupted using SDS, a detergent that consists of a hydrophobic 12-carbon chain and a polar sulphated head. The hydrophobic chain intercalates into the hydrophobic parts of the protein, leaving the sulfate group on the surface of the protein. SDS coats the protein with a uniform “layer” of negative charges which replaces the net intrinsic charge of the native protein. Once the SDS treated samples are placed into a gel and an electric field is applied, the proteins migrate towards the anode and get separated depending only on their size. The size depending separation is achieved through the pores of the gel that allow the small molecules to travel faster than the big ones. The hydrogen bonding between the different amide groups present in the peptide chain plays a crucial role in the formation of protein secondary structures. High concentrations of urea interrupt these hydrogen bonds, rendering the polypeptides highly water-soluble. Urea achieves this through its strong dipole moment. However, because it is uncharged, urea does not migrate in electrical field and does not interfere with the results of the electrophoretic separation.

The gels were prepared using the mini-gel instrument MiniProtean from Bio-Rad.
In the table 5 are presented the solutions for the separation gel and stacking gel.
The separation gel buffer contains 0.5 M Tris, 0.4% SDS pH 6.8 and the stacking gel buffer contains 1.5M Tris, 0.4% SDS pH 8.8. The power/PAC 1000 instrument from Bio-Rad at constant current in two steps was used for gel electrophoresis.

a. 60 V when the samples were in the stacking gel.
b. 120 V when the samples were in the separation gel.

The molecular weight of the proteins was assigned using different markers.

Table 5: Solution volumes required for gel casting.

Solutions	**Gel concentration**		
	5%	12%	10%
4× Stacking gel buffer	2.5 mL	-	-
4× Separation gel buffer	-	6 mL	6 mL
MilliQ	5.8 mL	8.4 mL	10 mL
Acrylamide solution	1.7 mL	9.6 mL	8 mL
10% Amonium peroxidsulphate	85 µL	125 µL	125 µL
N', N', N', N'-tetramethylethylenediamine	20 µL	20 µL	20 µL

3.4.2 Staining of the gels with Coomassie Brilliant Blue

Coomassie Blue staining is based on the non-specifically binding of the dye Coomassie Brilliant Blue G250 to virtually allproteins in acid solutions. This binding results in a spectral shift from reddish-brown (λ 465 nm) to blue (λ 610 nm). Coomassie Blue binds roughly stoichiometrically to proteins, allowing densitometric determinations. The proteins are detected as blue bands on a clear background, after fixing the gel with trichloro-acetic acid (TCA) for obtaining maximum sensitivity. Staining solution was prepared from: acetic acid 10% (v/v); methanol 40% (v/v); Coomassie Brilliant Blue G-250 0.1% (w/v). After the gel was fixed for 30 minutes in fixing solution (12% TCA in MilliQ), it was left overnight in a mixture of 80 mL buffer (10% $(NH_4)_2SO_4$, 2% H_3PO_4 in MilliQ) with 20 mL methanol and 2 mL Brilliant Blue G-Colloidal Concentrate, shaking. Afterwards the gel was washed with 25% methanol for 60 seconds and scanned with GS-710 Calibrated Imaging Densidometer from Bio-Rad. The image was acquired and saved with PD Quest software.

3.5 Inhibition assay of Aß-oligomerization by HCC peptides.

The gel was prepared using the mini-gel instrument MiniProtean from BioRad. Samples were dissolved in 50 µL of running buffer (25 % glycerol, 4 % SDS, Coomassie) and added to the 12 % gel (8.4 mL MilliQ, 6 mL 4X separation buffer, 9.6 mL acrylamide solution, 125 µl APS, 20 µL TEMED). The power/PAC 1000 instrument from Bio-Rad at constant current was used for gel electrophoresis in two steps: (a) 60V when samples were in the stacking gel; (b) 120V when samples were in the separating gel.
Thioflavine T (ThT) is a dye common used to visualize the amyloid fibrils. For the free dye excitation and emission occur at 385nm and 445 nm; for the bounded – excitation and emission occur at 450nm and 482nm. For this experiment 100µM ThT solution was prepared in 50mM Glycine, pH 8.5. The samples (50µl of supernatant and 10µl of suspention) were loaded on a 96-well black plate together with 50µl ThT, and measured on PerkinElmer Wallac VICTOR2 E.L.I.S.A. reader. The results for each experiment are presented on graphs and they are average from two measurements.

3.6 Immunoanalytical Methods

3.6.1 Enzyme linked immunosorbant assay (ELISA)

Indirect ELISA using antigen dilutions was carried out for the immunological characterization of Aβ and HCC peptides. Ninety-six-well ELISA plates (Bio-Rad) were coated overnight at room temperature with 100 µL/well of antigen (serial dilutions from 2.5 µM to 0.00001 µM of biotin G_5Aβ(…) peptides concentration). Afetr coating, the wells were washed with 200 µL/well PBS-T 0.05% Tween-20 v/v in PBS-phosphate buffer saline (Na2HPO4 5 mM, NaCl 150 mM, pH 7.) and the nonspecific adsorption sites were blocked with 200 µL 5 % BSA for 2 h at RT.After incubation and washing steps, mouse anti-Aβ(…), anti-Aβ (…) were added to the plate. After another 2 h of incubation at room temperature the unbound first antibody was washed away and 100 µL of peroxidase labeled goat anti-mouse IgC (Jackson Immuno Research) diluted 5000

times in 5% BSA was added to each well. After an additional incubation for 2 h, he wells were washed three tmes with PBS-T and once with 50 mM sodium phosphate-citrate buffer, pH 5. 100 µLwell of 0.1% o-phenylenediamine dihydrochloride (OPD) in sodium phosphate-citrate buffer at c1mg/mL and 2 µL of 30% hydrogen-peroxide per 10 mL of substrate buffer was added to the plates. The absorbance was measured at λ = 450 nm on a Wallac 1420 Victor^2 ELISA Plate counter (Perkin Elmer).

3.6.2 SAW Biosensor

The bioaffinity measurements were performed using a SAW biosensor K5 instrument from the firma Biosensor GmbH, Bonn. The instrument consists of the biosensor itself and an automated autosmapler. The sensing surface consisting of gold is found on a small chip made of quartz. Before performing the actual measurements, the active surface of the chip has to be cleaned and then chemically modified, in order to permit the immobilization of the antibodies. The gold chips can be reused after a thorough cleaning of the surface. This is achieved by washing the chip using Piranha solution which consists of equal volumes of concentrated sulphuric acid (98%) and hydrogen peroxide (30%). Practically, the solution was made by mixing 1 mL of each reagent in a small glass recipient (the hydrogen peroxide was added first, then the acid). In the cold mixture (room temperature, 10 minutes after mixing) the chip was left for 45 minutes. After this, the chip was washed with MQ and ethanol, and then dried. Accordingly to the producer's specifications, the chips can be reuse as many as 50 times, but in practice we observed a much faster degradation of the active surface (scratches and spots), after 10to 20 cycles of use and cleaning. The formation of the self assembled monolayer (SAM) occurs through chemical adsorption from a solution containing compounds with thiol groups. A solution of 16-mercaptohexadecanoic acid 10 µM is prepared by dissolving 5.77 mg of the acid in 2 mL of chloroform and ethanol, and then dried.

All reactions involved in the immobilization of the first partner of the affinity system are conducted in the micro-fluidic cell of the biosensors. The gold chip with a fresh SAM

build on its surface is inserted in the instrument and a flow of MQ is allowed to wash the chip. The flow rate is usually a small one, and kept relatively constant throughout the entire experiment. Typically used flow rates are 20 to 30 µL/min. The reactants are added as solutions that are injected using the autosampler. In general, for the immobilization of a compound (antibody or protein) three injections are required. The first one contains the reactants for the activation, a mixture of 200 mM EDC (1-ethyl-3-(3-dimethylaminopropyl)carbodiimide) and 50 mM NHS (N-Hydroxysuccinimide) solutions, I a volumetric ratio of 1:1. Since EDC is quite unstable at room temperature, samples with weightsclose to 11.502 mg (quantity needed for 300 mL solution 200 mM) are weighted and then stored at -20 ºC in the freezer. Short before he injection, the solution is made by adding the required volume of PBS (used to insure the right pH for the reaction) over the EDC sample. The NHS is more stable then the EDC, but the same procedure is followed as for the EDC. The samples with weights close to 1.726 mg (quantity required for 300 mL solution 50 mM) are stored also in the freezer (-20 ºC) or in the fridge (5 ºC) and the solutions are made short before the injection by adding the right volume of PBS. Generally, in the autosampler was introduced a sample glass with 200 µL mixture of both solutions and for the injection were use 150 µL.

The second injection contains the compound to be immobilized (peptides, proteins and antibodies). Different concentrations and volumes have been tested in order to achieve a maximum quantity of compound on the chip. However, given the purpose of the work, the focus was to determine the best conditions for the immobilization of the antibodies. A maximum quantity of antibody on chip can be achieved by injecting 150 µL (out of 200 µL inserted in the autosampler) of a 200 nM antibody solution in MQ. With a molecular weight of …, the solution was prepared diluting … µg peptide in 200 µL MQ. Practically we added 6 µL stock solution of peptide (which had a concentration of 1 µg/µL) to 194 MQ. The third injection the function of capping all the activated carboxyl groups that did not reacted with the compound to be immobilized. It consisted of a 1 M solution of ethanolamine, brought to pH 8.5. From the 200 µL inserted in the autosampler, were

injected only 100 or 120 µ. After the immobilization of a first compound, it's affinity partner was also injected. This was done when the signal of the biosensor was equilibrated back after the change of the buffer from MQ (used during the immobilization) to PBS. For the study of the affinity interactions PBS was used as buffer in order to adapt the conditions of the experiment as close as possible to those of the physiological environment. For the injection of the affinity partner were also different concentrations and volumes used, according to the purpose of the experiment.

For attempting the coupling of the biosensor with the ESI-ion trap mass spectrometer, it was aimed to bind through affinity to the chip as much compound as possible through a single injection. For eluting the affinity bound compounds from the chip, we used acidic conditions, injecting for example glycine 50 mM (with an adjusted pH 2) or acetic acid 1% (pH 1.8). However, for dissoaciation of the antigen/antibody complexeswe found the solution of HCl 0.1 M (pH 1.8) to be best suited. Active surface of a channel is 0.072 cm^2. The quantity of bound compound to the chip can be evaluated from the measured phase shift (recorded with the K15 software) using the following sensitivity calibration factor:

$515 = \varphi(^o)/(m(\mu g).A(cm^2)$

3.6.3 ZipTip desalting and clean up procedure

The ZipTip desalting procedure was performed using ZipTip® C_{18} and C_4 pipette tips from Millipore. The ZipTip pipette tips are modified pipette tips containing a small bed of reversed-phase chromatography media (0.6 µL) inside the cone end of the tip, with no dead volume. ZipTip C_{18} pipette tips are to be used for peptides and low molecular weight proteins. The procedure was performed according to the instructions of the producer, following five major steps: wetting the chromatographic media, equilibration of the ZipTip pipette tip, binding of the peptides and/or proteins to ZipTip pipette tip, washing off of the impurities nd slats, elution of the purified and

desalted protein/peptide sample. The used solutions are listed in Table 3.

Table 3: Solutions required for use with ZipTip pipette tips containing C_{18} media.

Solution	ZipTipC_{18} Pipette Tips	
Wetting solution	100% ACN in MilliQ water	
Sample preparation	0.1% TFA in MilliQ water (final sample solution pH<4)	
Equilibration solution	0.1% TFA in MilliQ water	
Wash solution	0.1% TFA in MilliQ water	
Elution solution	for MALDI	0.1% TFA/50% ACN
	for ESI	2% acetic acid/50% methanol

3.6.4 ESI-Ion Trap-MS

All mass spectra were obtained using an ESI ion trap mass spectrometer Esquire 3000 plus from Bruker Daltonik (Bremen, Germany). The scheme of its build-up is presented in Figure 34.

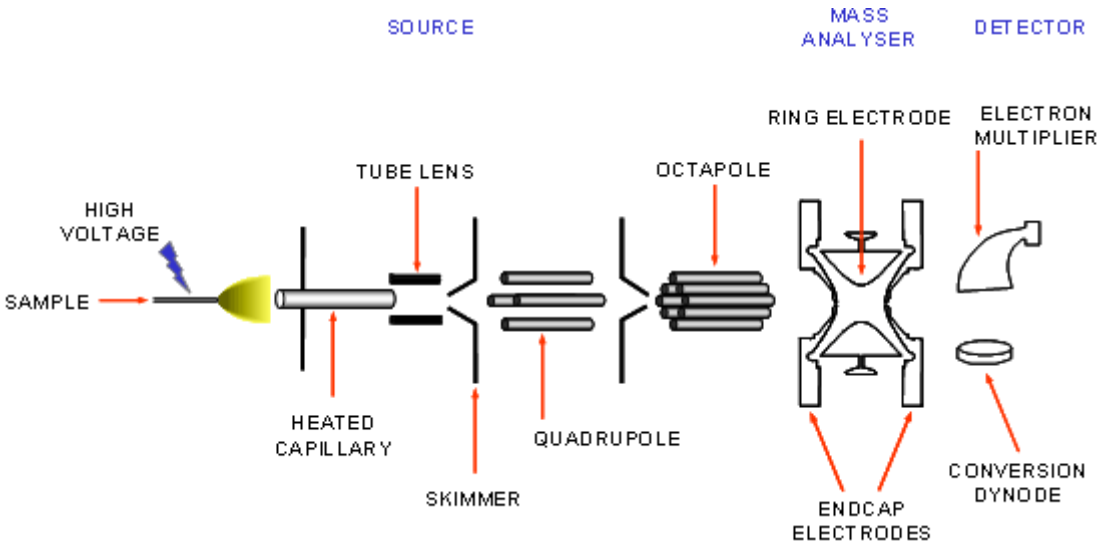


Figure 34: Schematic representation ESI ion trap mass spectrometer Esquire 3000 plus from Bruker. The ions are generated by the ESI source, are introduced in the mass spectrometerbthrough a capillary, are

focused by two octopoles and then allowed to enter the ion trap. After being separated according to their m/z values, the ions exit the trap and reach the detector.

The Aβ and HCC peptides, solubilized in 1% formic acid at a final concentration of 10 µM, were measured through direct infusion with an Esquire 3000 Plus ion trap device. The instrument was controlled by the equireControl software. For the direct bioaffinity, a binary gradient system consisting of solvent A (0.3% formic acid, 5% acetonitrile) and solvent B (95% acetonitrile, 0.3% formic acid in water) was employed. All MS results were obtained using atmospheric pressure chemical ionization (APCI) in the positive ion mode. Mass spectra were recorded in the full scan mode, scanning from m/Z 100 to 3000. Ion source parameters were 15 psi nebulizer gas and 6L/min of drying gas with a temperature of 200 ºC (Table 4).

Table 6: The ESI ion trap parameters:

Parameter	Value
Target mass	1000
Compound stability	100%
Trap drive	80%
Nebulizer	15 psi
Dry gas	6L/min
Dry temperature	200 ºC
Mode	Positive
Scann	100-3000
Average scans	6

4.7 Computer programs

4.7.1 GPMAW

The software GPMAW 5.0 (General Protein/Mass Analysis for Windows) from Lighthouse Data, Denmark, was employed in the theoretical calculation of molecular

weight of the peptides and proteins. The program was able to calculate both the monoisotopic and average masses of the peptides or proteins having as input their sequence. It also allowed to modify the sequence adding different chemical modifications, e.g. to the side chain of the amino acid rest. It was also possible to predict the average and the monoisotopic values for the single and multiple charge ions. Another feature of GPMAW 5.0 is the prediction of the secondary structure and the hydrophobicity of a given protein or peptide sequence.

4. SUMMARY

Alzheimer's disease (AD) and AD-related neurodegenerative disorders have become the predominant form of progressive cognitive failure in elderly humans, a development presently accelerating due to the significant increase in life expectancy in the last decades. A major constituent of amyloid fibrils in brain of patients with AD and AD-related diseases, as well as in aged individuals without any neurological disorder is the β-amyloid polypeptide (Aβ). Aβ arises from a large precursor, the amyloid precursor protein (APP); [1,2] it is produced by normal cells and detected as a circulating peptide in plasma and cerebrospinal fluid (CSF) of healthy humans.[3, 4]The accumulation of Aβ, a 39-42 amino acid proteolytic fragment of APP, in neuritic AD plaques is thought to be causative for disease progression. [3, 4]

Although amyloid plaques in brain of AD patients contain predominantly Aβ-aggregates, immunohistochemical studies have shown the co-deposition of several other proteins, such as the protease inhibitor cystatin C, apolipoprotein E, clusterin, transthyretin and gelsolin. [5-8] In particular, the presence of human cystatin C (hCC) in amyloid deposits has found much interest, [9, 10] and a wide spectrum of activities has been associated with hCC such as modulation of neuropeptide activation and neurite proliferation. [11, 12]

The 13 kDa protein hCC is the main cysteine protease inhibitor in mammalian body fluids [13, 14] and has been found with high concentrations in CSF. The presence of hCC in Aβ-plaques has been suggested to result from its binding to APP, or alternatively, hCC may bind to Aβ prior to the secretion or following the deposition in brain. [9] Sastre et al. found that the association of hCC with Aβ causes an inhibition of fibril formation, and suggested an N-terminal Aβ-sequence to be responsible for the interaction, with formation of a stoichiometric hCC-As complex. [11] The interaction of hCC with Aβ may be an important neuroprotective mechanism in brain, and may attenuate the oligomerisation of Aβ and play a regulating role in Aβ amyloidogenesis. The identification of the binding epitope of hCC in the central domain of Aβ confirmed the importance of this protease inhibitor for the aggregation process and

amyloidogenesis, since blocking the hydrophobic coremay inhibit Aβ oligomerisation and regulate fibril production.

Using proteolytic extraction and excision of the human cystatin C-Aβ(1-40) immune complex (e.g. trypsin, Glu-C proteases) in combination with electrospray ionization (ESI)- and MALDI- mass spectrometry, the epitope was identified at the middle-carboxy terminal domain of Aβ, Aβ(17-28). An almost identical minimal epitope to that of the VHH-anti-Aβ-antibody (Aβ(17-24)) was found, which binds to a specific C-terminal domain of HCC, HCC(101-114) [1, 2]. The identified HCC epitope peptide was found to specifically inhibit Aβ-oligomerization in vitro, in agreement with the Aβ-epitope domain interfering with the Aβ-aggregation. Other affinities studies using SAW and ELISA allowed to understand better the interaction between this two groups of peptides. The identified Aβ and HCC epitopes and the study of their affinity represent new lead structures for designing neuroprotective inhibitors of the Aβ-aggregation process, and for molecular AD diagnostics.

The discovery of soft ionization methods such as MALDI and ESI has the mass spectrometry analyses of biomacromolecules. Since then, mass spectrometry has become one of the most important analytical methods for the analysis of biopolymers. Biosensors have been recently developed as new analytical tools that rapidly found applications in the study of biomolecular interactions. The SAW biosensor is suitable to analyze samples in solution, being highly sensitive to mass loadings and viscosity changes. Therefore, the SAW biosensor is successfully used for affinity binding studies. However, a principal weakness of all bioaffinity methods is the lack of molecular structure information of ligand-binder interactions. The method was applied to several antigen-antibody systems related to a neurodegenerative disease of great impact worldwide, Alzheimer’s disease). The antibodies were covalently immobilized on the surface of the chip used by the SAW sensor and the interactions with the antigens (peptide or protein) present in solution were determined.

Several Aβ peptides were synthesized and their bioaffinity to HCC peptides was studied. The investigated antigen-antibody systems were: Aβ (1-40) with HCC (93-120), Aβ (12-40) with HCC (93-120), Aβ (17-28) with HCC (93-120); Aβ (1-40) with HCC (101-117), Aβ (12-40) with HCC (101-117), and Aβ (17-28) with HCC (101-117)as well as the inverse systems. The bioaffinities were comparatively investigated by ELISA and SAW, and the results were found to be in good agreement. It was demonstrated that all the systems (after a successful synthesis and purification) present affinity, and was possible to calculate all the constant dissociation basing on the affinity results.

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6. APPENDIX

6.1 Appendix 1 – Abbreviations

Abbreviations	Name
ACN	Acetonitrile
Ab	Antibody
APS	Ammoniumperoxodisulfat
CD	Circular dichroism
CDR	Complementary determining region
Da	Dalton
DHB	Dihydroxybenzoic acid
DTT	Dithiothreitol
EDC	1-ethyl-3-(3-dimethylaminopropyl) carbodiimide
ELISA	Enzyme liked immunosorbent assay
ESI-MS	Electrospray/ionizations- Mass spectrometry
HCCA	4-Hydroxy-α-cynamic acid
HPLC	High performance liquid chromatography
MALDI-MS	Matrix-assisted Laser desorptions-/ionizations-Mass Spectrometry
min	Minute
m/z	Mass over charge ratio
NHS	N-Hydroxysuccinimide
PBS	Phosphate buffered saline
PD	Parkinson disease
pH	Negative logarithmus of H_30^+-iones concentration
SAM	Self Assembled Monolayer
SDS-PAGE	Sodiumdodecylsulfat-Polyacrylamid-Gel electrophoresis
TEMED	N,N,N',N'-Tetramethylethylendiamine
TFA	Trifluoroacetic acid
TOF	Time of flight

Tween	Polyoxyethylen Sorbitan Monolaurat
UV	Ultraviolet
ºC	Grad Celsius

6.2 Appendix 2 – Abbreviations for amino acids

Table 5: Amino acids abbreviations.

Name	One letter code	Three letters code	Monoisotopic mass (Da)
Alanine	A	Ala	71.03711
Arginine	R	Arg	156.10111
Asparagine	N	Asn	114.04293
Aspartic Acid	D	Asp	115.02694
Cysteine	C	Cys	103.00919
Glutamine	Q	Gln	123.05858
Glutamic acid	E	Glu	129.04259
Glycine	G	Gly	129.04259
Histidine	H	His	57.021046
Isoleucine	I	Ile	113.08406
Leucine	L	Leu	113.08406
Lysine	K	Lys	128.09496
Methionine	M	Met	131.04049
Phenilalanine	F	Phe	147.06841
Proline	P	Ro	97.05276
Serine	S	Ser	87.03203
Threonine	T	Thr	101.04768
Tryptophan	W	Trp	185.07931
Tyrosine	Y	Tyr	163.06333
Valine	V	Val	99.05841