Scientific Report

Regarding implementation of project, October 2011 – October 2014

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"New mass spectrometric approaches for elucidation of oxidative modifications in proteins"

The research grant funded by the National Plan for Research-Development and Innovation II comprised three main goals: (i) the return and reintegration of young researcher Dr. Brînduşa Alina Petre in academic and romanian research environment; (ii) the set up of a research laboratory with international visibility and (iii) the action of research itself: purchase of instruments and reagents specific for the proposed topic, experimental work and dissemination of the obtained results. All these objectives were succesfully fulfilled; the research team is harmonized with a built altruistic team spirit revealed by the participating at national and international conferences and by publishing articles in high ranking journals. The scientific report based on the effective experiments and the obtained results is presented as following:

First stage of the Project (Stage I) consisted of a period of documentation and literature selection necessary for theoretical initiation of the new younger team members. Literature and protocols materials were prepared for relatively new techniques introduced in biochemical and analytical chemistry laboratories as they can be applied in the experimental part of the project. These analytical techniques constitute the ground for the research strategy called proteomics. Proteomics is a new research domain used for successful protein identification from complex biological sample mixtures and offers information about their structrure, post-translational modifications and their interaction with differents ligands. Technologies that allow succesfull analysis of hundreds of proteins in one experiment are used in proteomics. However, in many cases these investigations allow us the identification of a protein, but these investigations may not offer informations about the primary structure of the protein (the amino acid sequence). Full characterization of the protein's primary structure, including PTMS remains a fruitful domain for many years that will follow. Proteomics combines separation techniques for biological samples such as electrophoresis, chromatography and immunology with analysis techniques based on mass spectrometry. Identification of the proteins and of their modifications is usually realized by comparing the experimental data with already known aminoacid sequence that cand be found in an on-line accessible database (for example SwissProt or UniProt). As the manager of the project and superviser of experimental investigations, I considered absolutely necessary to prepare prior to the experimental work, materials available to the whole research team. These documents

contain basic notions and describe practicle principles of the analytical techniques that will be used. More exactly, I studied books and articles published in journals of international prestige and from these I selected the best examples [1-6]. These studied materials will help students to understand the importance of the techniques mentioned above as methods used for the identification of oxidative modifications in the investigated proteins. Also, I realized presentations using the PowerPoint program where I presented general notions such as: (i) principles and definitions, (ii) description of the used instruments, and (iii) examples of previously obtained results. Furthermore, I prepared the working protocols for the analytical techniques used by me in my previous research activity at high ranking laboratories from the University of Konstanz, Germany and Washington University, St. Louis, MO, USA.

The initial step for realizing the first objective of the next stage (Stage II) was the documentation about oxidative modifications of proteins (for example tyrosine nitration) and protocols for identification of these proteins in biological sputum samples. Proteins are biopolymers whose synthesis is encoded by genes. If we understand better the proteins implicated in cystic fibrosis, the oxidative modification of these proteins and as well the interactions that can happen in lungs, it would an important step to develop diagnostic tests and treatment for patients suffering of lung diseases. We realized a documentation study based on the information received from our co-workers from Clincal University of Tuebingen, Germany [7-10]. Our research group focused especially on documentation for identification of modified proteins as a result of oxidative stress, process that happened in sputum sample we had for research. Protein nitration at the tyrosine residue is one of the most frequently occurring oxidative modifications and can be associated with many patophisiological conditions such as: lung disease, cardiovascular disease, diabetes, neurological disease, allergies and cancer [11-15]. Only a small number of proteins are modified *in vivo* by nitration (in biological systems) and this selectivity can be determined by a combination of factors such as: (1) proteins are close to the place where the nitration agent is generated; (2) chemical selectivity of the nitration agent; (3) relative abundance of target proteins; (4) accelerated „turnover” of nitrated proteins; (5) proteins contain tyrosine residues in a specific aminoacid sequence and (6) proteins „cleaning” by putative enzymes called denitrases. Efficient, sensitive and high performant bio-analytical methods are necessary in order to identify nitrated proteins from biomedical samples and to correlate their involvement to some negative effects on health. Most important methods we can use for detection, identification and quantitation of 3-nitro-tyrosine are: high performance liquid chromatography (HPLC) in combination with different

detection systems such as UV/Vis, or fluorescence detection after a derivatisation step; gaz chromatography coupled with mass spectrometry (GC-MS); liquid chromatography coupled with mass spectrometry; (4) proteomics; and a variety af imuno-analitical methods [16- 27].

In past years the combination of 2D-gel electrophoresis, Western Blot and mass spectrometry has been used for analysing nitrated proteins in different conditions. Many studies reported only the identification of nitrated proteins, but showed no information about the tyrosine residue at which nitration occurred and this was due to extremly low level of nitrated peptides in biological samples. Elucidation of oxidative post-translational modifications of proteins is still an attractive and important research subject in the biochemistry and biomedicine fields due to the relevance of these proteins in pathophysiological processes.

In the second stage of the project (Stage II) all the proposed activities and objectives were fully realized. This was possible due to an old collaboration with University of Konstanz, Germany; new established connections for collaboration with Petru Poni Institute, Iasi and with University of Agricultural Sciences and Veterinary Medicine "Ion Ionescu de la Brad", Iaşi. The obtained results guided us to a new research subject, the oxidative modification of proteins by hydroxylation of tyrosine residues, results that will be presented in a research article that is now in preparation. The mentioned results are presented as follows: Cystic Fibrosis (FC) is one of the most common recesive monogenic diseases and is caused by the mutations in CFTR gene (Cystic Fibosis Transmembrane Conductance Regulator). The encoded protein by this gene controls the function and secretion of the exocrine glands of the bronhopulmonar, digestive, reproductive systems and of other organs. In respiratory tract the disease is characterized by: modification of Cl_2 epitelial level, modifications of the water delivery in body, high viscosity of mucus and low antibacterial defence. As a result FC patients are more susceptible to bacterial infections of the respiratory tract, frequently with Staphylococcus aureus, Haemophilus influenzae, and Pseudomonas aeruginosa and are predisposed to death because of respiratory failure caused by acute and chronic pulmonary infections [28-30]. In numerous biological studies were presented results that make a connection between FC and a high level of NO, but scientist are still investigating the identification of new biomarkers of the cellular oxidative stress. The most important objectives of this study consisted of separation, identification and characterization of nitrated proteins that can be considered biomarkers of cellular oxidative stress in sputum samples[31, 32]. For this, sputum samples were collected from a 28 years old patient with cystic

fibrosis and that also presented an infection with P. Aeruginosa. The samples were provided as a result of the collaboration with coleagues from Clinical University of Tuebingen, Germany.

The detection and the identification of nitrated proteins in sputum samples taken from the patient with cystic fibrosis was a project started in the research group supervised by Prof. Przybylski from University of Konstanz and continued at the University of Iasi. Initially, sputum sample was investigated by proteomics approaches. Proteomic approaches include: (i) separation of the proteins by 2D-gel, (ii) visualization of the nitrated proteins by Western blot (Wb), (iii) enzimatical digestion of proteins with trypsin, (vi) mass spectrometry analysis of resulted peptides and (v) identification of proteins/ possible biomarkers from sputum sample with the help of database search, UNIPROT (http://www.uniprot.org). Because of very low concentration of nitrated peptides detected by the immunological Western blot experiment, we couldn't detect the nitrated tyrosine site. Due to difficult extraction of proteins from this little spots, the analysis by mass spectrometry was deficitary. As previously shown, characterization of nitration sites is difficult to realize by mass spectrometry because of the low level of nitration in biological samples, where we do not interfere with any nitration agent as for *in vitro* experiments [35]. Therefore, it is difficult to identificate the nitration modifications by mass spectrometry if other oxidative modifications at tyrosine residue interfere (e.g. formation of hydroxi-tyrosine). For the first time in the literature the modification of tyrosine by hydroxylation was reported. All these results were recently published in JASMS, a journal of international prestige in the mass spectrometry field [36].

We got new sputum samples through our collaboration with Clinical University of Tuebingen, Germany and the primary objective was to identify proteins – biomarkers of oxidative stress. We got three types of samples: (i) sputum, (ii) sputum with high number of eosinophils and (iii) sputum with high number of neutrophils. Eosinophils and neutrophils are white blood cells and are involved in defence organism system against infections. In order to identify nitration in these granular proteins, in the first step the proteins were isolated from the sputum sample enriched in eosinophils and neutrophils. In normal physiological conditions, these white cells are in small quantities in blood, but their concentration is elevated in infections and inflammations. All samples were solubilized in a reducing buffer (using sodium-dodecil sulfate and urea) and then centrifugated for 20 minutes at 5000 rpm in order to determine the sedimentation of unsolubilized cellular material. The quantity of the extracted proteins in solution (1.8 - 2,7 mg/ml) was determined by classical Bradford method [33]. Approximately 15 μg of proteins mixture isolated

from sputum sample were separated by 1D-gel containing 12% polyacrilamide. Two gels were worked in the same time, one being colored by Coomassie Brilliant Blue for visualising separated protein (Figure1. A) and the second was used to transfer out the proteins separated in gel onto PVDF membrane, that was later used in an immunological Western blot experiment performed to localize possible nitrated peptides.

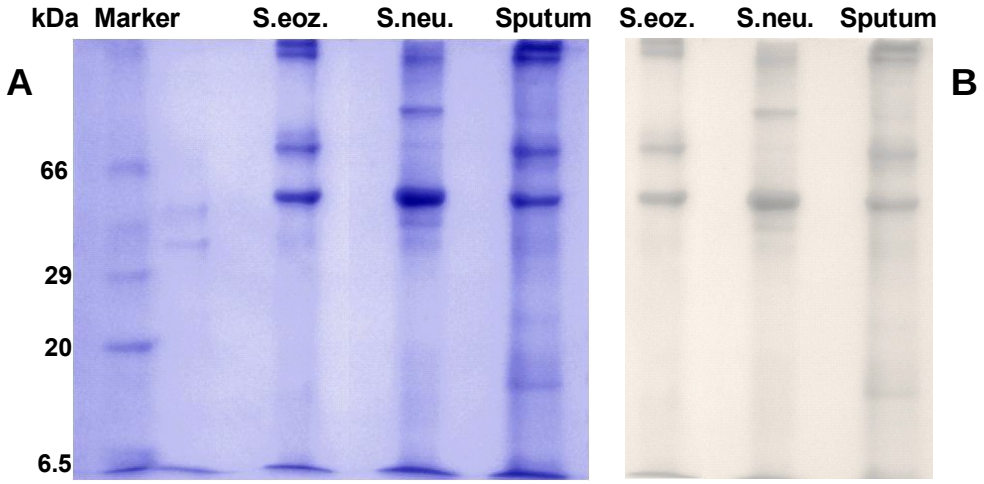


Figura 1. (A) 1-D SDS-PAGE electrophoresis of proteins from sputum of a patient who suffered of cystic fibrosis and who had an infection with P. aeruginosa. (B) Western blot experiment in which an anti-3-nitrotyrosine antibody was used to detect possible nitrated peptides.

The Western blot experiment indicates the presence of some nitrated proteins that will be analyzed by mass spectrometry. Next, we will verify the specificity of the antibody used in Wb experiment. Proteins separated by 1D-gel electrophoresis (Figure 1A) will be measured by mass spectrometry at University of Konstanz. We are optimistics that the results will be special and will be published in an next sciantific article, in which we will acknowledge the project for financial support.

Since during our research experiments hydroxy-tyrosine was also identified, we decided to investigate the specificity of the antibodies used in Western blot experiment towards peptides containing hydroxi-tyrosine. A number of model peptides (presented in Table 1) were synthesized by solid phase synthesis, using Fmoc (fluorenyl-9-metoxycarbonyl) strategy. The basic principle of solid phase peptide synthesis is the coupling of amino acids from the amino acid sequence from C-terminus to N-terminus. All the amino acids used in the synthesis were protected at N-terminus with Fmoc group and at the carboxyl group were free in order to be attach to the solid support called resin. The synthesis takes place within a few steps: (i) the coupling of the first aminoacid to the resin. The compound that assures the covalent interaction between the resin and peptide chain

is called a linker. This linker is important for resin's activation and for the linkage between the C-terminal aminoacid and the solid support (resin). (ii) In the next step the deprotection of the N-terminal aminoacid that is already coupled at the resin is performed. The deprotection is performed using N-metil-morfoline (NMM) disolved in di-metilformamide (DMF). (iii) In the third step, the next aminoacid is coupled. This step implies the activation of carboxyl group, that can be performed in the presence of compounds that present: (i) high reactivity, (ii) high coupling yield and (iii) high specificity. It is recomanded always to realize a double coupling. This cycle (deprotection of amino group of an aminoacid and coupling of the next aminoacid) is repeated until the wanted sequence of aminoacids is obtained. In the end the peptide is cleaved from the solid support using a mixture of 90% TFA (trifluoracetic acid), 5% TIS (triisopropil silan) and 5% water. After cleavage, peptide is precipitated overnight at -20^{o}C in diethylether and then is solubilized in 10% acetic acid.

Table 1. Characterization of model peptides by HPLC and *MALDI-ToF*

Peptide ligand			
No.	**Peptide sequence**	**HPLC** retention time (min) [c]	**MALDI-TOF** [d] m/z **[M+H]$^+$**
1	AMRAINN**Y**RWR[a]	27.88	1450.95
2	AMRAINN**Y(NO$_2$)**RWR[a]	32.90	1495.87
3	AMRAINN**Y(OH)**RWR[a]	27.35	1466.97
4	FAYG**Y**(**OH**)IEDLK[b]	25.23	1234.72

[a] peptide fragment (26-36) from Eosinophil cationic protein
[b] peptide fragment (204-213) from Leukocyte elastase inhibitor.
[c] RP-HPLC column: Vydac C_{18} (250 × 4 mm, 300Å, 5 μm); 365 nm for nitrotyrosine peptides and 220 nm for peptides with tyrosine and hidroxy-tyrosine
[d] Mass spectra were obtained with a Bruker Microflex MALDI-ToF mass spectrometer

These peptides were synthesized by solid phase synthesis and characterized by mass spectrometry. The synthesis was successfully performed, only some peptides needed purification by liquid chromatography. Two peptides were synthesized manually in the biochemistry Laboratory group at "Al. I. Cuza" University of Iași. Because of some difficulties (high reagents and time consumption, low yield), MSc. student Claudia Andrieș (research assistant in this project) realized a reseach stage in the Laboratory of Analitical Chemistry, University of Konstanz (July - august 2012). There she synthesized and characterized all the peptides included in Table 2. The pure

peptides were used in last stage of the project in order to study the interaction between the antibody anti-3-nitro-tyrsine and model peptides.

In the fird stage of the project (Stage III) we studied the aggregation of Abeta, a peptide involved in the patophisiology of neurodegenerative disease such as Alzheimer. Alzheimer disease is a progressive phenomenon of deterioration of cognitive functions that can lead to dementia. The main components of aggregates formed in Alzheimer disease brain are Abeta 1-42 and Abeta 1-40 polypeptides. Until now we investigated the Abeta 1- 40 peptide by a combination of methods such as H/D exchange & mass spectrometry (H/Dex-MS). This technique of exchange of hydrogens from a polypeptidic chain with deuterium atoms from D_2O is a method that can easily determine components that participate at the interaction of proteins with ligands (metals, antibodies, carbohydrates) and even at the protein-protein interactions (the case of the aggregation process). The result of the exchange of the hydrogen with deuterium can be easily observed by mass spectrometry as every exchanged hydrogen from the investigated molecule determines the modification of molecular weight with 1 Dalton (+1 Da) comparing to initial molecular weight. In proteic/ polypeptidic molecules are three types of hydrogens (those from amidic group, those from side chain and those from carboxi- and amino-terminal groups), but only the hydrogens from amidic groups have an exchange rate from some minutes to hours, while other hydrogens having a very small exchange rate (msec) can not be measured by mass spectrometry. H/D ex-MS method was optimized for Abeta 1-40 peptide study, a peptide with low stability in solution. Thus, Abeta 1-40 peptide was dissolved in hexafluoroisopropanol (HFIP) (stock solution), a solvent often used to dissolve this peptide which aggregates very fast in aqueous solutions. First by H/Dex-MS was investigated the global kinetics of Abeta (1-40) peptide (Figure 2.). Figure 2A indicates there is no big influence of the incubation time in D2O, an equilibrium is established after 15 minutes. Moreover Figure 2 B shows that the exchange of hydrogen with deuterium is not dependent on the peptide concentration. In order to detect the changes in Abeta (1-40) peptide during aggregation, we chose as experimental working conditions a concentration of peptide of 20μM and an incubation time in D_2O of 30 minutes.

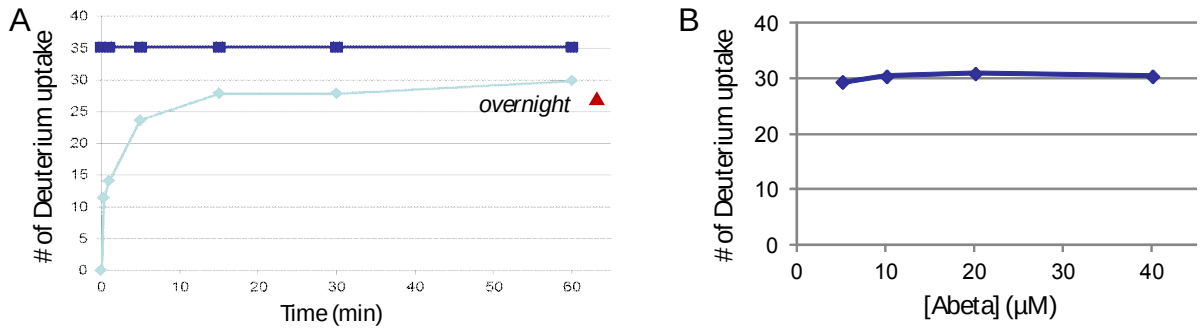
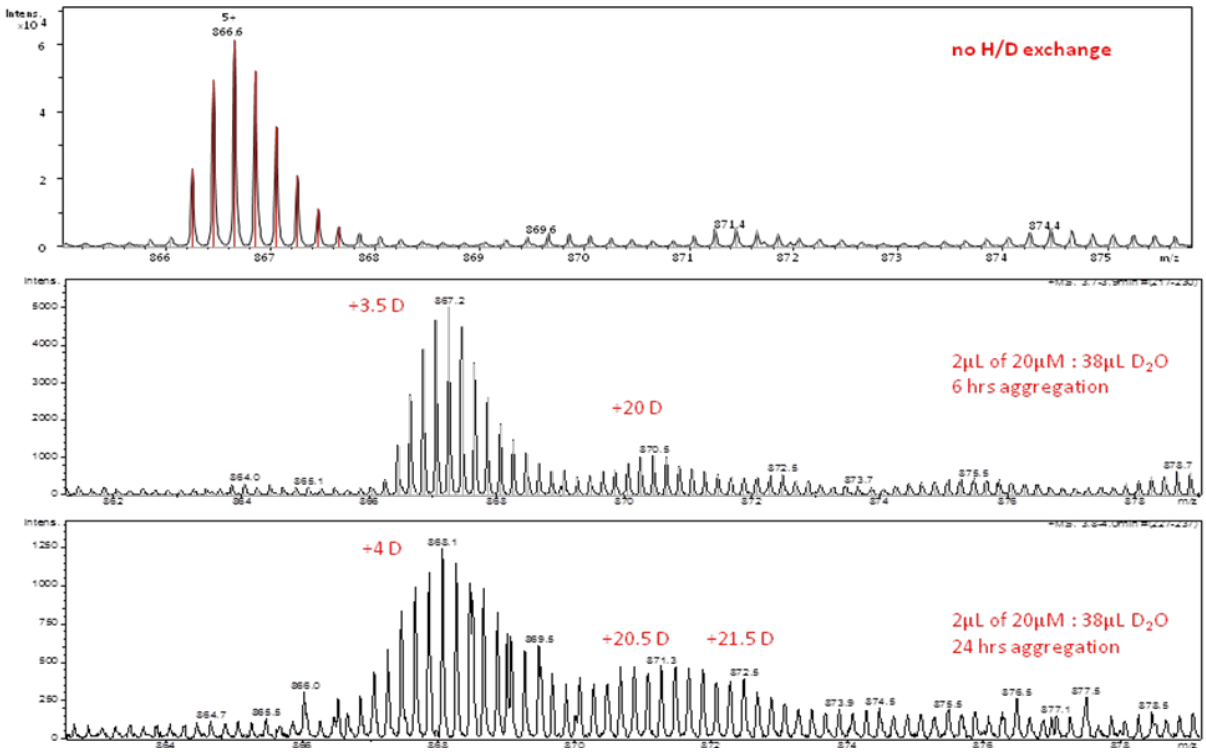


Figure 2. Global kinetics of Abeta(1-40) (A) depending on time and (B) depending on concentration, determined by H/D-ex – Ms experiments

From Abeta 1-40 stock solution, 50 µl were taken and diluted with PBS buffer and then the samples were incubated for different (6 hours, 24 hours, respectively 2 days, 4 days, 7 days). The results of these experiments are presents in Figure 2. For reproductibility all the experiments were realized in triplicates.



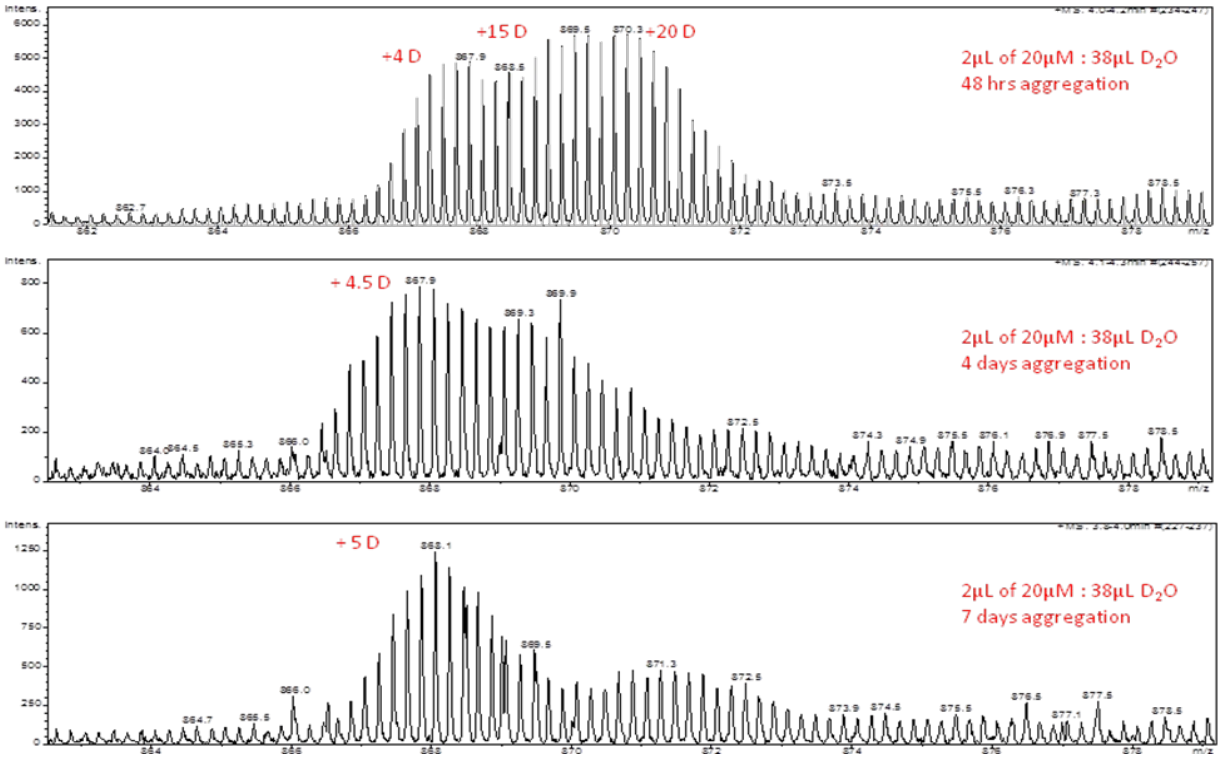


Figure 3. LC-MS spectra of Abeta (1-40) peptide generated after peptide's aggregation for different times in H/D exchange – MS experiments.

Mass spectra were recorded with an Electrospray – Mass Spectrometry (ESI-MS) instrument after the incubation of peptide in D_2O and inhibition of hydrogen/ deuterium exchange at 0^{o} C on ice by adding 1N HCl. In first spectrum we can notice multiple charged molecular ion $[M+H]^{5+}$ of Abeta 1-40 peptide, in next spectra is presented the multiple charged molecular ion $[\text{Abeta 1-40}]^{5+}$ at different incubation times. After 6 hours of aggregation we can notice an intense signal with 3-4 deuteriums incorporated in polypeptides sequence. It is interesting that after 48 hours of aggregation, in spectrum can be noticed more aggregate species and Abeta changes fewer hydrogens, it is not so dynamic in solution anymore and the solutions gets opalescent due to aggregation.

For confirmation of the results described above, for same aggregation times of 200μM Abeta 1-40 solution circular dicroism (CD) measurments were performed (Figure 4).

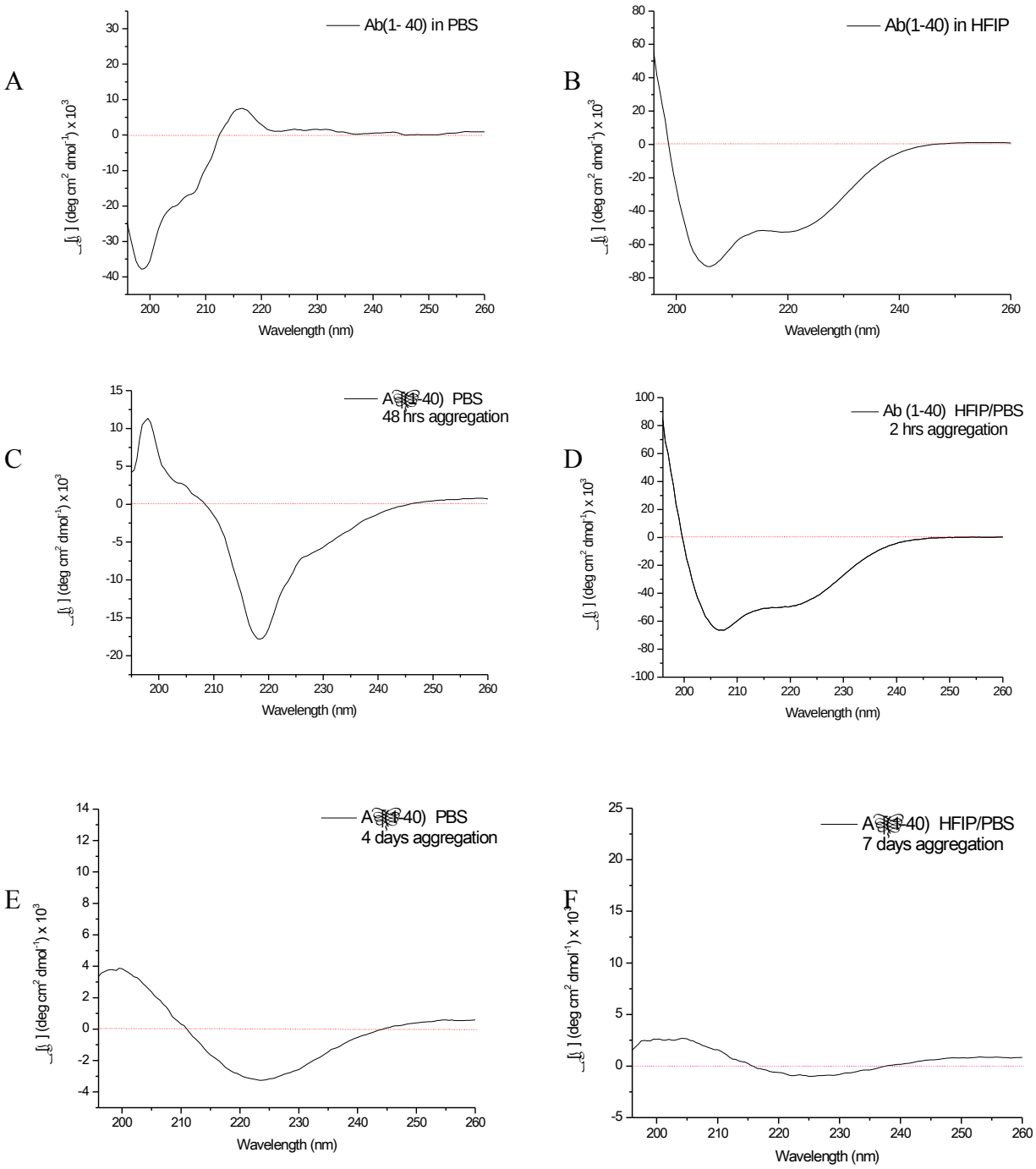


Figure 4. CD spectra of Abeta (1-40) peptide generated after the peptide's aggregation at different times. (A) Spectrum of peptide in PBS, (B) spectrum of peptide in HFIP that induces ordonated structure and (C-F) Abeta 1-40 at different aggregation times

The initial peptide in PBS buffer, at pH 7.4 presents a unordered structure. Incubation of Abeta 1-40 peptide in HFIP changes its native conformation into α-helicoidal shape. After the aggregation

process starts, for diluted solution in PBS from stock solution, peptides conformation becomes β-sheet, conformation described in literature as being the conformation in which Abeta-smyloid aggregates are formed progressively in the brain of the Alzheimer patients. Very interesting is the fact that after 4 and 7 days Abeta 1-40 peptide is found predominantly as aggregate and the measurments were difficult to realize because of the solution's opalescence. H/Dex-MS and CD experiments showed that in maximum 2 days the Abeta (1-40) peptide changes its dynamics and it's mostly included in aggregates formed *in vitro* experiments. These results will be combined with previously obtained results at the University Washington, St. Louis, USA and will be published in high ranking journal. Recent studies sugest that the oxidative modifications such as tyrosine nitration lead to increase of the formation of α-synuclein aggregates and oxidation of methionine residue in Abeta (1-40) peptide determines reduced neurotoxicity and formation of Abeta-amyloid aggregates is delayed. The perspective for future research is (i) to synthetize various polypeptide/protein fragments that are modified by oxidation and (ii) to induce by *in vitro* experiments the modifications mentioned above in order to study their behavior in aggregation process. For this we synthesized using Fmoc strategy some peptides presented in Table 2 [37]. Abeta 25-35 peptide was manually synthetized in Biochemistry Laboratory, "Al. I. Cuza" University, Iasi; humanine (presented in literature as a protective peptide against aggregation in Alzheimer pathology Alzheimer) [38], 1-19 sequence from Eosinophil cationic protein, another aggregating protein [39] and Abeta 1-40 were synthesized at University of Konstanz, Germany where C. Andries and L.Ion were visiting researchers (march – june 2013).

Table 2. *Characterization o new synthesized peptides by HPLC and MALDI-ToF*

Peptide	Sequence	HPLC Retention time (min)	MALDI-ToF m/z $[M+H]^+$ calc/exp
Aβ 25-35	GSNKGAIIGLM -OH	19.50	1060.28/ 1060.15
Humanine	MAPRGFSCLLLLTSEIDLPVKRRA	46.08	2687.3/ 2682.65
ECP (1-19)	RPPQFTRAQWFAIQHISLN	29.02	2310.6/ 2308.52
Aβ 1-40	DAEFRHDSGYEVHHQKLVFFAEDVGSNKGAIIGLMVGGVV	35.8	4329.9/ 4325.82

After synthesis, the peptides were characterized by high performance liquid chromatography (HPLC) in order to verify their purity and their molecular weight was determined by mass spectrometry. The separation of peptides by high performance liquid chromatography was performed on an analytical C_{18} column, and the detection wavelength was set to 220nm (specific to peptidic bond) and the purification of peptides was performed on a semi-preparativ column at

the same wavelength. For example Figure 5 shows the mass spectrum of ECP peptide immediately after synthesis (A), chromatogram for HPLC separation (B) and mass spectrum of ECP peptide after purification.

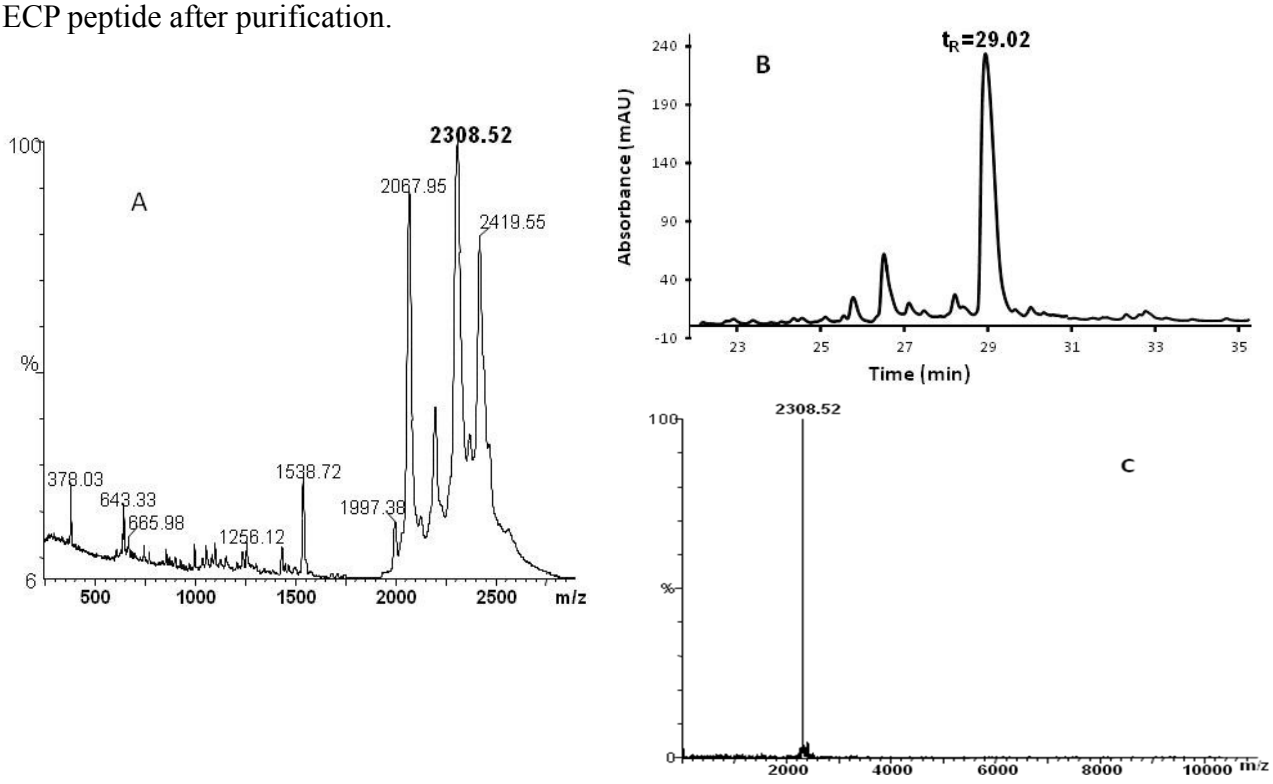


Figure 5. Characterization o ECP 1-19 peptide after synthesis; (A) MALDI-ToF mass spectrum immediately after synthesis; (B) HPLC chromatogram and (C) MALDI-ToF mass spectrum after purification.

Only one objective of Stage III couldn't be realized due to eosinophil cationic protein (ECP) degradation. We are looking for new samples from which we could extract native ECP and for this we established new connections with the Faculty of Medicine, UMF, Iasi. Eosinophils are white cells produced in the bone marrow and are found in blood stream and guts. Eosinophil granulocites contain three proteins (eosinophil cationic protein (ECP), eosinophil-derivated neurotoxin (EDN) and major basic protein (MBP)) and an enzyme eosinophil peroxidase (EPO) that is important for the defence of organism against infections. In healthy organisms, eosinophils are present in small quantities in blood, but they become predominant in association with different infections, inflammations and allergies. It is possible to isolate this white cells form blood only if their number is bigger than 10^{10} and for this we must find a patient with elevated number of eosinophils in blood.

In the third part of the project, we started the last activity of the first objective of the project related to the study of *in vitro* nitration of biological protein model. The proteins we have investigated were OmpA (outer membrane protein A) and VDAC (voltage dependent anion channel), received from our collaborators from Faculty of Biology, University of Konstanz. This two proteins were found to be nitrated in previous studies by using Western Blot, but because of the low level of nitration in biological samples, the nitration site could not be identified [40, 41]. Membrane proteins are conformational compact proteins which tend to keep this rigid conformation even when are isolated from membrane. The protein were nitrated by using peroxynitrite (PN) [42]. The proteins were first separated by 1D SDS-PAGE, followed by transferring to nitrocellulose paper in order to see the effect of peroxynitrite on the protein by Western blot technique using an antibody anti-3-nitrotyrosine (Figure 6). Very interesting was the fact that the VDAC protein was not found to be nitrated as the results of their exposure at two different PN concentrations (5μM şi 50μM) by Western Blot method. This may be due to the different localization of tyrosine in the sequence of the VDAC protein beside the OmpA which react with PN. The Western Blot experiment was performed in duplicate and the result was the same. Next we used a molecular modeling program, able to visualized tyrosine residue, the most reactive amino acids for nitration. Furthermore we nitrated proteins with PN and after in gel digestion of nitration site, was able to identify by mass spectrometry.

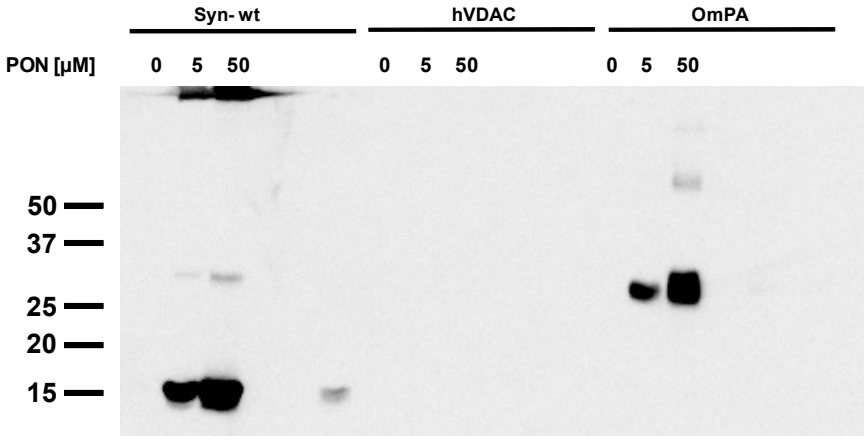


Figure 6. Western blot for nitrated peptide using different PN concentrations: synuclein, VDAC and OmpA (from left to right).

We consider also investigation of *in vitro* nitration of the two membrane porins with another oxidative agent SIN-1. 3-Morpholinosyndnomine (SIN-1) is used in biochemical studies as peroxynitrite donor ($OONO^-$) because after decomposition in aqueous solutions produce both

nitric oxide (NO) and superoxide anion (O_2^-), reactive species which contributes at *in vivo* nitration mechanism [43].

In the final stage of the project we used the peptide synthetized at the end of part II for affinity studies of the antibodies used in the immune-affinity experiments for sputum samples. The experiments were done at University of Konstanz, Germania, were Dr. Brindusa Alina Petre had in august 2013 a research internship. To avoid large consumption of large amount of antibody (100µg cost ~ 350 Euro) used in ELISA experiment initially proposed, we used SAW-biosensor which determine the affinity and affinity constants based on a piezoelectric mechanism. For a first viewing of antibody interactions against nitrated tyrosine we used Dot Blot technique (Figure 7). The peptide (1µg/µl) were placed on a nitrocellulose membrane using a micropipette, then the membrane was immersed in a block agent (Roti-block, Sigma - Aldrich, Germany) in order to prevent non-specific binding of the antibody to the membrane. After five washing steps using phosphate buffer solution, pH 7.5, the membrane was immersed in phosphate buffer after adding the anti-3 nitrotyrosine antibody (2500:1). Dot Blot experiment was performed for two types of antibodies, we notice that only one is specific to oxidative modified peptides (Figure 7. A) and the second one is non-specific, detecting the unmodified peptide (Figure 7.B). Only the specific antibody was used in the next SAW-biosensor studies.

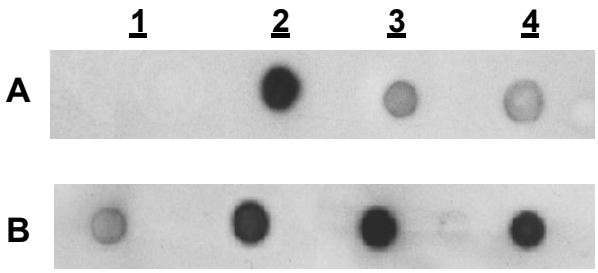


Figure 7. Dot blot experiment for nitrated peptides using a specific antibody (A) and a non-specific antibody (B).

SAW-biosensor technologies uses quartz chip with 5 independent channels which are cover with gold [44]. The piezoelectric acoustic sensor applied oscillating electric field to create a mechanical wave that propagates on surface and is then converted back to an electric field to be measured [45]. Initial on chip area is formed a SAM (self-assembled monolayer) by immersing the chip in 16-mercaptohexadecanoic acid, 12 h at 25°C. Immobilization of synthetic peptide on chip surface is done after prior activation of carboxylic group of SAM (self-assembled monolayer) using 200

mM N- (3-dimethylaminopropyl) carbodiimide and as coupling agent 50 mM N-hydroxysuccinimide (NHS). Each peptide (10µM) was immobilized manually on two adjacent channels; the one in the middle is left free to prevent possible contamination between peptides. After peptide immobilization, NHS groups remaining uncoupled were blocked with 1 mM ethanolamine for 1 h at room temperature. The chip is introduce into the biosensor and equilibrated at optimum pH for affinity studies (phosphate buffer, pH 7.5, for 30 minutes, 20 µl min^{-1}). After this time has passed over the chip is added 150 µl anti 3-nitro-tirosine antibody at different concentration (5 nM, 10 nM and 20 nM). The interaction between immobilized peptides and antibody was dissociated in the presence of 1mM glycine which is distributed on chip surface. The peptide affinities provided by the SAW biosensor are presented in Figure 8, for a 5 nM concentration. Can be notice that all peptides oxidative modified (nitrated and hydroxylated) present high affinity to the antibody used (Figure 8. B, C, D), while the peptide containing tyrosine showed no affinity (Figure 8.A).

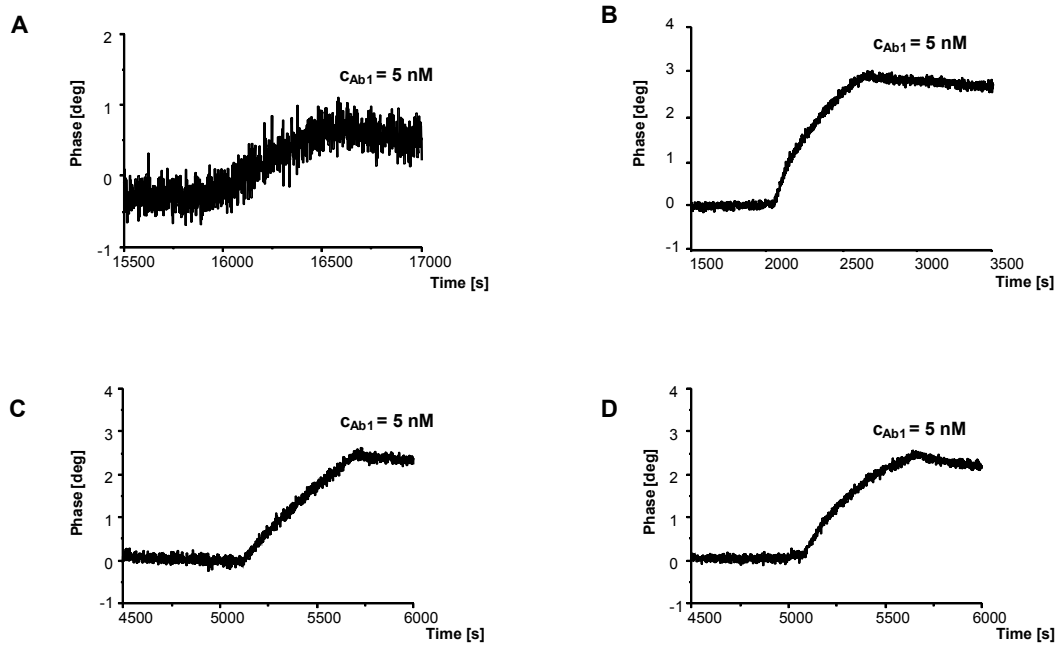


Figure 8. Affinity curve of model peptide (A) peptide 1, (B) peptide 2, (C) peptide 3, (D) peptide 4 to anti-3-nitro tyrosine antibody.

SAW – biosensor instrument was able to determine also the dissociation constants (**Kd**) of the antibody which were extracted from affinity data by using a macromolecular mathematic model from FitMater and OriginePro 7.5 and a linear regression $\mathbf{Kd} = K_{off}K_{on}^{-1}$**.** All data obtained from Dot Blot and SAW – biosensor instruments are summarized in Table 3.

Table 3. Characterization of model peptides and experimental data on their affinity to anti-3-nitro-tyrosine antibody

Peptide ligand				Antibody binder		
No.	**Peptide sequence**	**HPLC retention time (min) [c]**	**MALDI-TOF [d] m/z [M+H]$^+$**	**Anti 3-nitro Ab 1**		**Anti 3-nitro Ab2**
				Dotblot [e]	**Kd (10^{-9} M) [f]**	**Dotblot [e]**
1	AMRAINNYRWR[a]	27.88	1450.95	-	-	+
2	AMRAINNY(NO_2)RWR[a]	32.90	1495.87	+	**0,33**	+
3	AMRAINNY(OH)RWR[a]	27.35	1466.97	+	**5,65**	+
4	FAYGY(OH)IEDLK[b]	25.23	1234.72	+	**8,77**	+

[a] Fragment peptide (26-36) from Eosinophil cationic protein.
[b] Fragment peptide (204-213) from Leukocyte elastase inhibitor.
[c] RP-HPLC column: Vydac C_{18} (250 × 4 mm, 300Å, 5 μm); 365 nm for peptides with nitro – tyrosine and 220 nm for peptides with tyrosine and hydroxi – tyrosine.
[d] Mass spectrum were obtained using a Bruker Microflex MALDI-ToF.
[e] Affinity using Dot blot; (+) affinity for anti-3nitrotyrosine antibody; (−) no-affinity for anti-3nitrotyrosine antibody.
[f] Dissociation constant value (***Kd)*** determined by peptides immobilization on the chip surface. (-) peptide **1** showed no affinity for anti-3nitrotyrosine antibody **1**.

The data obtained in the last part of the project will be the subject of a new publication, early next year. In all submitted publications we will mention the financial support received from this project.

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