

ELECTROSPRAY MASS SPECTROMETRY EVIDENCE FOR THE NON-COVALENT COMPLEX FORMATION BETWEEN BETA- CYCLODEXTRIN AND BETA-AMYLOID (1-40) PEPTIDE AT NEUTRAL pH

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Abstract: Alzheimer’s disease is characterized by progressive cognitive decline and neuronal loss. Amyloid beta peptide consisting of 39-42 amino acids is prone to aggregation forming oligomers and amyloid plaques which are considered causes of neurotoxicity. One of the most studied strategies for preventing beta-amyloid aggregation is relying on the hypothesis that non-covalent bound substances may reduce the aggregation of beta-amyloid. Considering previous studies which demonstrate the formation of non-covalent complex between beta-amyloid (1-40) and beta-cyclodextrin using a denaturing chemical environment and considering the results regarding beta-cyclodextrin presence in the rat plasma and in organs after administration of certain medications containing beta-cyclodextrin the present study aims to evaluate the interaction of beta-cyclodextrin and beta-amyloid (1-40) at neutral pH in ammonium bicarbonate. The analysis was performed using an electrospray-triple quadrupole mass spectrometer coupled to a liquid chromatograph using the flow injection analysis technique. Additionally, the cleavage using trypsin of the beta-amyloid (1-40) peptide after non-covalent complex formation was performed for the purpose of identifying the minimal peptide sequence involved in the interaction with beta-cyclodextrin. The results indicate the formation of the non-covalent complex in the chemical environment employed. Further studies using transgenic Alzheimer’s Disease mice models expressing human beta-amyloid may clarify whether oral administration of beta-cyclodextrin has beneficial effects to the health of Alzheimer’s disease patients.

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Introduction

Alzheimer's disease represents a health concern for the society because at global level it affects more than 30 million people. It is estimated that the number of persons suffering from this disease will increase. The disease reduces life expectancy and the quality of the life for patients and their families.¹

The clinical picture and the histological characteristics of Alzheimer's Disease including amyloid plaques, neurofibrillary tangles, and arteriosclerotic changes were first described by the German psychiatrist Alois Alzheimer in 1906. In the last 20 years a special attention was given by the scientists to the progressive neurodegenerative effect exerted by beta-amyloid oligomers and amyloid plaques. The amyloid plaques are composed of fibrils resulted from the aggregation of many A β peptide molecules. A β peptide contains 39-42 amino acids which are covalently bound to form the amino acid sequence Asp-Ala-Glu-Phe-Arg-His-Asp-Ser-Gly-Tyr-Glu-Val-His-His-Gln-Lys-Leu-Val-Phe-Phe-Ala-Glu-Asp-Val-Gly-Ser-Asn-Lys-Gly-Ala-Ile-Ile-Gly-Leu-Met-Val-Gly-Gly-Val-Val-Ile-Ala. A β peptides are liberated in the extracellular medium after the cleavage of amyloid precursor protein by γ - and β -secretases.¹

Removal of A β peptide molecules from human blood by interaction with specific antibodies or low molecular weight molecules that inhibit oligomerization of the A β peptide could lead to a reduction of the amount of these peptides in the central nervous system followed by a decrease of oligomerization and fibrilization processes.^{2,3}

β -cyclodextrin is a cyclic oligosaccharide formed by seven D-glucopyranose monosaccharides which are linked through α -1,4 bonds (Figure 1). Beta-cyclodextrin is used often in medications due to its chemical properties of forming non-covalent complexes with certain drugs. Various administration routes were employed in different studies in the case of beta-cyclodextrin-drug nanoparticles and a biodistribution study was carried out by liquid chromatography coupled with mass spectrometry. The results of this study indicated the presence of β -cyclodextrin in blood and organs and its complete removal from blood after 36 hours.⁴ To the author knowledge this is the first quantification study of β -cyclodextrin in biological matrices by liquid chromatography coupled with mass spectrometry. The results obtained by the researchers could be employed for the design of future studies carried out using laboratory animals or humans. Furthermore, the presence in rat blood and organs of β -cyclodextrin raises the question whether similar absorption and biodistribution processes occur at human individuals and whether β -cyclodextrin would interact with human β -amyloid peptides at neutral pH.

Patrick Camilleri and his collaborators investigated the interaction between A β (1-40) and β -cyclodextrin using a mass spectrometer equipped with an electrospray source and a single quadrupole mass analyzer. Although A β (1-40) peptide and β -cyclodextrin were solubilized in a solvent containing water, methanol and acetic acid which are denaturing experimental conditions, a strong signal corresponding to the protonated molecular complex between one molecule of β -cyclodextrin and one molecule of A β (1-40) carrying four positive charges was observed in the mass spectrum. Addition of β -cyclodextrin in the medium of rat pheochromocytoma cells (PC12) decreased the neurotoxicity of A β (1-40).⁵

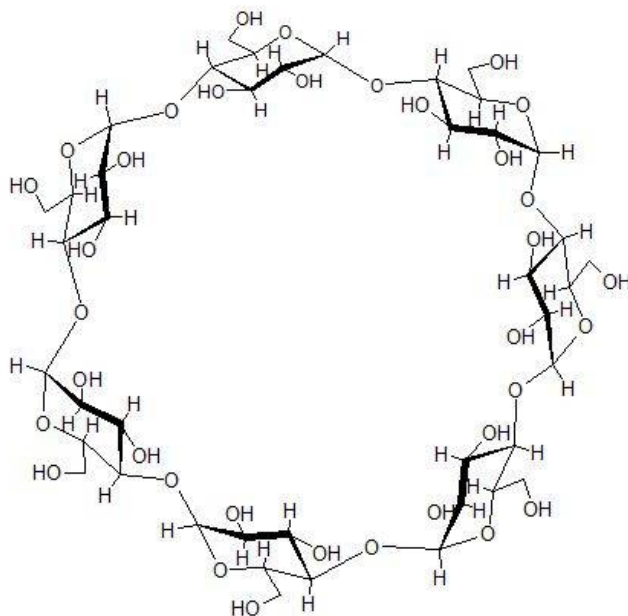


Figure 1. Molecular structure of beta-cyclodextrin.

The present study aims to investigate the non-covalent complex formation between molecules of β -cyclodextrin and A β at the pH of 7.35. The chemical environment employed is ammonium bicarbonate which is compatible with electrospray ionization mass spectrometry analysis.

Results and Discussion

Electrospray ionization in mass spectrometry is a gentle ionization technique which allows the analysis of intact protonated organic molecules. In the first experiment the mass spectrum of a 50 pmol/ μ L β -amyloid (1-40) prepared in 1 mM ammonium bicarbonate at the pH of 7.35 and incubated at 37°C for 30 minutes was obtained and the presence of multiply protonated molecules of the peptide was observed. Based on the mass spectrum the molecular structure of β -amyloid was assigned to the signals observed as shown in the annotations presented in the Figure 2 and

explained in table 1. An additional signal was observed in the mass spectrum recorded for pure β -amyloid (1-40) at m/z value 1237.3826. The signal was assigned to the protonated dimer of the $A\beta(1-40)$ peptide as explained in the table 1.

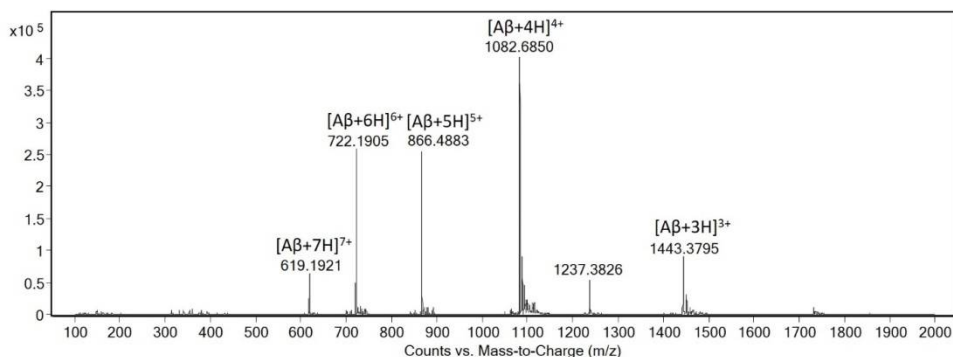


Figure 2. Mass spectrum of β -amyloid (1-40) peptide solubilized in 1 mM ammonium bicarbonate.

In a second experiment an equimolar mixture of 50 pmol/ μ L of β -cyclodextrin and $A\beta(1-40)$ were incubated in 1 mM ammonium bicarbonate at the pH of 7.35 and at 37°C for 30 minutes. The protonated molecules of $A\beta$ with 3, 4, 5, 6 and 7 protons were observed in the mass spectrum shown in the Figure 3a. The mass spectrum acquired indicates the presence of a protonated molecule of β -cyclodextrin denoted in the mass spectrum BCD. Additionally, a β -cyclodextrin adduct with potassium was observed as presented in the Figure 3b. The non-covalent complexes of one molecule of β -cyclodextrin and one molecule of $A\beta(1-40)$ protonated with 4 respectively 3 protons were identified at m/z 1366.28 and m/z 1821.37 (table 1). The signals which can be observed in the figures 3a) and 3b) at m/z values of 1237.3826 and 1732.1751 were not annotated in the figures. However in the table 1 is specified that these two m/z values are assigned to the protonated dimer of the peptide $A\beta(1-40)$.

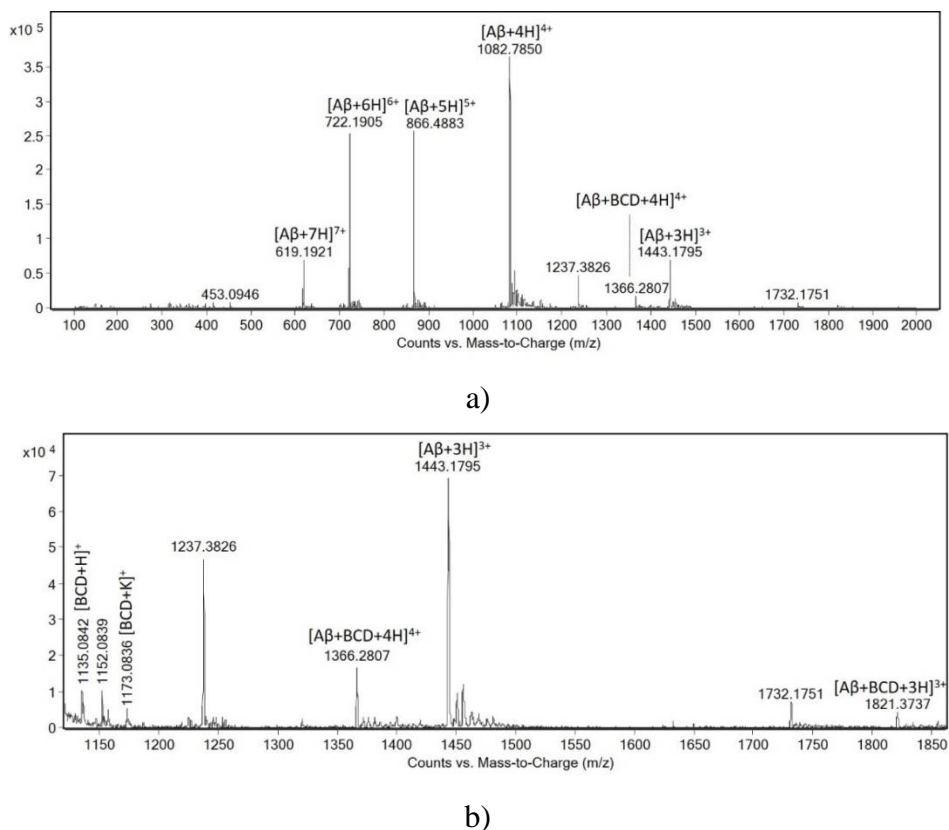


Figure 3. Mass spectrum of a 50 pmol/ μL equimolar mixture of beta-amiloid (1-40) peptide and beta-cyclodextrin in 1 mM ammonium bicarbonate : a) presentation of the range of m/z values 100-2000; b) detailed representation of the m/z values range 1100-1900.

The present study employed a method previously published for the observation of the molecular complexes of $\text{A}\beta(1-40)$ and oleuropein. In a further experiment the proteolysis with trypsin was performed according to the method described in the previous study.⁶ First the peptide $\text{A}\beta(1-40)$ was incubated with β -cyclodextrin and the formation of the complex was verified by flow injection analysis at the electrospray - triple quadrupole mass spectrometer. The presence of the non-covalent complex was confirmed and trypsin was added to the reaction mixture and the vial was incubated for 30 minutes at 37°C.

Table 1. List of protonated molecules and molecular complexes identified in the mass spectra.

Protonated molecule/ molecular complex	Annotation	Specification average/ monoisotopic mass	m/z experimental	m/z calculated
Aβ(1-40)	[M+3H] ³⁺	average	1443.3795	1443.0620
	[M+4H] ⁴⁺	average	1082.6850	1082.5483
	[M+5H] ⁵⁺	average	866.4883	866.2401
	[M+6H] ⁶⁺	average	722.1905	722.0346
	[M+7H] ⁷⁺	average	619.1921	619.0307
Aβ(1-40) dimer	[M+M+5H] ⁵⁺	average	1732.1715	1732.5462
	[M+M+7H] ⁷⁺	average	1237.3826	1237.8210
β-cyclodextrin	[BCD+H] ⁺	monoisotopic	1134.9891	1135.3770
	[BCD+K] ⁺	monoisotopic	1173.0836	1173.3329
Molecular complex	[A β +BCD+4H] ⁴⁺	average	1366.2807	1366.1408
	[A β +BCD+3H] ³⁺	average	1821.3737	1821.1853

The mass spectrum acquired includes protonated molecules which correspond to all the proteolytic fragments resulting after cleavage by trypsin in silico. β -cyclodextrin alone is present in the mass spectrum but a complex between intact A β (1-40) and β -cyclodextrin could not be observed. However the A β (1-40) sequence binding to β -cyclodextrin was not cleaved indicating a shielding effect of the cleavage sites for trypsin within the amino acid sequence of A β (1-40).

In the present study beta-cyclodextrin forms protonated molecular complexes with beta-amyloid (1-40) peptide. The molecular complexes contain 3 protons and 4 protons indicating that a more folded conformation of A β (1-40) interacts specifically with beta-cyclodextrin while the more

unfolded conformations of A β (1-40) do not present specific binding. A second possibility which does not exclude the previous explanation is that a domain of beta-amyloid is entering in the cavity formed by the beta-cyclodextrin molecule and the protonation sites are not accessible. According to the study published by di Cagno beta-cyclodextrin possesses a cavity which has hydrophobic properties and a hydrophilic external surface. Based on the results of the present study we hypothesize that the cavity might interact with part of the sequence (17-40) of beta-amyloid peptide and the hydrophilic functional groups at the external surface of beta-cyclodextrin might interact with the hydrophilic domain of beta-amyloid peptide.⁷

Experimental

Chemicals and materials

A β (1-40) peptide amidated at the carboxyl-terminal end, trifluoroacetate salt was purchased from Bachem AG, Bubendorf, Switzerland. β -ciclodextrin (purity higher than 99%), 1,1,1,3,3,3-hexafluoro-2-propanol (HFIP) and ammonium bicarbonate were purchased from Sigma-Aldrich, Saint Louis, Missouri, USA. Ammonium acetate was obtained from Honeywell Speciality Chemicals Seelze GmbH, Seelze, Germany. Acetonitrile was of HPLC, UHPLC and LC-MS purity and were obtained from VWR Chemicals (Rosny-sous-Bois, France). Formic acid was purchased from Merck, Darmstadt, Germany. Ultrapure water (0.055 μ S/cm c) was produced by an Arium Mini Plus UV system (Sartorius Lab Instruments GmbH & Co., Goettingen, Germany). Sequencing grade modified trypsin was obtained from Promega Corporation, Madison, Wisconsin, USA.

Stock solutions preparation

A solution of beta-amyloid (1-40) in 1,1,1,3,3,3-hexafluoroisopropanol at the concentration 1 mg/mL was prepared as described in a previous study. Beta-cyclodextrin was solubilized in ultrapure water at the concentration 1600 pmoli/ μ L and was stored at 4°C.

Non-covalent molecular complex formation at pH 7.35

For the investigation of the non-covalent complex between beta-cyclodextrin and A β (1-40) a solution containing 50 pmol/ μ L of each of the chemical compounds was prepared in 1 mM ammonium acetate. 10 μ L of A β (1-40) with the concentration 1 μ g/ μ L were pipetted in a Protein LoBind microreaction tube and was allowed to dry at room temperature for 2 hours after opening the lid. A 10 mM solution of ammonium bicarbonate was prepared and the pH was adjusted to 7.35 with 10% acetic acid in ultrapure water. 10 μ L of stock solution of beta-cyclodextrin were mixed with 310 μ L of a solution 1.03225 mM ammonium bicarbonate to produce a solution of 50 pmol/ μ L beta-cyclodextrin in 1 mM ammonium bicarbonate. 46.2 μ L of this solution was added to the microreaction tube containing 10 μ g of dried A β (1-40).

Flow injection analysis using LC-ESI-triple quadrupole

The mass spectrometry analyses of the non-covalent complex were performed by a 6410 triple quadrupole LCMS system equipped with an electrospray source and coupled to an Agilent 1200 liquid chromatograph containing a solvent compartment, a degasser (G1379B), a binary pump (G1312A), an autosampler (G1329A) a thermostatted column compartment (G1330B). Solvent A was 99.9% ultrapure water containing 0.1% formic acid and solvent B contained 80% acetonitrile, 19.9% ultrapure water, 0.1% formic acid. By mixing solvents A and B using the ratio of 12.5%

solvent B and 87.5 % solvent A a solution containing 10 % acetonitrile and 90% ultrapure with 0.1% formic acid was obtained. The solution was pipetted in the vial containing a 250 μL glass insert. A volume of 10 μL was injected in the mobile phase using a flow rate of 0.05 mL/min.

The electrospray triple quadrupole was operated in positive mode. The mass spectra were acquired in the mass range of m/z 100-2000. Source parameters were a nebulizer pressure of 35 psi, a drying gas temperature of 325°C, a capillary voltage of 4000 V.

Conclusions

The method presented relies on a previously developed method used for establishing the formation of a non-covalent complex at neutral pH in ammonium bicarbonate for beta-amyloid (1-40) and oleuropein. The usage of exactly the same experimental conditions led to fast observation of non-covalent complex between beta-cyclodextrin and $A\beta(1-40)$. However, the study of non-covalent complexes by liquid chromatography coupled to mass spectrometers equipped with electrospray sources require often variation of ammonium acetate or ammonium bicarbonate concentration depending on the chemical properties of the molecules involved in the interaction and more gentle ionization conditions.

The results presented in this study indicate that oral administration of beta-cyclodextrin may lead to formation of non-covalent complexes with free beta-amyloid (1-40) from blood. The presence of beta-cyclodextrin and beta-amyloid in urine was previously demonstrated. Further experiments could be performed which may clarify whether the non-covalent complex formation is beneficial for the organisms receiving medications containing beta-cyclodextrin.

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