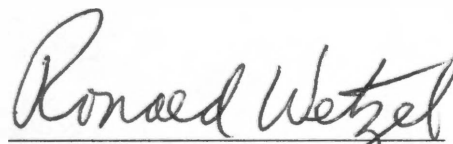
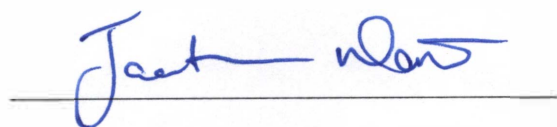


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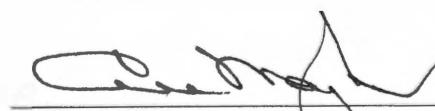

Ronald Wetzel, Major Professor

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**STIMULATORS OF A β AGGREGATION IDENTIFIED THROUGH DRUG
SCREENING**

**A Thesis
Presented for the
Master of Science
Degree
The University of Tennessee, Knoxville**

**Matthew Sega
May 2003**

Dedication

This thesis is dedicated to the memory of Andrew “Drew” Sega.

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Abstract

Alzheimer's disease (AD) is a neurodegenerative disorder characterized by extensive deposition of amyloid plaques in the brain. These plaques contain an amyloid fibril core that is formed through self-aggregation of the β -amyloid peptide ($A\beta$), a proteolytic product of amyloid precursor protein (APP). $A\beta$ aggregation is currently thought to play a causative role in the development of AD, and thus is an attractive target for potential therapeutics. A library of pharmaceutically active compounds (LOPAC) was screened for potential inhibitors of $A\beta$ aggregation using a 96-well microplate assay. No inhibitors were found in the library, but, surprisingly, several compounds exhibited a stimulatory effect on $A\beta$ deposition. Two of the most potent stimulatory compounds from the library (NPC 15437 and calmidazolium chloride) were chosen for more detailed studies, with the hope that they might offer some insight into mechanistic and structural aspects of $A\beta$ aggregation, as well as providing possible ideas for drug development. NPC 15437 was found to stimulate the nucleation phase of $A\beta$ aggregation in a concentration dependent manner through an interaction with monomeric $A\beta$ peptide. Calmidazolium chloride at a concentration of 100 μ M was observed to stimulate the formation of an aggregated state of $A\beta$ that is clearly distinct from mature amyloid fibrils. Based on these findings the compounds described here (a) could possibly offer insight into the mechanism by which amyloid is formed, as well as the chemical structures that are important to amyloid formation, (b) could be used to test the amyloid hypothesis in mouse models of AD, and (c) may be of some importance in the development of AD therapeutics

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I. Background

Amyloidosis is the term for a series of diseases involving soluble proteins aggregated into highly ordered structures termed amyloid fibrils [Sipe, 1992 #1]. Rudolf Virchow first applied the term “amyloid” – meaning “starchlike” – to these diseases in 1853 based on the staining properties of extracellular deposits in human tissue [Selkoe, 1991 #2]. However, it is now known that amyloid deposits are actually composed of proteins, and that they have several common features. They have a high content of β -pleated sheet secondary structure, as well as a high affinity for the histochemical stain Congo red and the fluorescent dye thioflavin T (ThT) [Sipe, 1992 #1]. They are also insoluble under physiologic conditions, and are resistant to proteolytic digestion [Sipe, 1992 #1].

Amyloidosis occurs in many human diseases, including type II diabetes, Creutzfeldt-Jakob Disease, rheumatoid arthritis, Huntington’s Disease (HD), and Alzheimer’s Disease (AD) [O’Brien, 2000 #3]. The peptide associated with the amyloid deposits in AD is the amyloid β -protein ($A\beta$) [Selkoe, 1991 #2]. $A\beta$ exists in many forms, ranging from 39 to 43 amino acids in length [Gandy, 1992 #4]. It is a product of the cleavage of the larger membrane bound amyloid precursor protein (APP) [Selkoe, 1989 #5]. The precise physiological function of APP is unknown, although there is a consensus sequence present at the carboxy terminus that is known to mediate endocytosis of cell surface proteins [O’Brien, 2000 #3]. It is possible that APP function is related to neuronal repair and regeneration [O’Brien, 2000 #3].

One hypothesis that has gained popularity is that $A\beta$ deposition in plaques in brain tissue is the causative factor in AD, and that all other pathological symptoms

(neurofibrillary tangles, cell loss, vascular damage, and dementia) follow as a result of this initial deposition [Hardy, 2002 #6]. Most of the strong evidence in favor of this hypothesis is genetic. Early onset forms of AD have been observed to cosegregate with mutations in APP [Hardy, 2002 #6]. Most of these mutations have been found to map at or near cleavage sites in the primary sequence of APP that are targeted by the proteases β - and γ - secretase, and therefore generate A β by favoring proteolytic processing of APP [Hardy, 2002 #6]. Other familial AD causing mutations that are internal to the A β sequence have been linked to changes in aggregation kinetics [Hardy, 2002 #6]. In addition, the gene encoding APP has been localized to chromosome 21, and individuals with trisomy 21 (Down's Syndrome) inevitably exhibit the neuropathology of AD [Masters, 1985 #7]). It is assumed that the third copy of chromosome 21 leads to higher levels of A β [Masters, 1985 #7]. Finally, the other known autosomal dominant gene mutations that cause early onset AD, the presenilin mutations, have been shown to increase the production of A β (1-42) [Hardy, 2002 #6]. The presenilin genes (PS1 and PS2) may alter APP metabolism by a direct effect on γ -secretase [Hardy, 2002 #6]. All of these observations are consistent with the theory that A β aggregation plays a causative role in AD.

However, there are some concerns with the amyloid hypothesis. For example, the number of amyloid deposits in AD brains does not always correlate with the amount of cognitive impairment, while deposition of tau protein in neurofibrillary tangles does seem to correlate with it [Hardy, 2002 #6]. However, recent findings have suggested that tau alterations occur after alterations in APP processing, and also that A β toxicity is tau

dependent [Hardy, 2002 #6]. These observations have led to a theory that smaller aggregated forms of A β may in fact be the true disease causing agents [Hardy, 2002 #6]. Thus, it seems reasonable that drug design strategies that target either A β production or A β aggregation are viable therapeutic approaches [Esler, 1997 #8].

To be able to target A β aggregation as a therapeutic approach, it is necessary to understand the stages and kinetics of A β fibrillization. The pathway of aggregation of A β from peptide to amyloid fibril appears to involve quaternary structural intermediates [Kelly, 1998 #9]. Circular dichroism (CD) has shown that A β fibrils are rich in β -sheet secondary structure [Harper, 1997 #10] of a specific type called a “cross- β ” conformation [Serpell, 2000 #11]. Fibrils also show a high amount of protection against hydrogen-deuterium exchange of the amide protons along the peptide backbone, which has been attributed to extensive hydrogen bonding in the β -sheet core of the fibrils [Kheterpal, 2000 #12].

Analytical ultracentrifugation, electron microscopy, atomic force microscopy, and light scattering have all shown that A β (1-40) at concentrations well above the critical concentration (C_r) (see below) exists as both high weight fibrillar oligomers, and also slightly lower weight oligomers [Harper, 1997 #10]. The presence of the smaller oligomeric form of A β (1-40) suggests that the fibril assembly pathway of A β (1-40) involves discrete intermediates [Harper, 1997 #10]. These intermediates have been visualized by electron microscopy [Nybo, 1999 #14]. The earliest observed intermediates are globular aggregates about 4-5 nm in diameter [Nybo, 1999 #14]. These intermediates then associate to form short protofilaments (10-20 nm in diameter), which in turn form

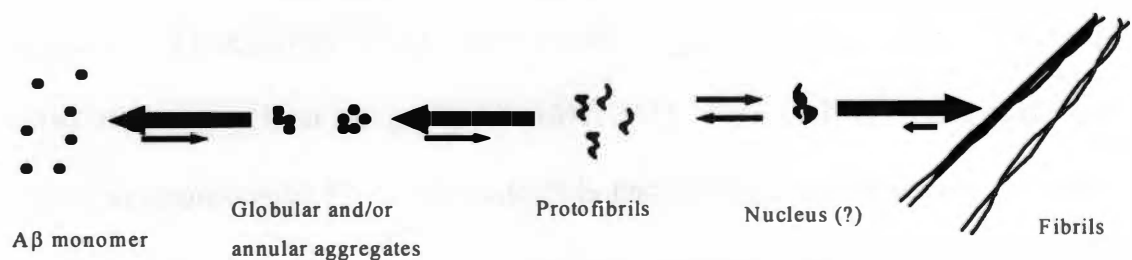
protofibrils, and subsequently amyloid fibrils through lateral association [Nybo, 1999 #14].

Protofibrillar structures have been observed in the polymerization of both A β (1-40) and A β (1-42), suggesting that they are a common intermediate in all A β fibrillogenesis [Walsh, 1997 #15]. Protofibrils have also been shown to be less resistant to hydrogen-deuterium exchange (I. Kheterpal and H. Lashuel, unpublished) than fibrils [Kheterpal, 2000 #12], suggesting that early intermediates are not as rich in β -sheet structure as fully formed fibrils. It has also been shown that formation of an oligomeric, α -helix rich intermediate is common to fibril formation in 18 forms of A β peptide [Kirkitaдзе, 2001 #16]. It is not clear which intermediate on the fibril formation pathway, if any, this α -helical structure represents and its significance in fibrillogenesis remains a matter of debate [Kirkitaдзе, 2001 #16]. It should be noted that all of the observed intermediates (early globular aggregates, protofilaments, and protofibrils) have only been seen *in vitro*. Even so, there is still a possibility that these non-amyloid oligomeric intermediates may be the toxic species in A β amyloidosis [Walsh, 1997 #15]. If so, protofibrils would be an attractive target for potential therapeutic agents.

The presence of a protofibrillar intermediate has been interpreted as being consistent with the theory that A β aggregation follows a nucleation dependent pathway [Harper, 1997 #10]. Several features characterize nucleation dependent polymerization. First, there is no aggregation at peptide concentrations below the critical concentration (C_r) [Harper, 1997 #10]. The critical concentration is defined as the concentration of peptide at which the rates of fibril formation and fibril dissolution are equal [Harper,

1997 #10]. Second, at peptide concentrations that are slightly higher than C_r , there is a lag time before aggregation begins [Harper, 1997 #10]. Lag time is defined as the time before amyloid formation occurs during which the peptide remains soluble [Harper, 1997 #10]. Finally, during the lag time, addition of a small amount of fibril seed results in immediate aggregation. Nucleation dependent aggregation is thermodynamically similar to micelle formation with respect to the critical concentration, but kinetically is much slower, possibly because the process is more complex, and there is a much greater entropic barrier to fibril organization [Harper, 1997 #10].

There are thus three main steps involved in nucleation dependent pathways (see Scheme 1). The first step, also known as the “nucleation” step, involves conversion of the monomer peptide to a thermodynamically unstable “nucleus” that is presumed to be oligomeric [Harper, 1997 #10]. During this step, molecules collide to form transitory, highly unstable complexes [Harper, 1997 #10]. The nucleus is the least stable oligomer on the assembly pathway. As more monomer is incorporated in elongation steps, that oligomer becomes more stable. Once the oligomeric nucleus contains more than the critical number of molecules, its continued growth leads to the formation of more and more stable aggregates, featuring less and less tendency to dissociate to monomer [Harper, 1997 #10]. After the formation of this “critical nucleus” comes the “elongation”



Scheme 1 Amyloid fibril formation

step of the pathway [Harper, 1997 #10]. During this step, where addition of further monomers is thermodynamically favorable (see above), rapid extension of the aggregates occurs [Harper, 1997 #10] according to pseudo first order kinetics (first order in both monomer and fibril growing ends, with the latter unchanging over the course of the reaction). The final phase of the pathway occurs when the peptide monomer is depleted to the point where fibril growth and dissolution are in a dynamic equilibrium [Harper, 1997 #10].

Fibril growth is more complex *in vivo*, however. For instance, factors such as chemical damage and binding of other proteins can render fibrils stable to dissociation [Harper, 1997 #10]. For example, apolipoprotein E2 (apoE2) is an endogenous protein that has been shown to inhibit amyloid fibril formation [Harper, 1997 #10]. Also, cellular concentrations of A β are much lower *in vivo* than those typically used *in vitro*, which makes it unclear how nucleation can occur *in vivo* [Harper, 1997 #10]. It may be possible that nucleation occurs in cellular organelles with a supersaturated A β concentration, and fibrils formed in this way can then be released from the cellular compartment and act as seeds for additional fibril formation [Harper, 1997 #10].

A number of assays have been developed for following amyloid fibril growth *in vitro* [Levine, 1999 #17]. One assay that can be used to monitor spontaneous aggregation of A β in solution monitors the change in fluorescence that accompanies ThT binding to fibrillar aggregates [Levine, 1999 #17]. This assay can be used to monitor either unseeded A β aggregation (nucleation and elongation phases of aggregation), or seeded A β aggregation (elongation phase of aggregation) [Naiki, 1999 #18]. Another assay [Esler, 1997 #8] that has been developed uses a 96 well microplate to monitor deposition

of A β monomer onto an immobilized fibrillar template. The microplate assay is similar to the ThT assay of seeded A β aggregation, except that the fibril seeds are immobilized on a plastic surface. One benefit of the microplate assay is that it can be used with peptide concentrations on the order of 10 nM, as opposed to the ThT assay, which uses peptide concentrations of 30 μ M. Also, the microplate assay offers a much higher throughput than the ThT assay.

High throughput assays such as the microplate assay described above [Esler, 1997 #8] lend themselves well to drug screening. In the drug screening process, a large number of pharmaceutically active chemicals can be tested in a biological system to determine which, if any, exhibit an effect on that system [Esler, 1997 #8]. For example, high throughput screening identified a small molecule that activates caspase-3 in cancer cells, and this discovery in turn revealed information about the regulatory pathways in tumor suppressor activity and apoptosis in these cancer cells [Jiang, 2003 #20]. High throughput screening has also been used to look for potential therapeutics in Alzheimer's disease [Reixach #21, Esler #8] by looking for compounds that affect A β fibril formation.

Inhibition of A β aggregation is a very appealing target for drug design [Lansbury, 1997 #22]. Studies have shown that A β immunizations in murine models of Alzheimer's disease reduce both the level of fibrillar A β deposition in the brain and the level of cognitive decline [Janus, 2000 #23]. These findings suggest that there is a link between A β aggregation/deposition in the brain and cognitive decline in Alzheimer's disease. Therefore, screening libraries of compounds for inhibitors of A β aggregation and/or deposition could lead to the discovery of novel drug candidates for treating Alzheimer's.

Interpretation of the results of such screens is complicated, however, due to the uncertainty about what the toxic aggregate is (i.e., the possibility that an intermediate on the fibril assembly pathway may be the toxic species, while mature amyloid fibrils may actually be protective) [Hardy, 2002 #6].

The high throughput microplate assay described previously [Esler, 1997 #8] can be used to screen a large number of compounds for potential inhibitors of A β deposition. A similar assay has been developed to screen for inhibitors of aggregation of the amyloid peptide polyglutamine (polyQ) [Berthelie, 2001 #24]. The biggest advantage of this assay over the assay described in [Esler, 1997 #8] is that it avoids the use of radioactivity. This assay has been adapted to identification of inhibitors of A β aggregation (R. Wetzel, unpublished).

Using this assay, we set out to identify inhibitors from a collection of compounds that had been prepared for use in drug screening. The Library of Pharmaceutically Active Compounds (LOPAC) from Sigma was screened for potential inhibitors. The LOPAC library contains 640 pharmaceutically active compounds dissolved in dimethyl sulfoxide (DMSO). The choice of DMSO in the library is because of its “universal solvent” properties. If the solubility limit of each compound in the library had to be determined individually, the overall assay process would bog down, despite the existence of a high throughput assay. In fact, not all compounds are soluble in 100% DMSO, and some compounds have limited solubility when diluted from DMSO into an aqueous buffer. However, the loss of some potentially useful compounds due to solubility issues is outweighed by the gains in sheer numbers that accompany high throughput analysis.

When the LOPAC library was screened for potential inhibitors of polyQ aggregation, several were found (V. Berthelie, T. Richey, unpublished). The use of the A β version of this microplate assay to screen the LOPAC library is described here. In fact, no inhibitors of A β were identified. Instead, several compounds were found that apparently stimulate deposition of A β *in vitro*. These compounds, whose discovery and preliminary characterization are also described here, may offer insight into the mechanism of A β aggregation, and possibly help elucidate the three dimensional structure of A β aggregates. Compounds in these classes may also prove to be important therapeutics, if they are stimulating the formation of a non-toxic aggregated state of A β .

II. Materials and Methods

Peptides. A β (1-40) peptide was obtained by custom solid phase synthesis from the Keck Biotechnology Center at Yale University. The biotinylated form of the peptide was prepared (R. Wetzel, unpublished) by alkylation of the sulfhydryl group of Cys⁻¹-A β (1-40) with PEO-iodoacetyl biotin (Pierce, Rockford, IL).

Disaggregation and solubilization of A β (1-40) peptides. Disaggregation and solubilization of A β (1-40) peptide was performed as described previously [Jao, 1997 #25]. Thus, A β (1-40) peptide was dissolved in trifluoroacetic acid (TFA) (Pierce, Rockford, IL) at a concentration of 1 mg/ml and sonicated for 15 minutes to remove any remaining aggregates. The TFA was then evaporated under a stream of argon in a fume hood. The peptide residue was then solubilized in 1,1,1,3,3,3-hexafluoro-2-propanol (HFIP) (Sigma, St. Louis, MO) at a concentration of 1 mg/ml and incubated for one hour at 37°C. At this point, the HFIP was evaporated under a stream of argon in a fume hood, and the peptide residue solubilized once more in HFIP at 1 mg/ml, and the solution aliquoted into test tubes with 0.5 mg of A β (1-40) per test tube. The solvent in each test tube was evaporated under argon, and the peptide residue was placed under vacuum for one hour to remove any remaining TFA and HFIP. The peptide residue was then dissolved at a concentration of 0.5 mg/ml in 1X PBS at a pH of 7.4 and this solution was centrifuged at 100,000g for 17 hours to remove any aggregated peptide. The supernatant was collected, and the exact concentration of A β (1-40) determined by reverse phase high performance liquid chromatography (RP-HPLC) (Hewlett-Packard, Palo Alto, CA) using a Zorbax SB-C3 column on an Agilent system 1100. Thus, 10 μ L of solubilized peptide,

diluted 10 times in 0.1% TFA, was injected onto the column and analyzed with a 0-50% (v/v) acetonitrile gradient in 0.05% TFA applied at a rate of 2% per minute. The peak area obtained at 215 nm was compared to a standard curve previously established with A β (1-40) whose concentration had been determined independently by amino acid composition analysis. All peptides were snap-frozen and stored at -80°C prior to use.

Preparation of A β (1-40) fibrils. A β (1-40) peptide solubilized as described above was adjusted to a concentration of 28.9 μM (0.125 mg/ml) in buffer containing PBS 1X and NaN₃ 0.05% and incubated at 37 $^{\circ}\text{C}$. After 14 days, the fibrils were collected by centrifuging at 20,000g for 25 minutes and resuspended in extension buffer (PBS 1X, 0.01% tween 20, and 0.05% NaN₃). The quality of the fibrils was checked by thioflavin-T (ThT) fluorescence and electron microscopy (EM). An aliquot of the fibrils was dissolved in TFA, and the exact concentration of the fibrils was determined using RP-HPLC to determine the amount of A β in the aliquot as described above. The fibrils were then sonicated for 5 minutes, snap-frozen in liquid nitrogen, and stored at -80°C .

Microplate preparation. A β (1-40) fibrils prepared as described above (or other A β aggregates, as described below) were fixed to activated ELISA 96-well microtiter plates (EIA/RIA Plates, Costar, Atlanta, GA) by passive adsorption. The microplates were incubated uncovered at 37 $^{\circ}\text{C}$ for 17 hours with 100 μL of A β (1-40) fibrils diluted to a concentration of 1 $\mu\text{g}/\text{ml}$ in 72 wells, which gave 100 ng of A β (1-40) fibrils per well, and 100 μL of extension buffer in the remaining 24 wells as a negative control to measure any background signal. After the 17 hour incubation, the wells were washed three times with extension buffer, incubated for one hour at 37 $^{\circ}\text{C}$ with 100 μL of 0.3%

(w/v) gelatin in extension buffer to block any remaining surfaces in the wells not bound with fibrils, and again washed three times with extension buffer. The microplates were used immediately following this treatment.

Kinetics assay. Prior to the assay, 90 μ L of extension buffer was added to each well. For kinetic measurements with compounds, 80 μ L of extension buffer was added to each well, followed by 10 μ L of compound dissolved in DMSO (at an appropriate concentration) prior to starting the extension assay. For each replicate of each time point, 10 μ L of 100 nM biotinyl-A β (1-40) was added to the wells, after which the plate was covered with an adhesive overlay and incubated at 37°C. Each time point was analyzed in triplicate (three wells) in reverse temporal order. Thus, the wells which have biotinyl-A β (1-40) added at the start of the assay will give the longest time point of the data. At each subsequent time point, the plate was removed from the incubator and uncovered, 10 μ L of 100 nM biotinyl-A β (1-40) was added to the next set of wells, and the plate was re-covered and returned to the incubator. This process was repeated until all of the intended time points had been set up.

Microplate signal generation. After the incubation for the final time point, the extension reactions were stopped simultaneously by washing each well three times with extension buffer using the stream from a wash bottle. To generate the signal for the microplate, each well was then incubated for one hour at room temperature with 100 μ L of a 100 ng/mL europium-streptavidin (EG&G Wallac, Gaithersburg, MD) solution in extension buffer containing 0.5% bovine serum albumin (Sigma) (BSA). After this incubation, the wells were washed three times with extension buffer, and the europium was released

from the streptavidin by adding 100 μL of an enhancement solution (EG&G Wallac) [Hemmila, 1984 #26]. After 5 minutes, the europium was measured by time-resolved fluorometry [Dimandis, 1988 #27] in a Victor 2 (EG&G Wallac) microtiter plate reader using the programmed parameters for counting europium. Europium counts obtained by this method were converted to fmoles of europium using a standard curve obtained with a standard europium solution from EG&G Wallac (not shown). Fmoles of europium were then converted to fmoles of biotinyl-A β (1-40) using the manufacturer determined ratio of 7 Eu^{3+} ions per streptavidin molecule.

Primary screen of LOPAC library. The LOPAC library (Sigma, St. Louis, MO) is provided in eight 96 well plates. Each plate has 80 wells containing pharmaceutically active compounds dissolved at concentrations of 1 mM in 100% dimethyl sulfoxide (DMSO), and 16 wells containing pure DMSO as controls. For the screening of each plate in the library by the extension assay, three microplates were coated with A β (1-40) fibrils, as described above, for triplicate measurements. 80 μL of extension buffer was added to all of the wells in each plate, followed by 10 μL of the LOPAC library solutions in the wells corresponding to their position in the LOPAC library plates, giving a final concentration of 100 μM compound in each well. Next, 10 μL of 100 nM biotinyl-A β was added to each well. The microplates were then incubated at 37°C for thirty minutes. The extension reaction was then stopped by washing each well three times with extension buffer, and the signal for each plate was generated as described above.

Dose response studies. Microplates were coated with A β (1-40) fibrils as described above, and 90 μL of compounds that showed a stimulatory effect on A β (1-40) extension

in the primary screen of the LOPAC library were added to the wells at concentrations ranging from 0 to 100 μM in extension buffer containing 10% DMSO. Each concentration of compound was added to three wells for triplicate measurements. Next, 10 μL of 100 nM biotinyl- $\text{A}\beta$ was added to each well. The microplates were then incubated at 37°C for thirty minutes. The extension reaction was then stopped by washing each well three times with extension buffer, and the signal for each plate was generated as described above.

Washout assay. Microplates were coated with $\text{A}\beta$ (1-40) fibrils as described above, and 90 μL of compounds that showed a stimulatory effect on $\text{A}\beta$ (1-40) extension in the primary screen of the LOPAC library were added to the wells at a concentration of 100 μM in extension buffer containing 10% DMSO. The microplate was incubated at 25°C for 60 minutes. The compounds were removed from the microplate by washing each well three times with extension buffer. In one set of three wells, the kinetic assay was conducted as described above. 100 μL of extension buffer was added to another set of three wells, incubated at 25°C for 120 minutes, and washed three times with extension buffer. The kinetic assay was then conducted as described above.

Solution phase aggregation assays. The most potent compounds from the LOPAC library as determined by the dose response studies were purchased from Sigma for more detailed study (see Results section). To determine the effect of these compounds on $\text{A}\beta$ (1-40) aggregation kinetics in solution, $\text{A}\beta$ (1-40) rigorously disaggregated as described above was incubated at 28.9 μM in PBSA (1X PBS, 0.05% NaN_3) buffer with compound concentrations ranging from 0 to 100 μM . As a control, $\text{A}\beta$ (1-40) was also incubated at

28.9 μ M in PBSA at 37°C in the presence of fibril seeds at 0.05% the weight of A β (1-40) monomer in the reaction. The aggregation kinetics were followed by ThT fluorescence measurements [Levine, 1999 #17], by RP-HPLC analysis [Naiki, 1999 #18], and by 90° light scattering at 450 nm [Wood, 1996 #19] as described previously. Thus, a ThT fluorescence signal was generated by diluting a 10 μ L aliquot of each reaction solution to a final volume of 400 μ L in PBSA containing 15 μ M ThT, and measuring fluorescence of the sample in a fluorometer with the excitation wavelength set to 442 nm and the emission wavelength set to 482 nm. The fluorescence data for each time point was collected over one minute, and then averaged. Each time point was analyzed in triplicate. For RP-HPLC analysis, a 30 μ L aliquot of each reaction was diluted 5-fold into ice-cold PBSA to stop the aggregation reaction, put on ice, and centrifuged at 100,000g for 25 minutes at 4°C. The upper 100 μ L of the supernatant was collected, and TFA added to a concentration of 1% (v/v). The sample was then analyzed for the amount of soluble A β (1-40) by RP-HPLC as described above. 90° light scattering measurements for each sample were obtained by diluting a 10 μ L aliquot of each reaction solution to a final volume of 400 μ L in PBSA and measuring the light scattering with excitation and emission wavelengths both set to 450 nm and slit widths set to 10 nm. To determine the effect of the compounds on the structure of the A β (1-40) aggregation products, aliquots of each reaction mixture were collected after 2 days and after 14 days and analyzed by electron microscopy. All electron micrographs were generated by the Life Sciences Electron Microscopy Facility at the University of Tennessee, Knoxville.

Collection and storage of aggregates. Aggregates were collected by centrifuging aliquots of various time points for each solution phase reaction at 20,000g for 25 minutes at 4°C, removing the supernatant, and resuspending the pellet in PBSA. This process was repeated three times to remove any remaining unbound compound from the reaction mixture. The exact concentration of the A β (1-40) aggregates was determined by centrifuging an aliquot of the resuspended pellet, and dissolving in TFA as described above, and measuring the concentration of A β (1-40) by RP-HPLC as described above. The resuspended pellet was then snap-frozen, and stored at -80°C until used.

III. Results

Since A β aggregation is implicated in the disease mechanism of Alzheimer's disease, compounds that influence A β aggregation may be potential therapeutics. A microtiter plate assay for A β extension has been developed (R. Wetzel, unpublished) which provides a high throughput way of screening large numbers of potential inhibitors. Figure 1 shows the typical kinetic time course observed in this assay¹. The time course is characterized by an initial fast phase of extension, and a second slow phase of extension. We decided to conduct the primary screen of the Library of Pharmaceutically Active Compounds (LOPAC) by stopping the extension reaction after 30 minutes, which corresponds approximately to the midpoint of the fast phase. The LOPAC library contains 640 pharmaceutically active compounds dissolved in dimethyl sulfoxide (DMSO) at a concentration of 100 μ M. Thus, dilution of the compound solutions into aqueous buffer will produce a mixed aqueous solvent containing a small amount of DMSO which could potentially influence the assay results. DMSO has been shown, however, to have no effect on the analogous kinetic determination of polyglutamine elongation as observed in the microtiter plate assay [Berthelie, 2001 #24]. Figure 2 shows that similarly there is no significant effect of DMSO up to a 10% concentration on A β extension in the microtiter plate assay after 30 minutes.

Figure 3 shows the results of the primary screen of the LOPAC library, with each bar on the graph representing one compound. The percent inhibition of each compound is calculated by the equation

$$(1) \quad I = [(C_n - C_i)/C_n] * 100$$

¹ All figures are located in the Appendix.

where

I = Percent inhibition

C_n = Amount of biotinyl-A β deposited in absence of compound

C_i = Amount of biotinyl-A β deposited in presence of compound

Surprisingly, none of the compounds in the LOPAC library showed any significant inhibition (greater than three standard deviations from the average) of A β deposition in the microplate assay. However, seven compounds actually stimulated (showed a negative percent inhibition of) deposition of A β . The compounds – amiloride HCl, phenamil methane sulfonate, naftopidil dihydrochloride, NPC 15437 dihydrochloride, calmidazolium chloride (R24571), 5-(nonoxyl)-tryptamine hydrogen oxalate, and L- α -methyl DOPA – have a wide variety of structures and biochemical properties (see Discussion section). The structures of these seven compounds are shown in Figure 4, along with the percent stimulation observed in our experiments. Even though the only hits observed in the initial screen exhibited stimulatory effects, they could still prove useful in gaining information about the biochemical process of A β aggregation (see Discussion).

Interestingly, several catechol-containing compounds, similar in structure to L- α -methyl DOPA, were initially identified as modulators of α -synuclein fibrilization, and were also identified as inhibitors of polyglutamine aggregation (V. Berthelie, unpublished). In both cases, it was subsequently determined that the species acting as the inhibitor was actually the oxidized quinone form of the compound [Conway, 2001 #28]. Since the LOPAC library had been stored prior to the screen, it may be possible that a

similar oxidized form of L- α -methyl DOPA is the active species in A β aggregation stimulation as well (see Discussion section). However, no experiments were performed to test this hypothesis. One reason for this is that the stimulation exhibited by L- α -methyl DOPA was marginal, compared to some of the other compounds (see below).

As part of the overall screening process, it is important to examine the concentration dependence of the hits. Figure 5 shows the results of such dose response studies (see Materials and Methods) for each of the seven compounds. The concentrations of the compounds in the dose response studies ranged from 0.05 to 100 μ M. Interestingly, only calmidazolium chloride showed a saturation effect for stimulation in this concentration range (Figure 5C). Calmidazolium chloride shows maximal stimulation at a concentration of 50 μ M, while the stimulation of the other six compounds still appear to be increasing even at concentrations of 100 μ M.

Compound potencies are described as EC₅₀ values. The EC₅₀ value of a compound is defined as the compound concentration at which the measured effect is half-maximal. The transition region of each dose response curve is identified as the region in which the percent stimulation begins to increase from values corresponding to no effect. Fitting the data points in the transition zone to a straight line through a linear regression gives an equation that can be used to calculate an EC₅₀ value for each compound. However, because calmidazolium was the only one of the seven compounds that showed a saturation effect (and hence a plateau such that a maximal effect could clearly be seen), it is the only compound for which a true EC₅₀ could be calculated. That is to say, since the effects of the other six compounds at concentrations higher than 100 μ M were never

determined, it may be possible that the calculated EC_{50} values are underestimates of the true EC_{50} values. Figure 5 shows the straight line fits for the transition region of each dose response curve, while the apparent EC_{50} values for each compound are given in Figure 4. Based on the calculated EC_{50} values of the seven compounds, calmidazolium chloride ($EC_{50} = 21.4 \mu M$), NPC 15437 ($EC_{50} \geq 27.2 \mu M$), phenamil methane sulfonate ($EC_{50} \geq 35.5 \mu M$), and 5-(nonyloxy)-tryptamine hydrogen oxalate ($EC_{50} \geq 30.3 \mu M$) are the most potent stimulators of A β deposition. L- α -methyl DOPA ($EC_{50} \geq 34.2 \mu M$) is the least potent stimulator.

Three of the four most potent compounds – calmidazolium chloride, NPC 15437, and phenamil methane sulfonate - are readily available from Sigma, and were therefore chosen for more detailed study. Amiloride HCl was also chosen for additional study because structurally (a) it very closely resembles phenamil methane sulfonate (Figures 4A and 4B), and (b) both compounds have structural elements suggestive of peptide bonds, which play a significant role in the formation of the β -sheet structure of fibrils. Once compounds have been identified in the initial screening process, it is important to obtain fresh compounds for further testing for two main reasons. First, testing fresh compounds makes sure that no mix-ups occurred in the original screening, and second, it can identify problems with long term storage of the compounds in DMSO (e.g. oxidation of dihydroquinones to quinones mentioned above). All four compounds to be retested were purchased from Sigma.

First, we set out to confirm the behavior of these apparent stimulators by conducting full kinetic analyses of each compound (rather than one time point) in the microplate assay. Figure 6A shows the kinetic time course of A β deposition in the

microplate assay in the presence and absence of calmidazolium chloride. The results show that calmidazolium chloride significantly increases the rate of deposition, as well as the amount of A β deposited. Figure 6B shows the kinetic time course of A β deposition in the microplate assay in the presence and absence of NPC 15437. These results show that NPC 15437 also stimulates both the rate and amplitude of the kinetic curve, although not as potently as calmidazolium.

Figures 6C and 6D show similar kinetic time course data for amiloride HCl and phenamil methane sulfonate. These two figures show that freshly dissolved amiloride HCl and phenamil methane sulfonate appear to have an inhibitory effect on the kinetics of A β deposition in the microplate assay. The contradiction between this result (inhibition) and the initial screening result (stimulation) for these two compounds may be due to the fact that they were not stored in DMSO for any length of time prior to the assay, in contrast to the compounds in the LOPAC library that had been stored for several months in DMSO prior to the primary screen. The storage of the compounds for an extended period of time may have led to a degradation product of amiloride and phenamil, which are highly similar structurally, and the degradation product may be the species responsible for the stimulation of A β deposition in the primary screen. In any case, the apparent inhibition by these two compounds was not great enough to warrant further investigations, and they were not studied further.

The solid phase microtiter format allows an assay modification that can be used to probe which molecular species is the target of the compounds' activity. This modification, termed the "washout assay" (see Materials and Methods) can be used to test whether the stimulating effects of calmidazolium chloride and NPC 15437 in the

microplate assay were due to interactions with the preformed fibrillar A β on the plate or with the solution-phase biotinyl-A β . The washout assay has been used successfully in studying inhibitors of polyglutamine aggregation to show that the inhibitors act through interactions with the polyglutamine aggregates rather than the monomer (V. Berthelie, unpublished). The washout assay results for NPC 15437 are shown in Figure 7. These results show that washing the wells that had been incubated with NPC 15437 immediately prior to starting the extension assay gives a kinetic time course that is virtually indistinguishable from the kinetic time course of A β deposition in the absence of the compound. However, in the continual presence of NPC 15437 an amplified kinetic curve is observed. The rates and amplitudes of both phases of this kinetic curve are significantly greater than those observed in the absence of NPC 15437. Also, absolutely no deposition of A β is observed when biotinyl-A β is incubated with NPC 15437 in the absence of a preformed fibrillar template (Figure 7B). These results suggest that NPC 15437 is stimulating deposition of A β through some sort of interaction with the A β monomer, and that if there is any non-covalent binding between NPC 15437 and fibrillar A β aggregates then that binding is very weak.

Figure 8 shows the results of the washout assay for calmidazolium chloride. The results of the washout assay indicate that calmidazolium chloride stimulates A β deposition even if it is removed from the solution phase prior to the extension assay. However, the stimulatory effect is decreased when the wells are incubated in extension buffer for two hours after the removal of the compound before starting the assay. Together these results suggest that (a) calmidazolium acts by binding to the fibrillar A β

aggregates and (b) that this binding is not extremely tight, since some compound can dissociate after incubation of the plate in extension buffer for two hours.

As a control in the washout assay, some microtiter plate wells without immobilized fibril seeds were incubated for 4 hours at 37°C with a solution of biotinyl-A β plus calmidazolium. Surprisingly, strong two phase kinetics are obtained even in the absence of a preformed fibrillar template, as shown in Figure 9. There are a number of possible explanations for this unexpected effect. For example, it is possible that calmidazolium interacts with the gelatin blocking agent in the microplate wells, converting the gelatin to a fibril-like seed capable of being elongated with A β . However, Figure 10 shows that even without a preformed fibrillar template, A β deposition is stimulated in the presence of calmidazolium regardless of whether the microplate wells are blocked with 0.3% gelatin buffer or with 0.5% BSA buffer. However, calmidazolium does not stimulate A β deposition as greatly in microplate wells blocked with 0.5% BSA, and it does not show strong two phase kinetics. The fact that calmidazolium still stimulates A β deposition in microplate wells blocked with 0.5% BSA suggests that calmidazolium does not act through a direct effect on the blocking agent. Another possibility is that calmidazolium is acting via an interaction with the monomeric A β . It should be noted, however, that to produce this effect calmidazolium must not only stimulate A β aggregation, but it must also stimulate the deposition of the aggregates on the plate. In addition, these aggregates must be elongation competent, but it is not clear from these experiments whether or not this is the case. To better understand the nature of

the effect that calmidazolium has on A β aggregation, it is necessary to use additional assays.

The microplate assay acts as an *in vitro* model of the elongation phase of A β aggregation. Solution phase assays have also been described [Levine, 1999 #17] that act as *in vitro* models of *spontaneous* aggregation (both the nucleation and elongation phases) of A β . These solution phase assays are important for several reasons. First, they can be used to confirm that the microplate results are accurate. Second, they can reveal whether the effect of the compounds on the nucleation phase of aggregation is different than the effect on the elongation phase. And finally, they offer a way to further explore the mechanism of calmidazolium action on A β .

These assays depend on the ability to monitor fibril growth either directly – by monitoring ThT fluorescence [Levine, 1999 #17] – or indirectly – by monitoring the depletion of A β monomer by HPLC [Naiki, 1999 #18]. To use the ThT assay, we first had to confirm that the test compounds have no effect on fluorescence under assay conditions. Figure 11 shows that calmidazolium chloride has no significant effect on ThT fluorescence at concentrations ranging from 0.1 μ M to 1 mM in the presence or absence of A β fibrils. Similarly, Figure 12 shows that NPC 15437 has no significant effect on ThT fluorescence over the same concentration range. Therefore, fibril growth in the presence of each compound can be monitored by ThT fluorescence without having to correct for high background signals due to direct fluorescence effects of the compounds.

Figure 13A shows the kinetic time course of A β aggregation monitored by ThT fluorescence in the presence of NPC 15437 at concentrations ranging from 0 to 100 μ M,

while Figure 13B shows the kinetic time courses for the same aggregation reactions monitored by RP-HPLC. Interestingly, the ThT results show that as the concentration of NPC 15437 is increased, the lag time to aggregation is decreased. This suggests that NPC 15437 is acting by stimulating nucleation of amyloid fibril formation by the A β monomer. However, even at NPC 15437 concentrations of 100 μ M there is still a lag time of approximately 4 days. As the ThT signal increases to a maximum, the level of A β left in solution drops to a base level. These results suggest that NPC 15437 does indeed stimulate the nucleation stage of A β aggregation.

It is also interesting that, contrary to previous observations [Wood, 1996 #19] when measured by RP-HPLC the level of A β monomer decreases well before any ThT signal develops, even in the absence of NPC 15437. One possible explanation for this result is that the reaction aliquots were centrifuged at 100,000g for 25 minutes to remove any aggregated A β (see Materials and Methods) as opposed to 14,000g as described previously [Wood, 1996 #19]. Thus, it is possible that early intermediates on the pathway from A β monomer to fibril are only removed by extremely rigorous centrifugation and that the formation of these intermediates, if they are ThT negative, would account for the decrease in monomeric A β . Non-fibrillar aggregates are clearly present at the 2-day point, as shown in Figure 14. The secondary structure of A β fibrillogenesis intermediates is difficult to measure, and there is currently no consensus on the extent to which protofibril substructure resembles that of fibrils [Kirkitadze, 2001 #16]. At the end of the lag phase the ThT and RP-HPLC results shown in Figures 13A and 13B seem to agree. The correspondence between the results of the ThT and RP-HPLC assays after the lag

phase suggests that the bulk of the aggregates formed in the presence of NPC 15437 are indeed fibrillar, in direct contrast to the results for calmidazolium (see below).

Figures 16, 17, and 18 show EM images of A β aggregates grown for fourteen days in the presence of NPC 15437 at concentrations ranging from 0 to 100 μ M. These aggregates show structures very similar to those of fibrils grown in the absence of any compound (Figure 15). In fact, the aggregates grown in the presence of 10 μ M NPC 15437 (Figure 17) appear to be even larger than untreated aggregates (Figure 14). It may be possible that NPC 15437 is somehow acting to stabilize the fibrillar state of the A β aggregates and enabling them to grow larger than usual. It may also be possible that NPC 15437 is somehow stabilizing key intermediates in the fibrillization pathway, thereby altering their steady state concentrations and in turn changing the observed rate. Isothermal titration calorimetry experiments did not indicate a titration between NPC 15437 and A β up to a molar ratio (NPC/A β) of 50 (data not shown). It is also possible that this compound is destabilizing off-pathway A β assemblies, effectively increasing the concentration of A β monomer or of some other critical on-pathway intermediates.

Figure 19 shows the kinetic time course of A β aggregation monitored by ThT fluorescence in the presence of calmidazolium chloride at concentrations ranging from 0 to 100 μ M. Interestingly, after 14 days the ThT signal of fibrils grown in the presence of 100 μ M calmidazolium chloride has an intensity that is approximately 25% of the intensity of the ThT signal of fibrils grown in the absence of calmidazolium. Furthermore, as the concentration of calmidazolium is lowered, the intensity of the ThT signal increases. This suggests that the A β aggregates formed in the presence of

calmidazolium are significantly less fibril-like than fibrils grown normally, since ThT fluorescence is proportional to the amount of fibrils present [Levine, 1999 #17].

Figure 20 shows the kinetic time course of A β aggregation monitored by RP-HPLC in the presence of calmidazolium chloride at concentrations ranging from 0 to 100 μ M. These results show that calmidazolium at a concentration of 100 μ M completely depletes the A β monomer in solution after only one day. However, Figure 19 shows that at the same time point under these reaction conditions, no appreciable ThT signal can be seen. This suggests that calmidazolium is driving A β into an aggregated state that is not amyloid. This profile is similar to that implied by the lack of ThT signal observed for early A β assembly intermediates in the absence of added compound (see above).

Figure 21 shows the kinetic time course of A β aggregation monitored by 90° light scattering at 450 nm. It is interesting that only A β aggregates grown in the presence of 100 μ M calmidazolium show any appreciable light scattering signal. The light scattering of these aggregates appears after one day, which fits with the data obtained by RP-HPLC. It also supports the theory that A β is rapidly aggregating in the presence of 100 μ M calmidazolium.

To get a more detailed look at the early events due to the effect of calmidazolium, the reactions were repeated and hourly time point measurements taken. Figure 22A shows the hourly time course of A β aggregation monitored by light scattering at 450 nm, while Figure 22B shows the same hourly time course as monitored by RP-HPLC. Interestingly, after only two hours the RP-HPLC measurement of the A β monomer

remaining in solution decreases from an initial value, while at the same time point, the light scattering signal increases from a baseline.

Based on the RP-HPLC data, A β monomer in the presence of 100 μ M calmidazolium is significantly depleted almost within 1-2 hours (Figures 22B). This depletion of monomer is accompanied by a simultaneous increase in light scattering (Figures 21 and 22A). Also, after one day at these reaction conditions a precipitate is observed. However, this precipitate is not the test compound, since no precipitate is formed when calmidazolium is incubated in buffer without A β present (not shown). These results indicate that calmidazolium is indeed stimulating aggregation of A β in solution. However, there is a significant reduction in the ThT signal of these aggregates compared to an equal weight of bona fide fibrils (Figure 19). One possibility that could reconcile these apparently contradictory observations is that calmidazolium is acting somehow to drive the A β monomer into a non-fibrillar aggregated state. The implication that calmidazolium stimulates aggregation of A β in a non-fibrillar form is not inconsistent with the original microplate result, because the microplate generates a positive signal for A β deposition regardless of whether or not the deposited product is fibrillar (see Materials and Methods).

Electron microscope (EM) images of A β aggregates grown for two days and A β aggregates grown for fourteen days at calmidazolium chloride concentrations ranging from 0 to 100 μ M are shown in Figures 23 through 28. Figure 14 shows that after two days, aggregates grown in the absence of calmidazolium show globular structures similar to those reported by others for A β amyloid assembly (see Background), and very few

fibrils. However, Figure 23 shows that after two days, aggregates grown at a calmidazolium chloride concentration of 100 μM are very large, and do not appear to be fibrillar (although these globular assemblies appear to contain short, filamentous substructures). Figure 24 shows that after two days, aggregates grown at a calmidazolium concentration of 10 μM have a more ordered structure than aggregates grown without calmidazolium, but these structures are not as large as those seen in aggregates grown in 100 μM calmidazolium. Compared to the EM images of the control fibrils (Figure 15), none of these aggregates appear to be fibrillar, which agrees with the ThT data (Figure 19). However, Figure 25 shows that after two days, A β aggregates grown in the presence of 1 μM calmidazolium appear to be highly fibrillar. This suggests that calmidazolium at concentrations below 10 μM enhances A β fibril formation, perhaps by stabilizing key intermediates in the amyloid assembly pathway. Circular dichroism experiments to determine the amount of β -sheet secondary structure in the non-fibrillar aggregates proved unsuccessful, due to the large amount of light scattering associated with them (data not shown).

Figure 15 shows the structure of fibrils grown in the absence of calmidazolium chloride after fourteen days. Figure 26 shows the structure of aggregates grown in the presence of 100 μM calmidazolium after the same amount of time. Interestingly, the large aggregated structures present after two days at these reaction conditions are still present, but fibrillar aggregates are now also present. At calmidazolium concentrations of 10 and 1 μM , the aggregated structures all appear to be fibrillar (Figures 27 and 28). From the EM images, it seems as though 100 μM calmidazolium is indeed driving A β into a non-

fibrillar aggregated state after only one day. However, after fourteen days A β fibrils are also present under these conditions, as seen in Figure 26. This could possibly be caused by the non-fibrillar A β aggregates rearranging to form fibrils, either by a direct transformation within the solid phase, or mediated by a small amount of solution phase A β .

If the aggregates that form rapidly in the presence of calmidazolium are indeed non-fibrillar, then they should exhibit no ability to seed wild type A β fibril formation/elongation. We tested the seeding ability of aggregates formed in the presence of calmidazolium using the microplate assay. Aggregates grown in the presence of calmidazolium at concentrations of 0, 10, and 100 μ M were collected as described in Materials and Methods after two days and fourteen days. These aggregates were washed, calibrated for weight concentration, and immobilized on a 96 well microplate (see Materials and Methods) to test their seeding ability. Figure 29A shows the kinetic time course of A β deposition onto equal weights of the aggregates grown in the presence of 100 μ M calmidazolium for two days and fourteen days, compared to the kinetic time course of A β deposition on equal weights of standard A β amyloid fibrils grown for the same periods of time. These results show that after two days aggregates grown in the presence of 100 μ M calmidazolium cannot seed deposition of A β in the microplate assay. After fourteen days these aggregates show some seeding ability, but it is very limited compared to the seeding ability of normal A β fibrils grown for fourteen days. This lack of seeding ability agrees qualitatively with the results of the ThT assay. It is also consistent with the hypothesis that calmidazolium is driving A β monomer into a non-

fibrillar aggregated state. The seeding ability of the calmidazolium aggregates after 14 days could possibly be due to the presence of a small amount of fibrillar A β that forms either from A β in solution that does not interact with calmidazolium, or by a rearrangement of the A β that is associated with calmidazolium. The partial seeding of A β elongation by 14-day A β /calmidazolium aggregates fits with the fibrillar and globular structures observed by EM in the 14 day calmidazolium aggregates (Figure 26).

Figure 29B shows the kinetic time course of A β deposition onto the aggregates grown in the presence of 10 μ M calmidazolium for two days and fourteen days compared to the kinetic time course of A β deposition onto normal fibrils grown for the same periods of time. These results show that aggregates grown in the presence of 10 μ M calmidazolium for two days also cannot seed A β deposition in the microplate assay. After fourteen days, however, these aggregates do show some seeding ability, although it is not nearly as great as that of normal fibrils grown for the same amount of time.

We also set out to test the hypothesis that calmidazolium stimulates deposition of elongation competent aggregates in the microplate assay (see above) by incubating gelatin blocked microplate wells at 37°C for 1 hour with either both 10 nM A β (non-biotinylated) and 100 μ M calmidazolium chloride, only 10 nM A β (non-biotinylated), or only 100 μ M calmidazolium chloride, then washing the wells with buffer, and conducting the microplate assay as described in Materials and Methods (Figure 30). The results of this test showed no A β extension after incubating the microplate wells with any combination of A β and calmidazolium, which suggests that calmidazolium is simply stimulating deposition of biotinyl-A β in the microplate wells, and the method of signal

generation in the assay (see Materials and Methods) creates a signal for the deposited biotinyl-A β , regardless of whether or not it is fibrillar.

In addition to testing the seeding ability of these A β /calmidazolium aggregates, we also checked the level of protection from hydrogen/deuterium exchange of the aggregates. It has been shown that, in A β (1-40) fibrils, about 50% of the 39 backbone amide protons of A β are strongly protected from deuterium exchange, indicating that about 19 of the protons are involved in stable β -sheet structure. [Kheterpal, 2000 #12, Kheterpal, 2003 #13]. Figure 31A shows that A β aggregates grown in the presence of 100 μ M calmidazolium even after 14 days undergo hydrogen exchange much more readily than do A β fibrils. This result suggests that the amide groups of the A β peptides in these A β /calmidazolium aggregates are much more exposed to solvent than those of fibrils, and that the network of hydrogen bonds is not as great as in fibrils. This might be attributed to there being less β -sheet secondary structure present in these aggregates compared to A β fibrils (see Background). The amount of protection seen for these calmidazolium aggregates is essentially identical to that seen for A β protofibrils isolated from the normal *in vitro* assembly reaction (I. Kheterpal and H. Lashuel, unpublished). As shown in Figure 31B A β /10 μ M calmidazolium aggregates also exchange to the same degree as the A β /100 μ M calmidazolium aggregates and protofibrils up to four days, but after fourteen days, they more closely resemble A β fibrils in terms of the amount of exposure to solvent. Could calmidazolium be stimulating the formation of a protofibrillar intermediate which is less strongly blocked from rearranging into fibrils at lower calmidazolium concentrations? If so, it could prove to be a valuable research tool in

further exploring the A β fibril assembly pathway, as well as the amyloid hypothesis (see Background).

IV. Discussion

Inhibition of the aggregation of amyloidogenic peptides is an attractive target for potential drugs to treat the diseases associated with those peptides [Lansbury, 1997 #22]. High throughput assays have been developed to screen a very large number of compounds for potential inhibitors of these amyloidogenic systems ([Bertheliet, 2001 #24], [Esler, 1997 #8]). However, these assays can also identify compounds that stimulate the aggregation of amyloid, as is the case with this work. Such stimulatory compounds have a number of potential uses. First, these compounds are of interest to researchers because studying their interaction with the amyloidogenic species can possibly offer insight into the mechanism by which amyloid is formed, as well as the chemical structures that are important to amyloid formation. Second, these compounds could be used to test the amyloid hypothesis. Do mouse models treated with these compounds develop Alzheimer's Disease pathology and/or cognitive defects more rapidly than normal? Alternatively, if the compounds stimulate deposition of a non-toxic form of A β , do they slow disease progress in the mouse model? If so, they could potentially be useful as therapeutics.

The compounds identified in this study that stimulate A β aggregation *in vitro* can be divided into four classes based on their chemical structures (Figure 4). The first class is comprised of compounds containing cyclical structures of carbon and nitrogen. These compounds are amiloride HCl, phenamil methane sulfonate, and naftopidil dihydrochloride. Interestingly, amiloride and phenamil are structural analogs. Phenamil has the basic chemical structure of amiloride with a benzene ring attached at one of the nitrogen atoms. Also, based on the EC₅₀ values, phenamil is approximately as potent as

amiloride ($EC_{50} \geq 38.5 \mu M$ and $35.5 \mu M$ respectively). Another interesting aspect of these two compounds is the fact that portions of their structures resemble a peptide bond (an α -carbon atom between C-N and C=O atoms). Unfortunately, the stimulating effect of these compounds is not reproducible when they are freshly dissolved in DMSO (see Figures 5A and 5B). This could be due to the fact that the compounds in the LOPAC library were stored in DMSO at $4^{\circ}C$ for several months prior to the primary screen. It is possible that degradation products of these two compounds were responsible for the stimulation of aggregation observed in the primary screen. This possibility was not explored in this work.

The second class of stimulators contains L- α -methyl DOPA. The catechol containing structure is similar to those of other compounds that have been identified as modulators of aggregation of the amyloid α -synuclein [Conway, 2001 #28] and inhibitors of polyglutamine aggregation (V. Berthelie, unpublished). In this previous work it was shown that the oxidized quinone forms of the catechol containing compounds are the active species [Conway, 2001 #28]. It may be possible that a similar oxidized form of L- α -methyl DOPA is responsible for the results in the primary screen of the LOPAC library. However, it is worth noting that the LOPAC library contained 12 catechol-like structures very similar to L- α -methyl DOPA, none of which had any effect on A β deposition in the microplate assay in our primary screen. Therefore, L- α -methyl DOPA may indeed be interacting with A β in the microplate assay, but due to the fact that it is not a very potent stimulator ($EC_{50} \geq 34.2 \mu M$) and the possibility that an oxidized form of

the compound is indeed the active stimulating species, the uses of L- α -methyl DOPA in further studies of A β aggregation would be very limited.

The third class of stimulators contains NPC 15437 and 5-(nonyloxy)-tryptamine hydrogen oxalate. These compounds both have amphipathic characteristics. They each contain long hydrophobic carbohydrate chains and primary amino groups that are presumably protonated at pH 7 (see Figures 4A and 4B). They are also both very potent ($EC_{50} \geq 27.2 \mu\text{M}$ and $30.3 \mu\text{M}$ respectively). The amphipathic molecule hexadecyl-N-methylpiperidinium (HMP) has been found to inhibit *in vitro* fibril formation [Wood, 1996 #29]; this work was conducted in a pH 6 aggregation system, however, and is therefore difficult to compare to results described here. It is unclear whether the amphipathic characteristics of NPC 15437 and 5-(nonyloxy)-tryptamine hydrogen oxalate are primarily responsible for their stimulatory effects on A β deposition. As compounds likely to form micelles, it is possible that such micelles are the actual species responsible for stimulating A β aggregation *in vitro*. However, light scattering experiments to determine a critical micelle concentration (CMC) for NPC 15437 proved unsuccessful (data not shown). NPC 15437 seems to stimulate the nucleation phase of A β aggregation (see Figure 13A), but it is unclear from the present data what the exact mechanism of stimulation by NPC 15437 might be. One possibility is that NPC 15437 alters the stabilities of key intermediates in the fibril assembly pathway, thereby altering the steady state concentrations of intermediates, which in turn would affect the observed rate of aggregation.

The final class of compound contains calmidazolium chloride. Calmidazolium is highly hydrophobic, with four phenyl groups attached to a central imidazole ring (see Figure 4C). Calmidazolium is also the most potent of the seven stimulators presented here ($EC_{50} = 21.4$). Interestingly, when the LOPAC library was screened for inhibitors of polyglutamine (polyQ) aggregation, calmidazolium had no effect on the rate of aggregation up to a concentration of 100 μ M (V. Berthelie, T. Richey, unpublished). This result suggests that there is an intrinsic mechanistic and/or structural difference between polyQ and A β amyloid aggregation, despite the many similarities also seen for these aggregates.

It is noteworthy that even though some compounds in the LOPAC library have been found to inhibit polyglutamine aggregation in the microplate assay (V. Berthelie, T. Richey, unpublished), none of them showed any inhibitory effect on A β aggregation in the same assay. Even more interesting is the fact that there were quite a few compounds in the LOPAC library that actually stimulated A β aggregation in the microplate assay, and none in the polyglutamine screen, again suggesting that the mechanisms of assembly and/or structural stabilization of different amyloid proteins, or the roles of various amino acid residues of the peptide in their assembly, are quite different from one another.

At least two different mechanisms of stimulation of A β aggregation have been characterized here, both of which are apparently due to interactions with non-amyloid states of A β (monomer and/or protofibrils). This result is exciting, as it could provide valuable insight into the basic mechanism of A β assembly. Also exciting is the fact that based on RP-HPLC and light scattering data, A β in the presence of calmidazolium forms

aggregates after only one day while EM images and ThT fluorescence measurements show that these aggregates do not appear to be fibrillar in nature. Is calmidazolium driving A β into a non-toxic, non-amyloidogenic conformation? If so, are there any other compounds that have the same properties as calmidazolium chloride (i.e., the ability to stimulate formation of a non-fibrillar A β aggregate)? It may be likely that there are other compounds similar to calmidazolium, since this work only looked at approximately 600 out of millions of chemical compounds. If there are compounds with properties similar to calmidazolium, then there may be potential therapeutic applications of them. However, much work must be done to determine if this is a viable option or not. For instance, if calmidazolium truly is a potential therapeutic then it must be determined if it can act at physiologically relevant concentrations of A β . The microplate assay is conducted at 10 nM A β , while the concentration of A β in blood and cerebrospinal fluid is in the low pM range [Harper, 1997 #10]. It is also necessary to determine whether calmidazolium is stimulating the formation of a toxic aggregated state of A β .

Additionally, the results from the microplate assay indicate that calmidazolium in the presence of A β at a concentration of 10 nM stimulates aggregation instantaneously, but in the ThT assay at A β concentrations of 30 μ M there is a delay before fibrils can be seen. The most likely explanation for this result is that the microplate assay detects all A β deposited (see Materials and Methods), regardless of whether or not it is fibrillar. The signal in the ThT assay only appears once fibrils are present. Also, as shown in Figure 22B, A β in the presence of 100 μ M calmidazolium in the solution phase assay is depleted

after only a few hours, which is consistent with the instantaneous effect seen in the microplate assay.

Calmidazolium chloride could prove to be a valuable tool in testing the amyloid hypothesis in a mouse model of AD. For instance, if protofibrils are the toxic species in the amyloid pathway and calmidazolium is stimulating their formation, would mouse models treated with calmidazolium be more prone to the neurodegenerative effects of AD, or would calmidazolium keep the protofibrils clustered in a large aggregated state that is non-toxic to cells? Also, if the toxic species is an intermediate in the amyloid assembly pathway, and calmidazolium is actually stimulating fibril formation as it originally appeared in the microplate assay, then it may actually prevent the accumulation of these intermediates. Of course, there is still the possibility that mouse models that are treated with calmidazolium will develop AD more rapidly than normal. The results of these mouse model experiments will undoubtedly give additional insight into the action of calmidazolium, and they will be important in developing potential therapeutics, but will likely not be immediately interpretable. In any case, calmidazolium could eventually give useful information about the role that A β plays in AD.

List of References

List of References

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Appendix

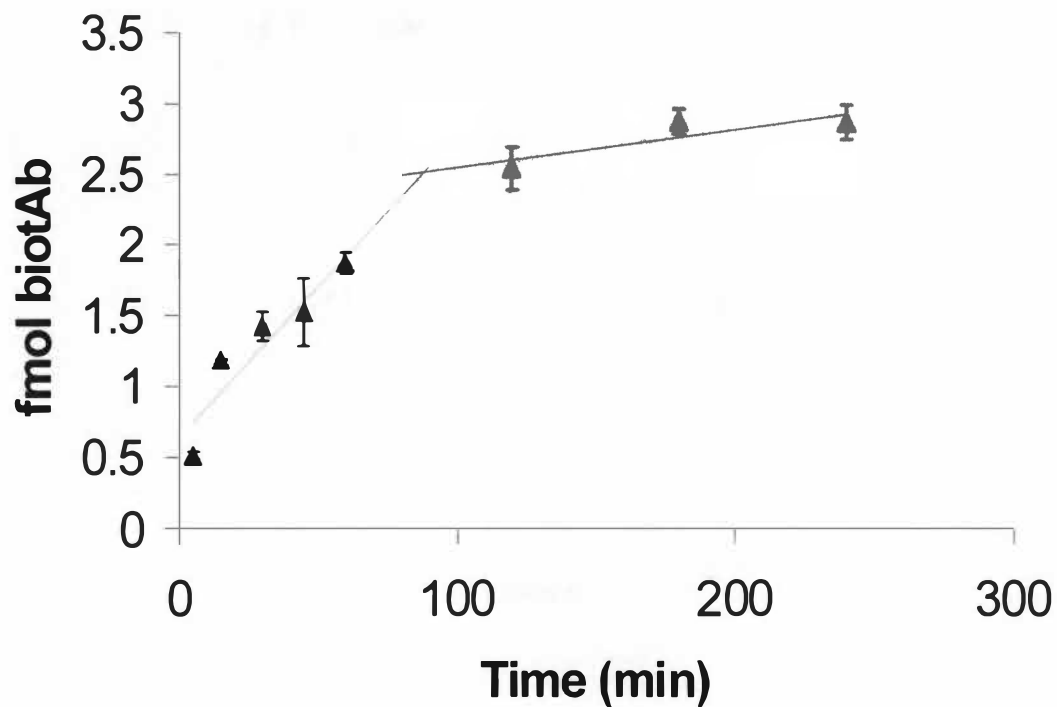


Figure 1 Kinetic time course of A β deposition in microplate assay (conducted as described in Materials and Methods). The “fast phase” of elongation occurs over the first 60 minutes, followed by the “slow phase”.

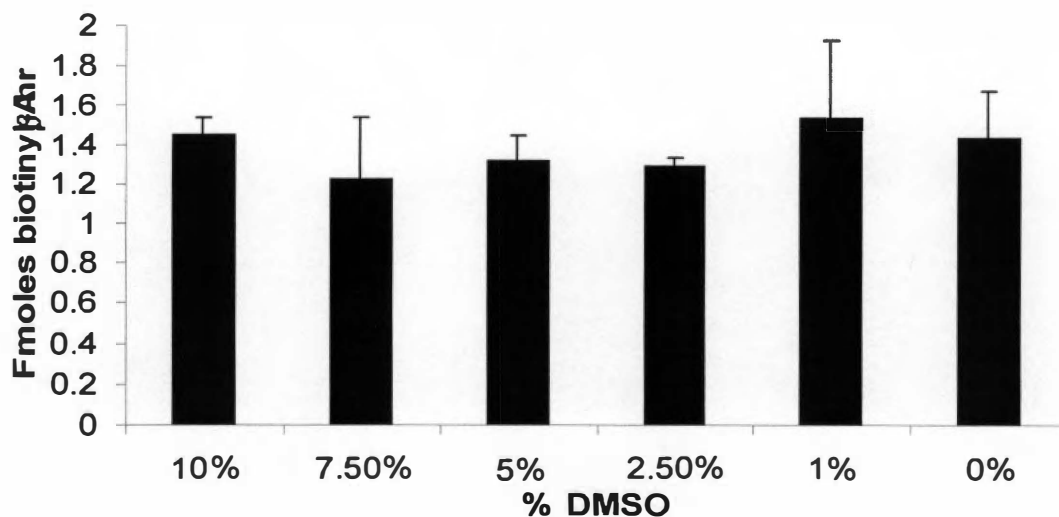


Figure 2 Effect of DMSO on A β microplate extension assay (as described in Materials and Methods) at 30 minute time point. Results shown are average of three replicates.

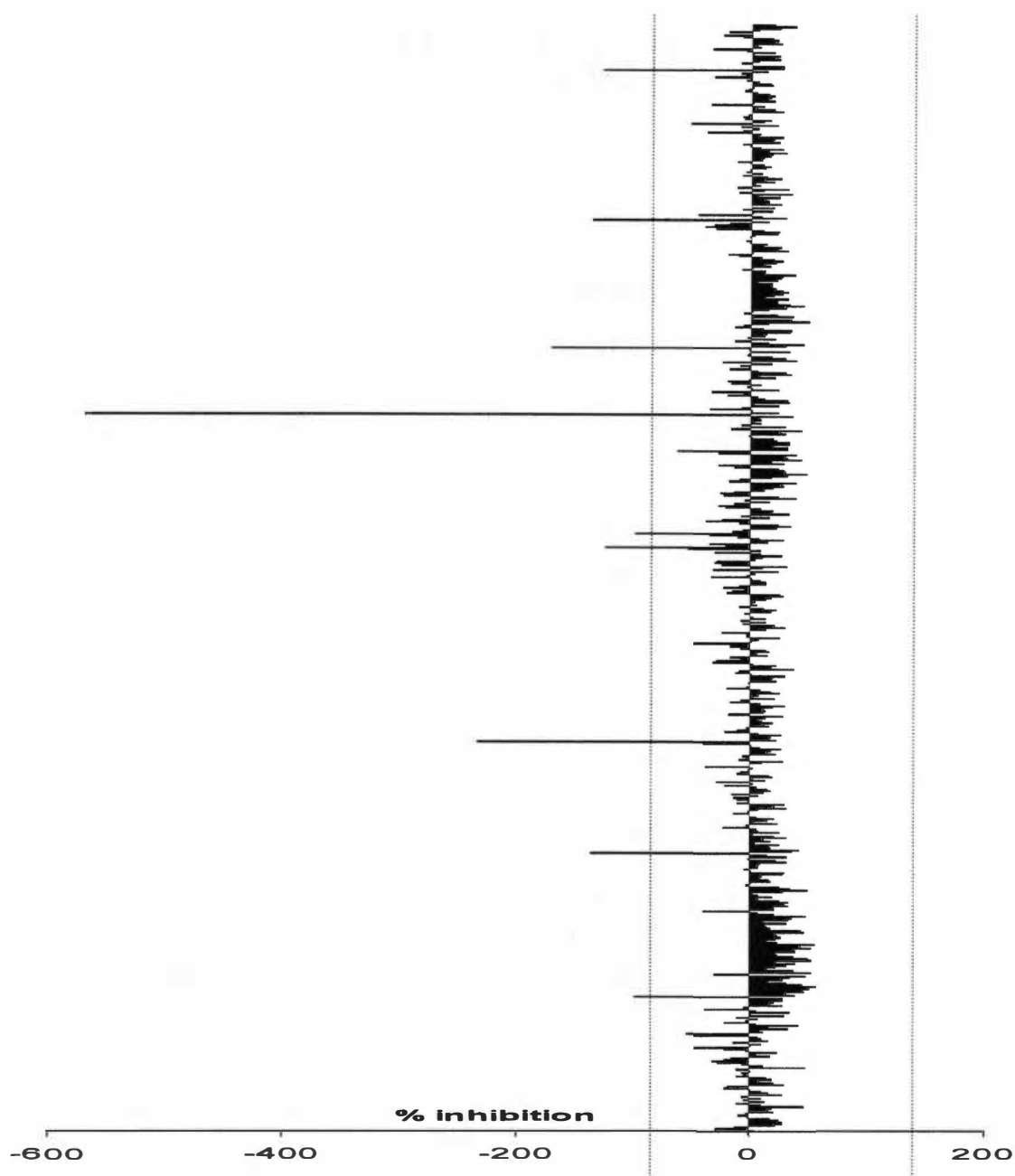


Figure 3 Results of primary screen of LOPAC library. Results shown are average of triplicate measurements for each compound after 30 minutes. Stimulatory compounds exhibit negative inhibition. Vertical lines represent three standard deviations.

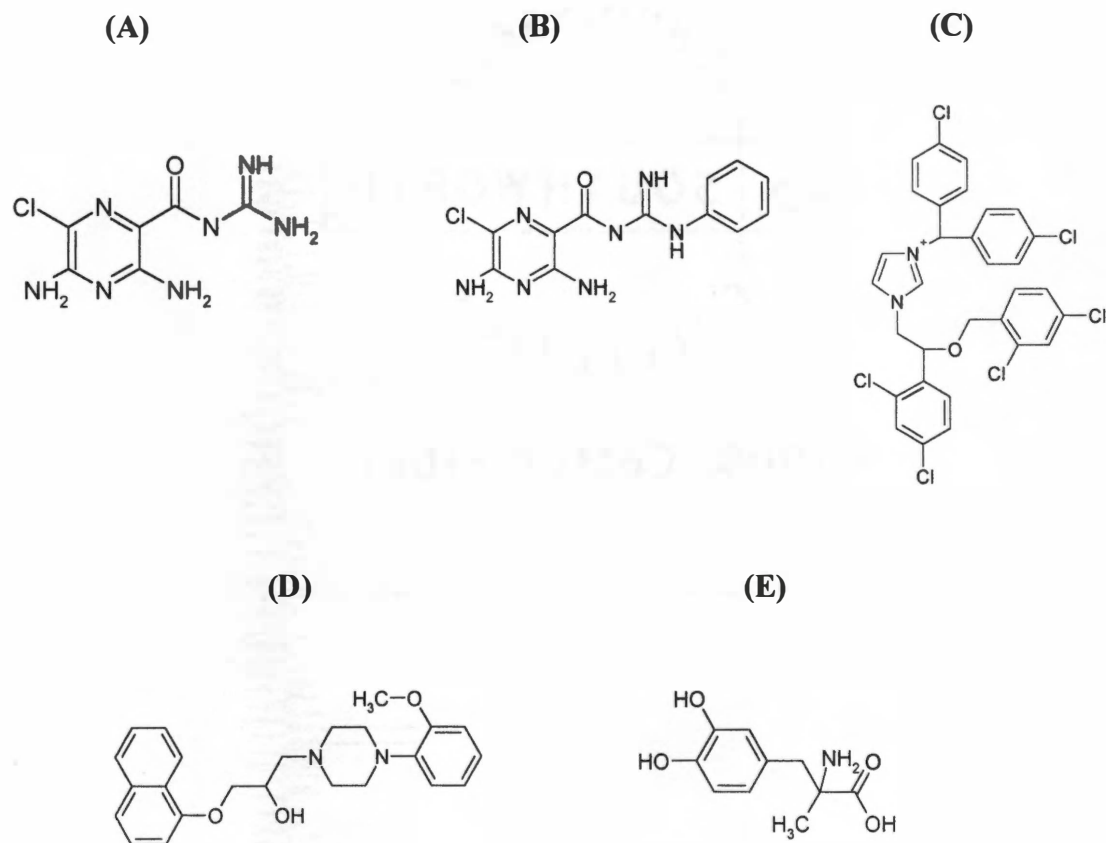
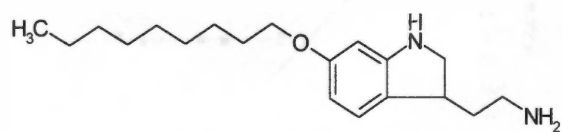


Figure 4 Compounds exhibiting stimulation of A β aggregation in initial screen of LOPAC library (see Materials and Methods). **A** – Amiloride HCl. Initial stimulation observed = 96%; EC₅₀ $\geq$ 38.5 μ M. **B** – Phenamil methane sulfonate. Initial stimulation observed = 242%; EC₅₀ $\geq$ 35.5 μ M. **C** – Calmidazolium chloride. Initial stimulation observed = 598%; EC₅₀ = 21.4 μ M. **D** – Naftopidil dihydrochloride. Initial stimulation observed = 150%; EC₅₀ $\geq$ 36.2 μ M. **E** – L- α -methyl DOPA. Initial stimulation observed = 127%; EC₅₀ $\geq$ 34.2 μ M.

(F)



(G)

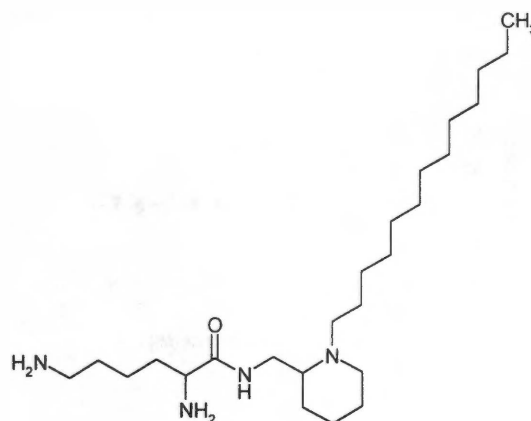


Figure 4 (cont.) F – 5-(nonyloxy)-tryptamine hydrogen oxalate. Initial stimulation observed = 136%; $EC_{50} \geq 30.3 \mu\text{M}$. G – NPC15437. Initial Stimulation observed = 149%; $EC_{50} \geq 27.2 \mu\text{M}$.

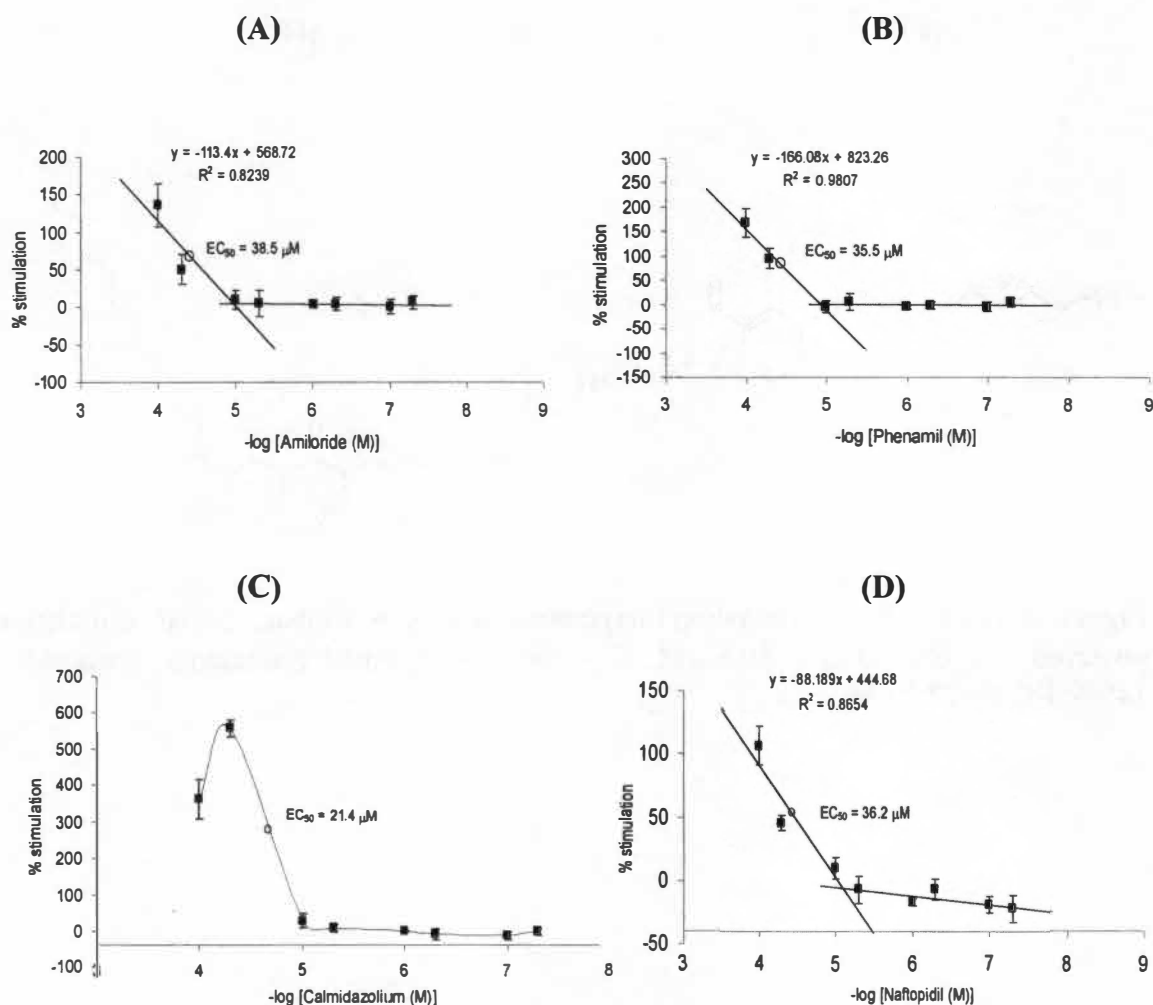


Figure 5 Dose response studies (conducted as described in Materials and Methods). EC₅₀ values represented on each curve by (o). **A** – Results for amiloride HCl. Data points within the $-\log[\text{Amiloride}]$ range of 4 – 5 were used for the linear fit. **B** - Results for phenamil methane sulfonate. Data points within the $-\log[\text{Phenamil}]$ range of 4 – 5 were used for the linear fit. **C** – Results for calmidazolium chloride. **D** – Results for naftopidil dihydrochloride. Data points within the $-\log[\text{Naftopidil}]$ range of 4.3 – 6 were used for the linear fit.

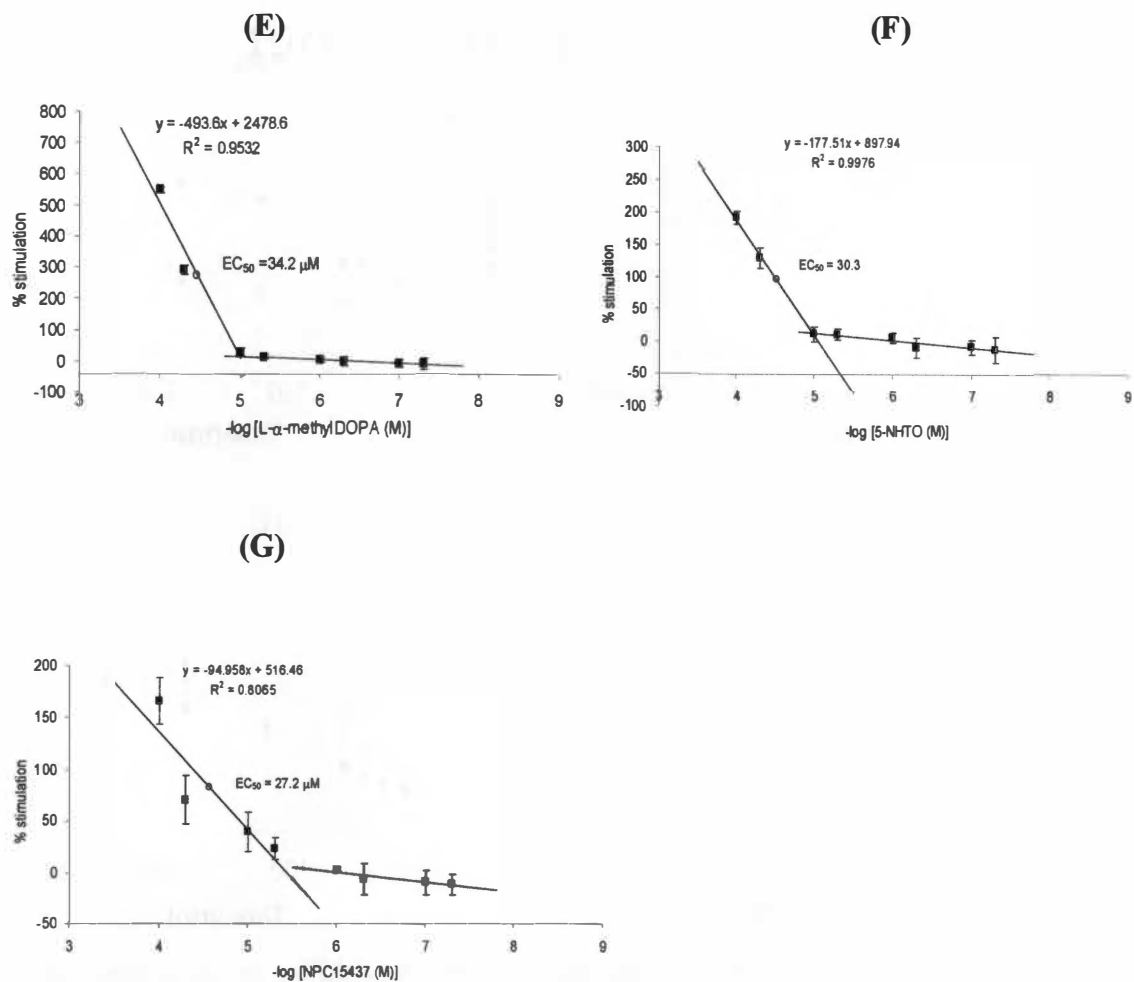


Figure 5 (cont.) **E** – Results for L- α -methyl DOPA. Data points within the $-\log[\text{L-}\alpha\text{-methyl DOPA}]$ range of 4 – 5 were used for the linear fit. **F** – Results for 5-(nonyloxy)-tryptamine hydrogen oxalate. Data points within the $-\log[5\text{-NHTO}]$ range of 4 – 5 were used for the linear fit. **G** – Results for NPC 15437. Data points within the $-\log[\text{NPC15437}]$ range of 4.3 – 6.3 were used for the linear fit.

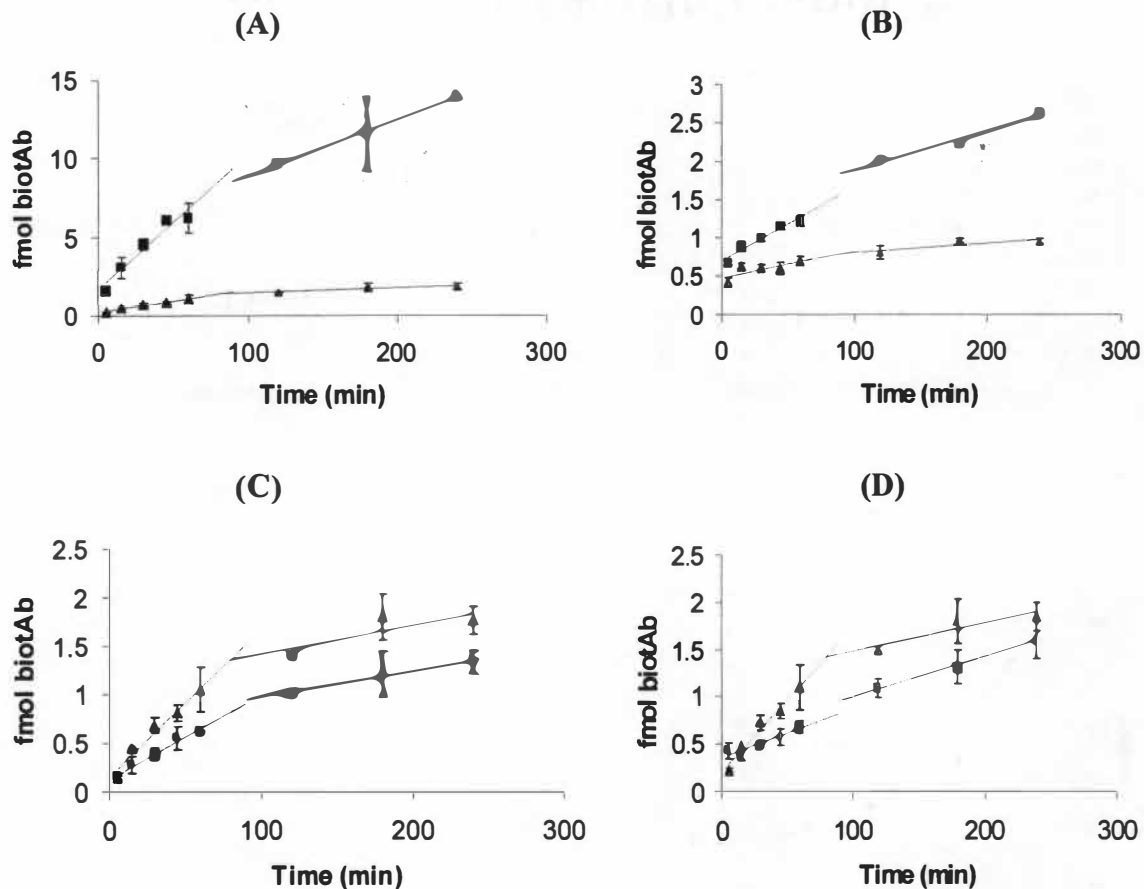


Figure 6 Effect of stimulatory compounds on A β deposition in microplate assay (conducted as described in Materials and Methods). **A** – Kinetic time course of A β deposition in presence (■) and absence (▲) of 100 μ M calmidazolium. **B** – Kinetic time course of A β deposition in presence (■) and absence (▲) of 100 μ M NPC 15437. **C** – Kinetic time course of A β deposition in microplate assay in presence (■) and absence (▲) of 100 μ M amiloride HCl. **D** - Kinetic time course of A β deposition in microplate assay in presence (■) and absence (▲) of 100 μ M phenamil methane sulfonate. The kinetic time courses shown in the absence of compound were obtained on one microplate for **A** and **B**, and a separate microplate for **C** and **D**.

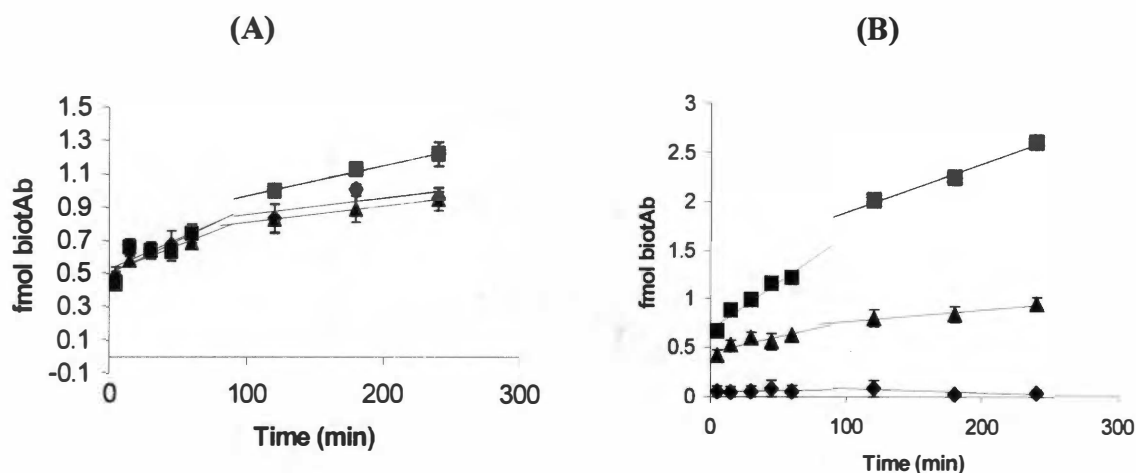


Figure 7 Washout assay results for NPC 15437. The washout assay was conducted as described in Materials and Methods. **A** – Kinetic time course of A β deposition in microplate wells incubated with 100 μ M NPC 15437 for 60 minutes followed by incubation in buffer for 2 hours prior to the assay ($\blacklozenge$), in wells incubated with 100 μ M NPC 15437 for 60 minutes immediately prior to the assay ($\blacksquare$), and wells with no NPC 15437 present ($\blacktriangle$). **B** – Kinetic time course of A β deposition in microplate wells with 100 μ M NPC 15437 and fibrils present ($\blacksquare$), wells with 100 μ M NPC 15437 and no fibrils present ($\blacklozenge$), and wells with fibrils and no NPC 15437 present ($\blacktriangle$).

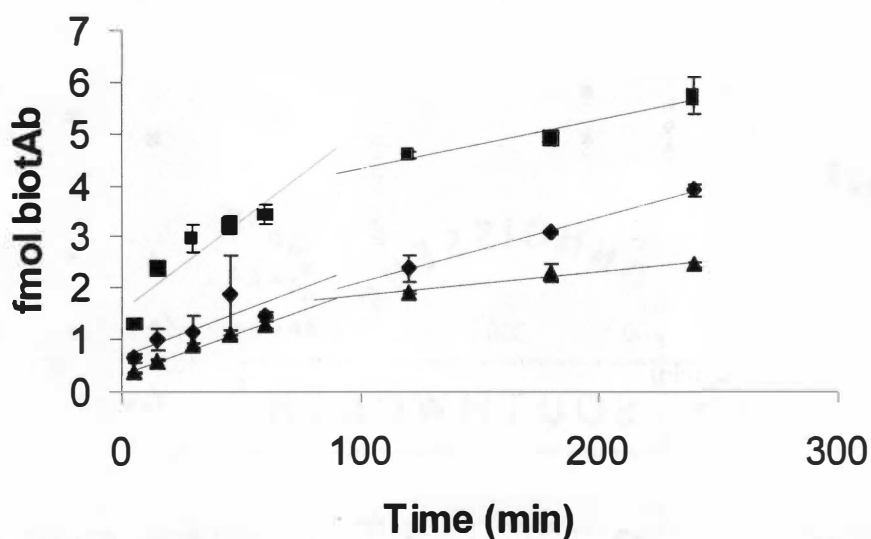


Figure 8 Washout assay results for calmidazolium chloride (conducted as described in Materials and Methods). Kinetic time courses shown represent A β deposition in wells incubated with 100 μ M calmidazolium for 60 minutes followed by a 2 hour incubation in buffer prior to the assay (♦), wells incubated with 100 μ M calmidazolium for 60 minutes immediately prior to the assay (■), and wells with no calmidazolium present during the assay (▲). The kinetic time courses shown here were obtained on the same day, and from the same microplate. The time course shown here in the absence of calmidazolium was obtained separately from that shown in Figure 5A.

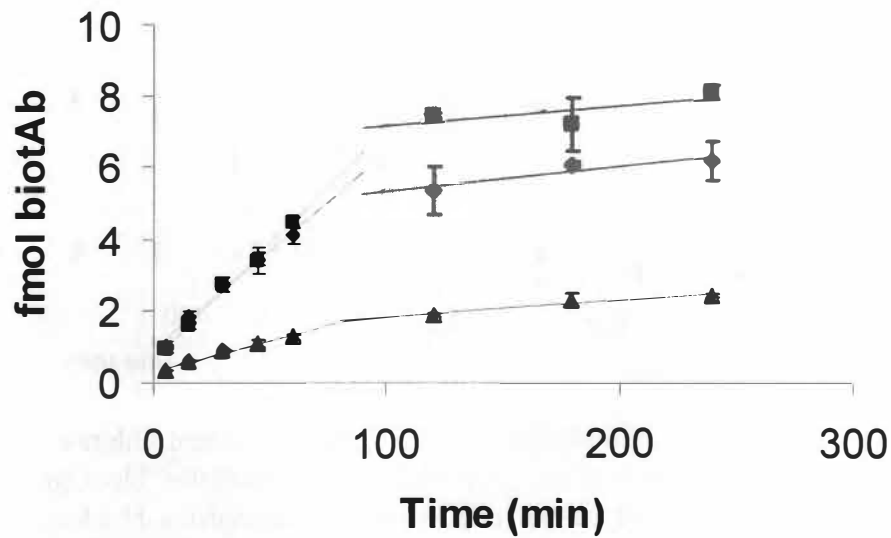


Figure 9 Lack of requirement of fibril seeds for the stimulatory effect of calmidazolium chloride. The microplate assay was conducted as described in Materials and Methods using fibrils coated wells in the presence and absence of 100 μ M calmidazolium, and wells without fibrils (blocked with 0.3% gelatin) in the presence and absence of calmidazolium. Data points shown represent the kinetic time course of A β deposition with 100 μ M calmidazolium and fibrils present ($\diamond$), 100 μ M calmidazolium with no fibrils present ($\blacksquare$), and fibrils with no calmidazolium present ($\blacktriangle$).

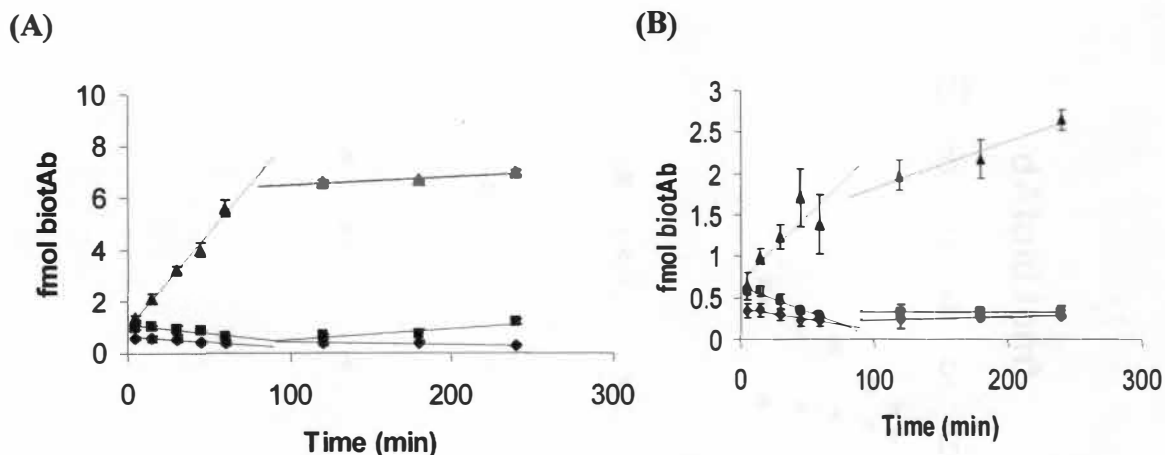


Figure 10 Lack of influence of blocking agent on calmidazolium chloride stimulatory effect. **A** – Kinetic time course of Aβ deposition on microplates blocked with 0.3% gelatin. **B** – Kinetic time course of Aβ deposition on microplates blocked with 0.5% BSA. No fibrils were present in any of the microplate wells. The washout assay was conducted for each microplate as described in Materials and Methods. Results are shown for wells incubated with 100 μM calmidazolium for 60 minutes followed by a 2 hour incubation in buffer prior to the assay (◆), wells incubated with 100 μM calmidazolium for 60 minutes immediately prior to assay (■), and 100 μM calmidazolium continuously present in the wells (▲).

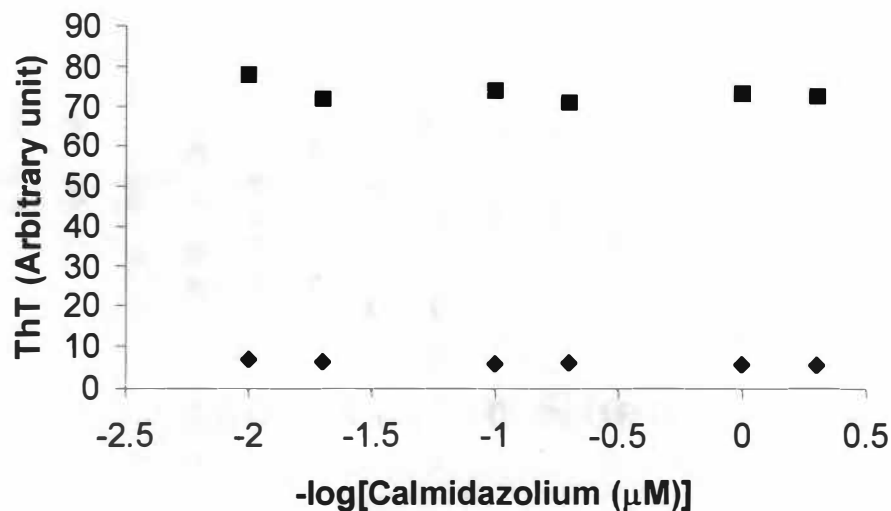


Figure 11 Effect of calmidazolium chloride on ThT fluorescence in presence (■) and absence (♦) of Aβ fibrils. ThT fluorescence values in absence of calmidazolium are 6.2 ± 0.4 for ThT alone and 71.7 ± 1.4 for ThT in the presence of fibrils.

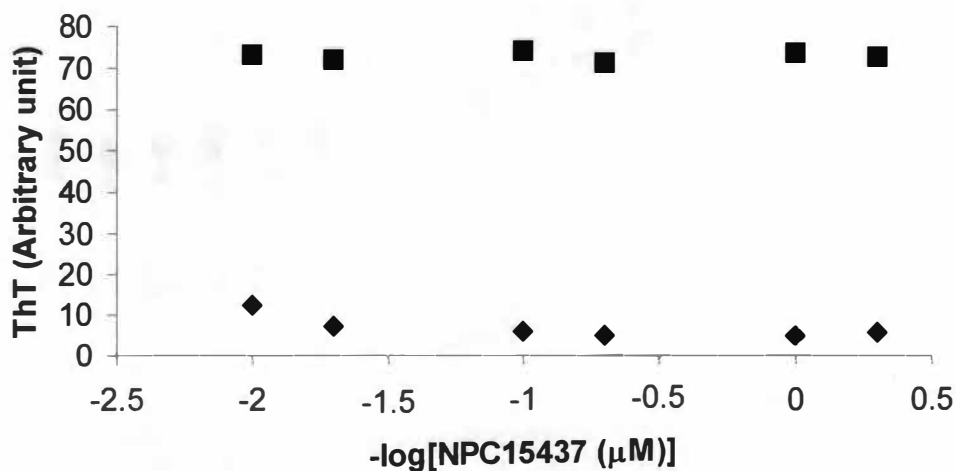


Figure 12 Effect of NPC 15437 on ThT fluorescence in presence (■) and absence (♦) of Aβ fibrils. ThT fluorescence values in absence of NPC 15437 are 6.2 ± 0.4 for ThT alone and 71.7 ± 1.4 for ThT in the presence of fibrils

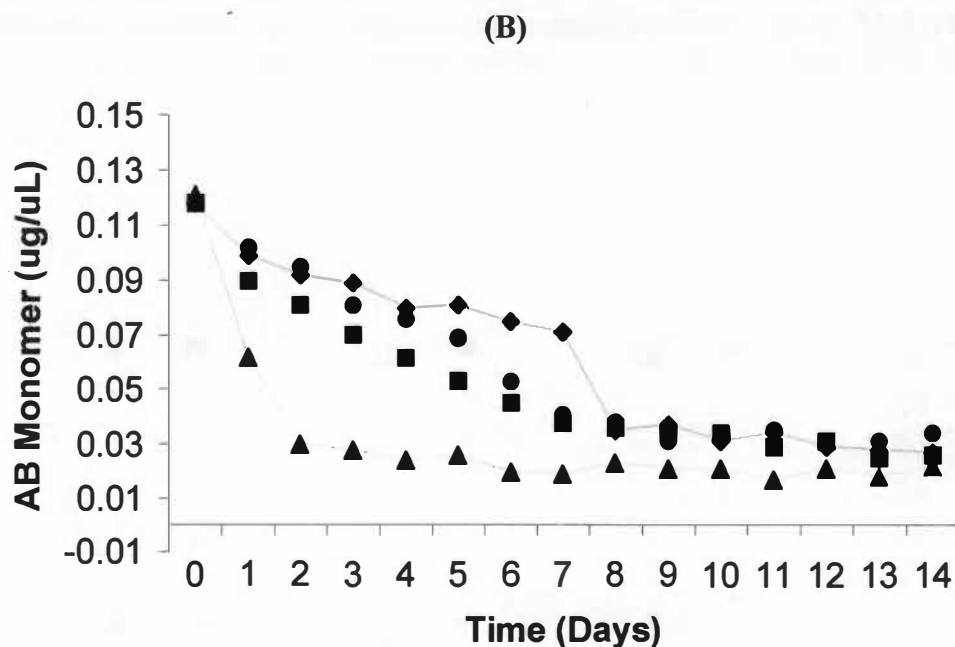
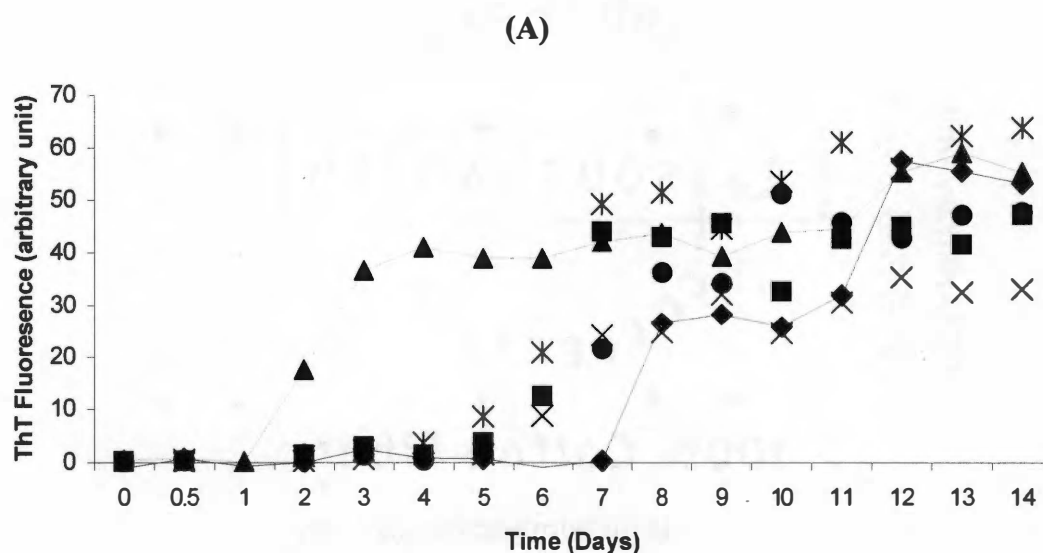


Figure 13 Effect of NPC 15437 on A β aggregation. **A** – As measured by ThT fluorescence (see Materials and Methods). **B** – As measured by RP-HPLC (see Materials and Methods). 30 μ M A β in 1X PBS present in each aggregation reaction. Unseeded (◆) and seeded (▲) control A β aggregation reactions (no NPC 15437 present) shown in **A** and **B**. **A** – Data points represent unseeded A β aggregation reactions in the presence of 100 μ M NPC 15437 (*), 50 μ M NPC 15437 (■), 10 μ M NPC 15437 (X), and 1 μ M NPC 15437 (●). **B** – Data points represent unseeded A β aggregation reactions in the presence of 100 μ M NPC 15437 (■), and 1 μ M NPC 15437 (●).

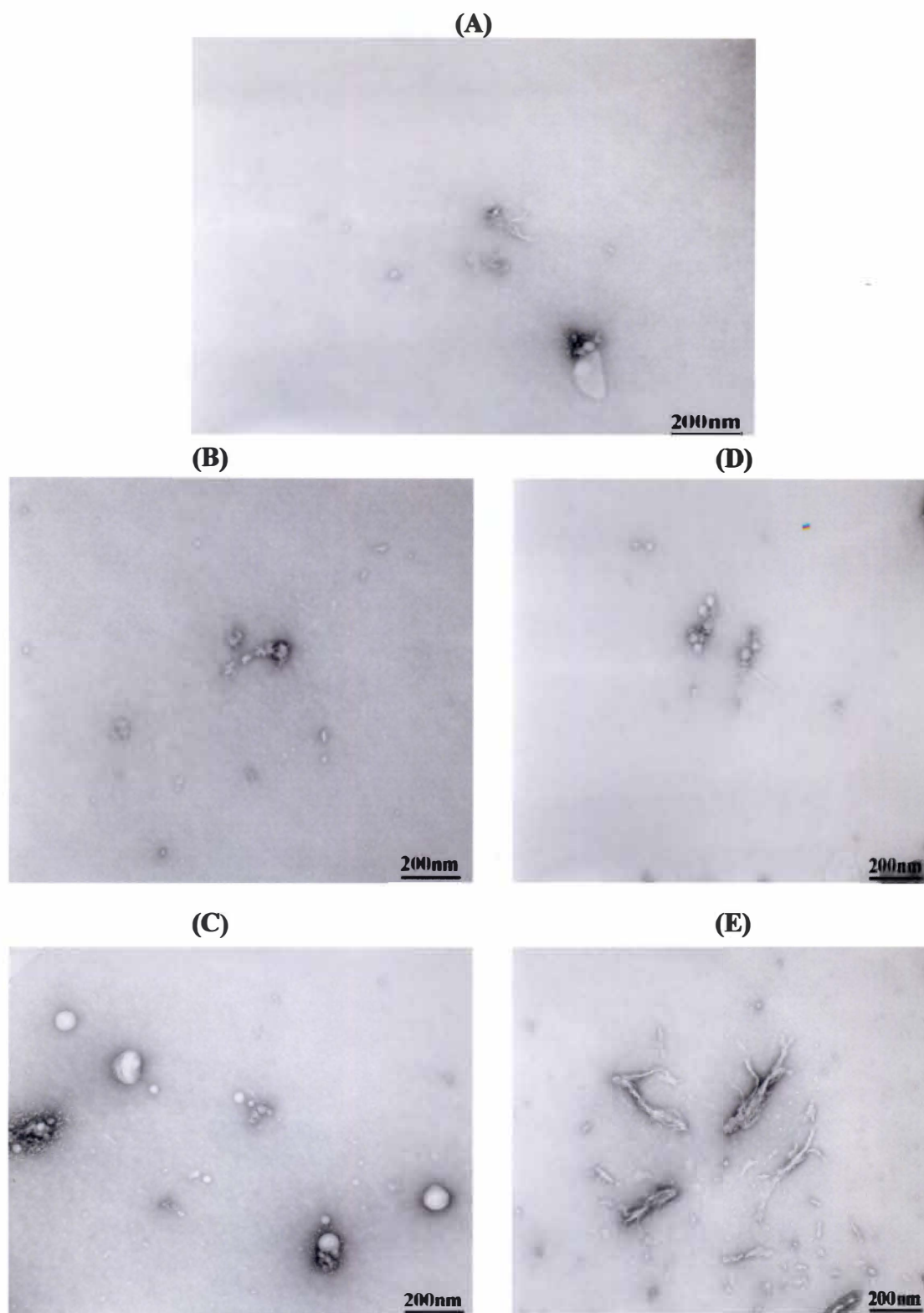


Figure 14 A β aggregates grown in absence of compound after 2 days.

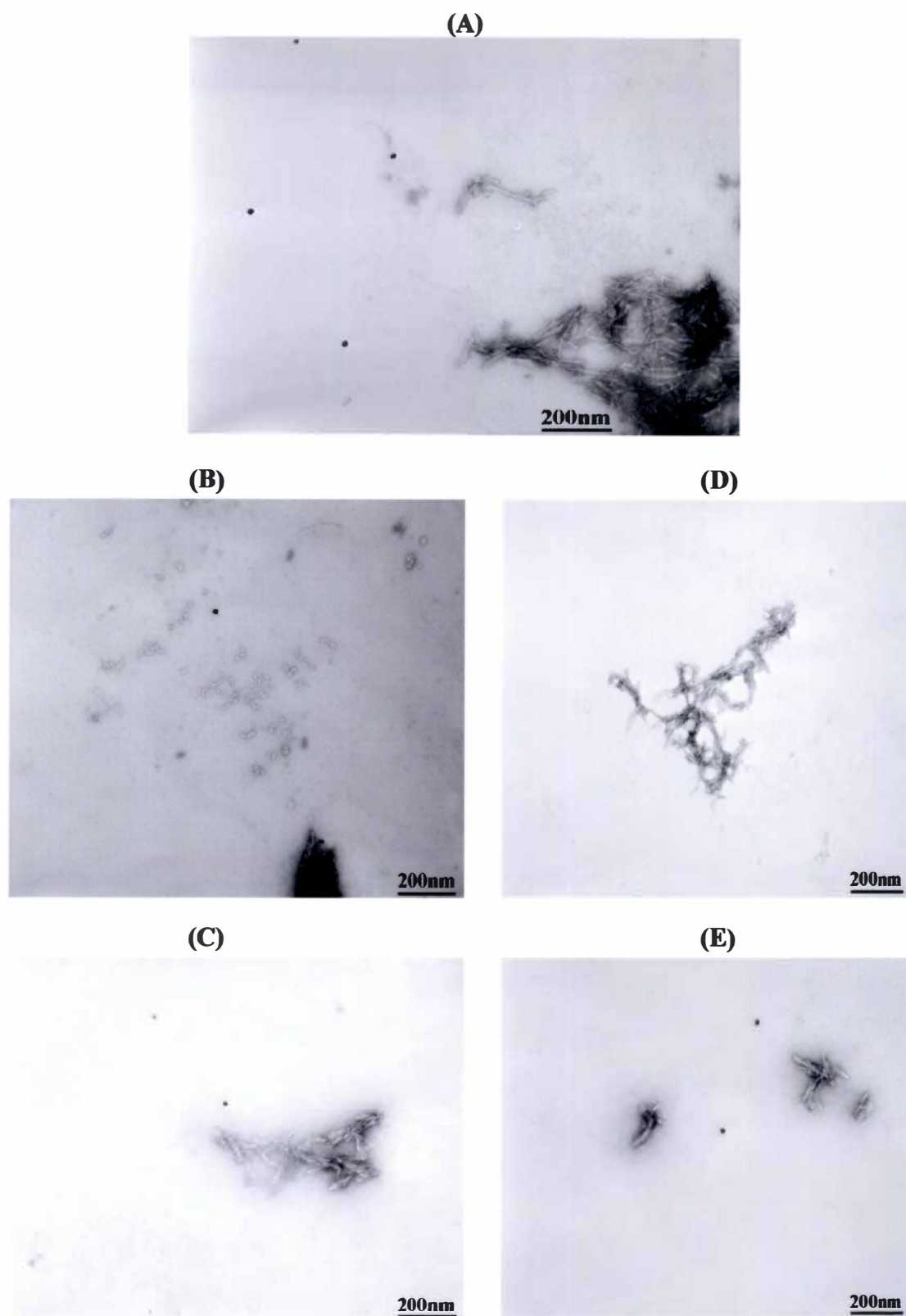


Figure 15 A β aggregates (fibrils) grown in absence of compound after 14 days.

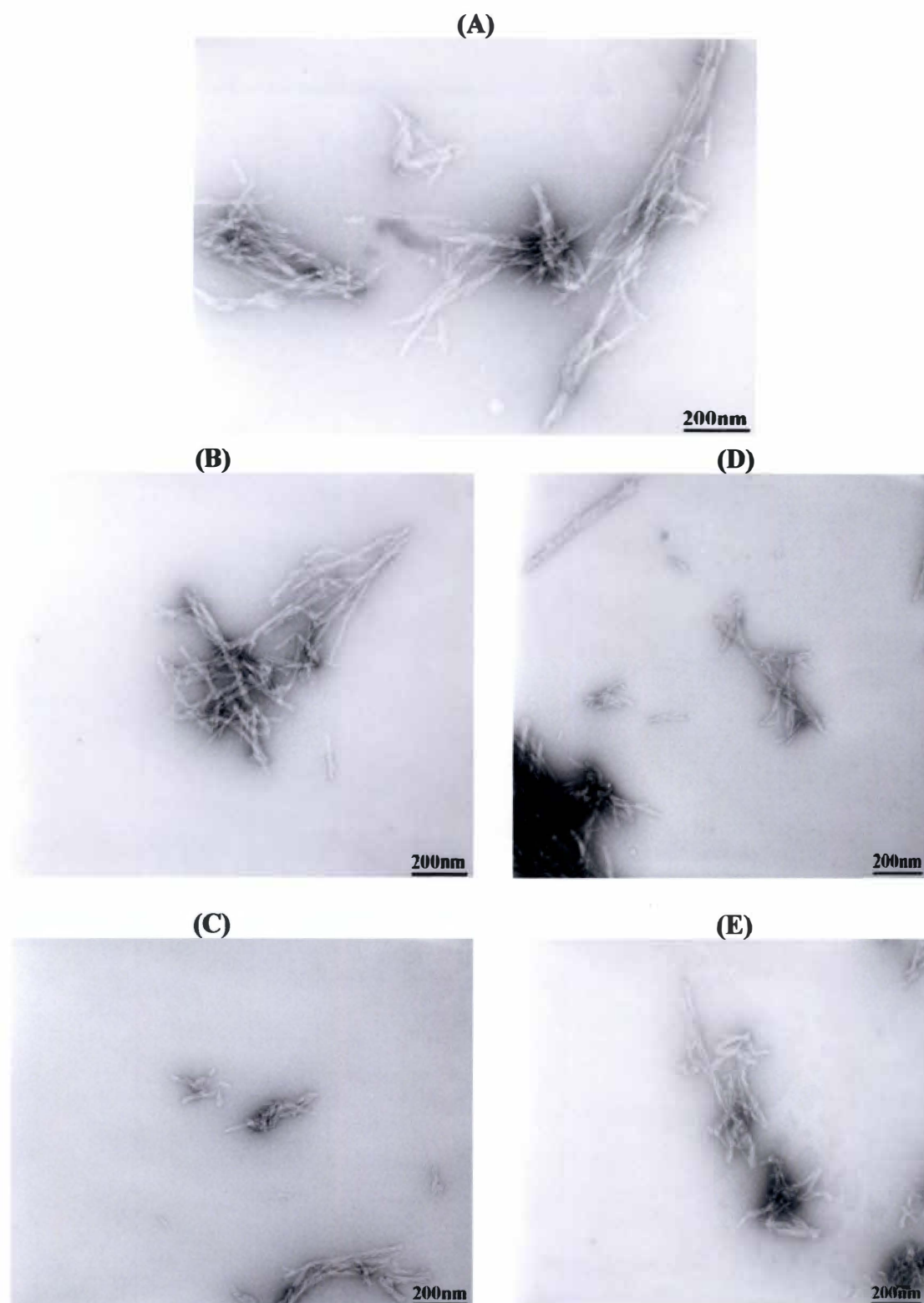


Figure 16 Aβ fibrils grown in presence of 100 μM NPC 15437 after 14 days.

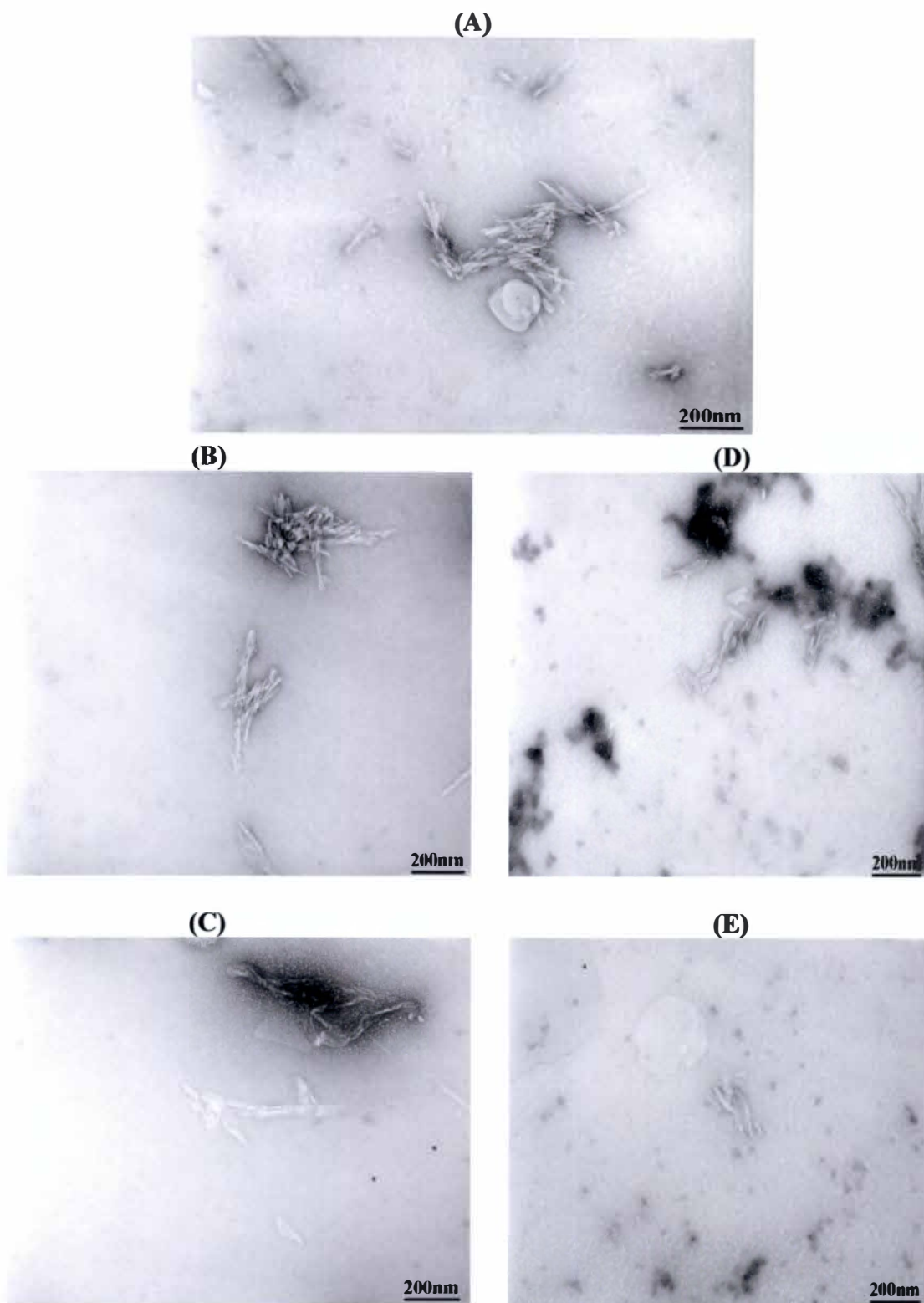


Figure 17 Aβ fibrils grown in presence of 10 μM NPC 15437 after 14 days.

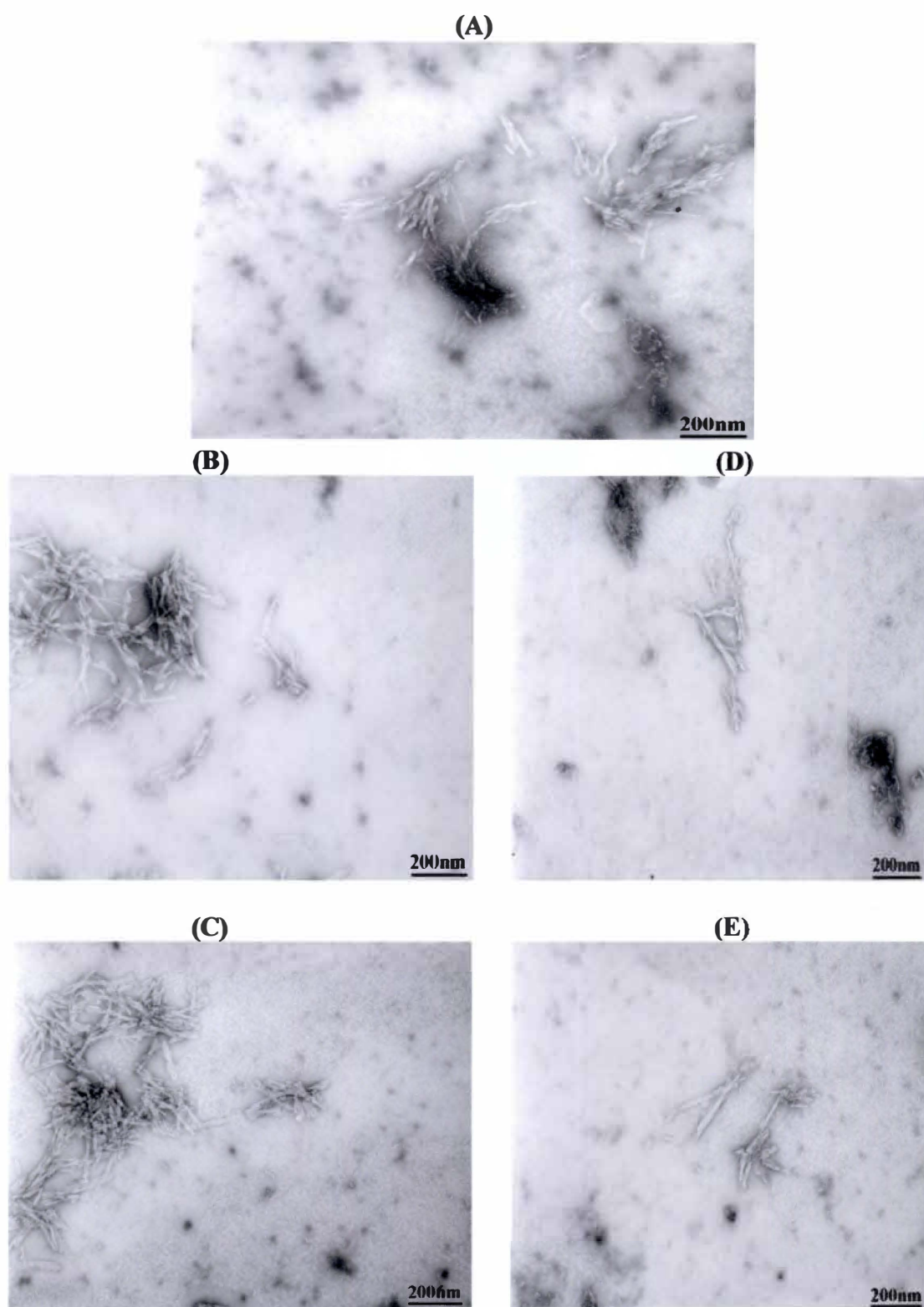


Figure 18 Aβ fibrils grown in presence of 1 μM NPC 15437 after 14 days.

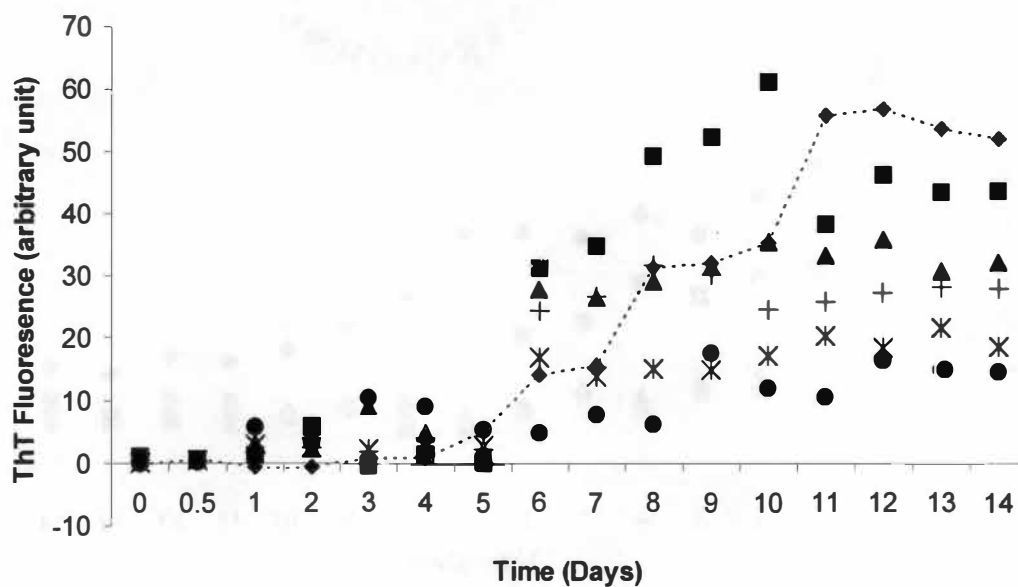


Figure 19 Effect of calmidazolium chloride on unseeded A β aggregation as measured by ThT fluorescence (see Materials and Methods). 30 μ M A β in 1X PBS present in each aggregation reaction. Data points shown represent reactions with no calmidazolium present (◆), 100 μ M calmidazolium present (●), 30 μ M calmidazolium present (*), 20 μ M calmidazolium present (+), 10 μ M calmidazolium present (▲), and 1 μ M calmidazolium present (■).

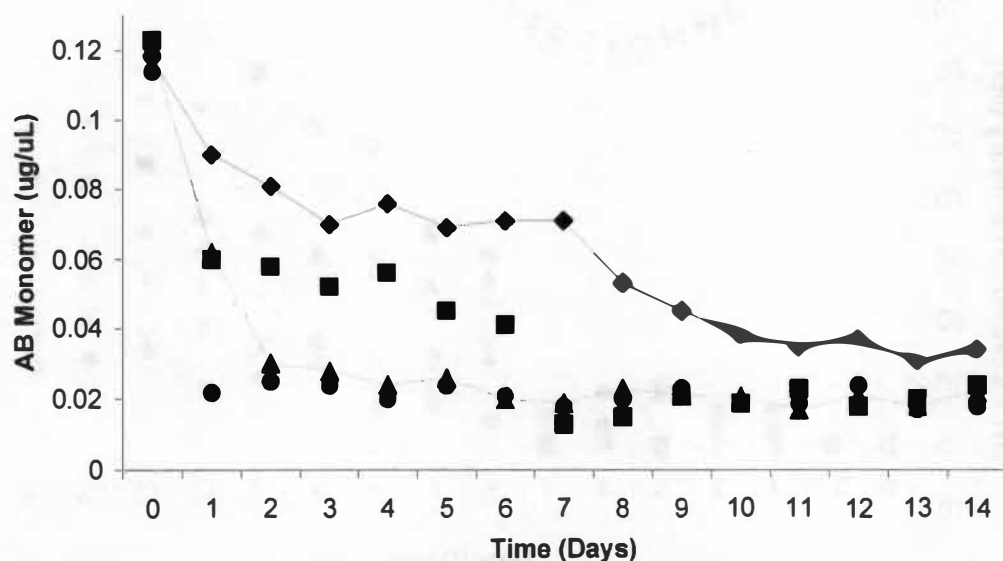


Figure 20 Effect of calmidazolium chloride on A β aggregation as measured by RP-HPLC (see Materials and Methods). 30 μ M A β in 1X PBS present in each aggregation reaction. Data points shown represent unseeded (◆) and seeded (▲) controls with no calmidazolium present, and unseeded reactions with 100 μ M calmidazolium (●) and 1 μ M calmidazolium (■) present.

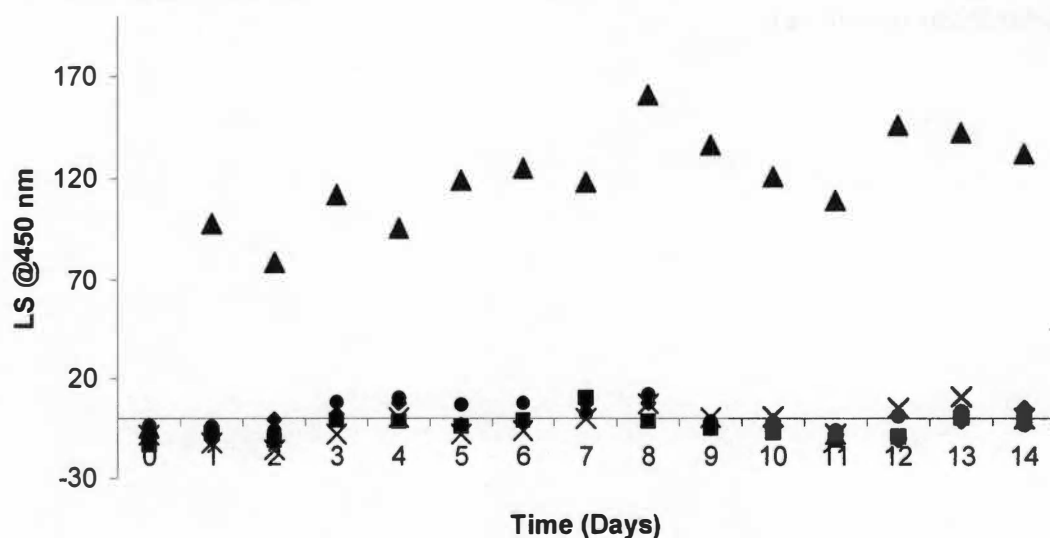


Figure 21 Effect of calmidazolium chloride on unseeded A β aggregation as measured by 90° light scattering at 450 nm (see Materials and Methods). 30 μ M A β in 1X PBS present in each aggregation reaction. Data points represent A β aggregation in the absence of calmidazolium (◆), in the presence of 100 μ M calmidazolium (▲), in the presence of 20 μ M calmidazolium (●), in the presence of 10 μ M calmidazolium (■), and in the presence of 1 μ M calmidazolium (X).

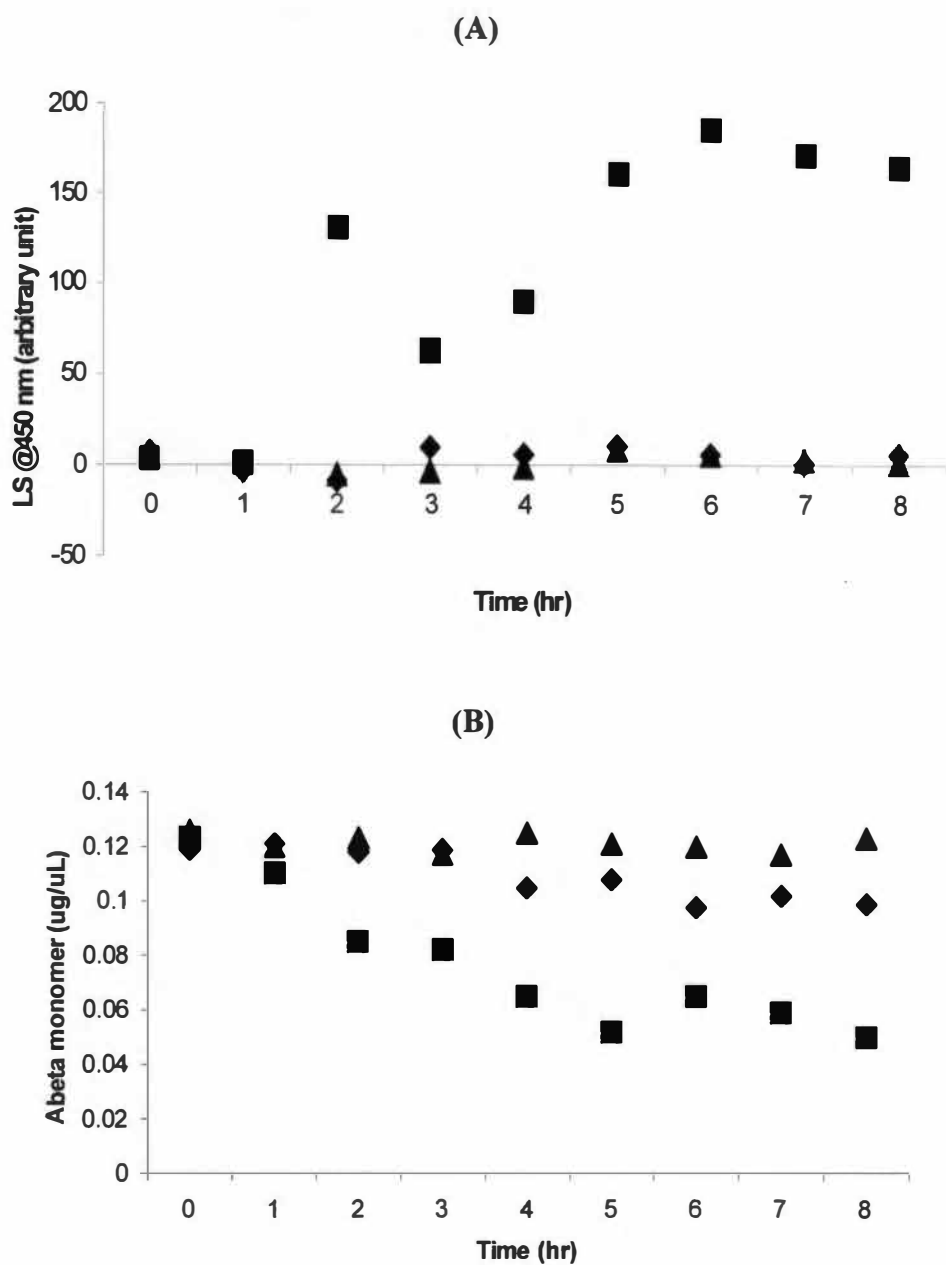


Figure 22 Effect of calmidazolium chloride on early stages of A β aggregation. **A** – A β aggregation as monitored by light scattering at 450 nm (conducted as described in Materials and Methods). **B** – As monitored by RP-HPLC (conducted as described in Materials and Methods). Data points shown represent unseeded (▲) and seeded (◆) A β aggregation reactions, and unseeded A β aggregation in the presence of 100 μ M calmidazolium (■).

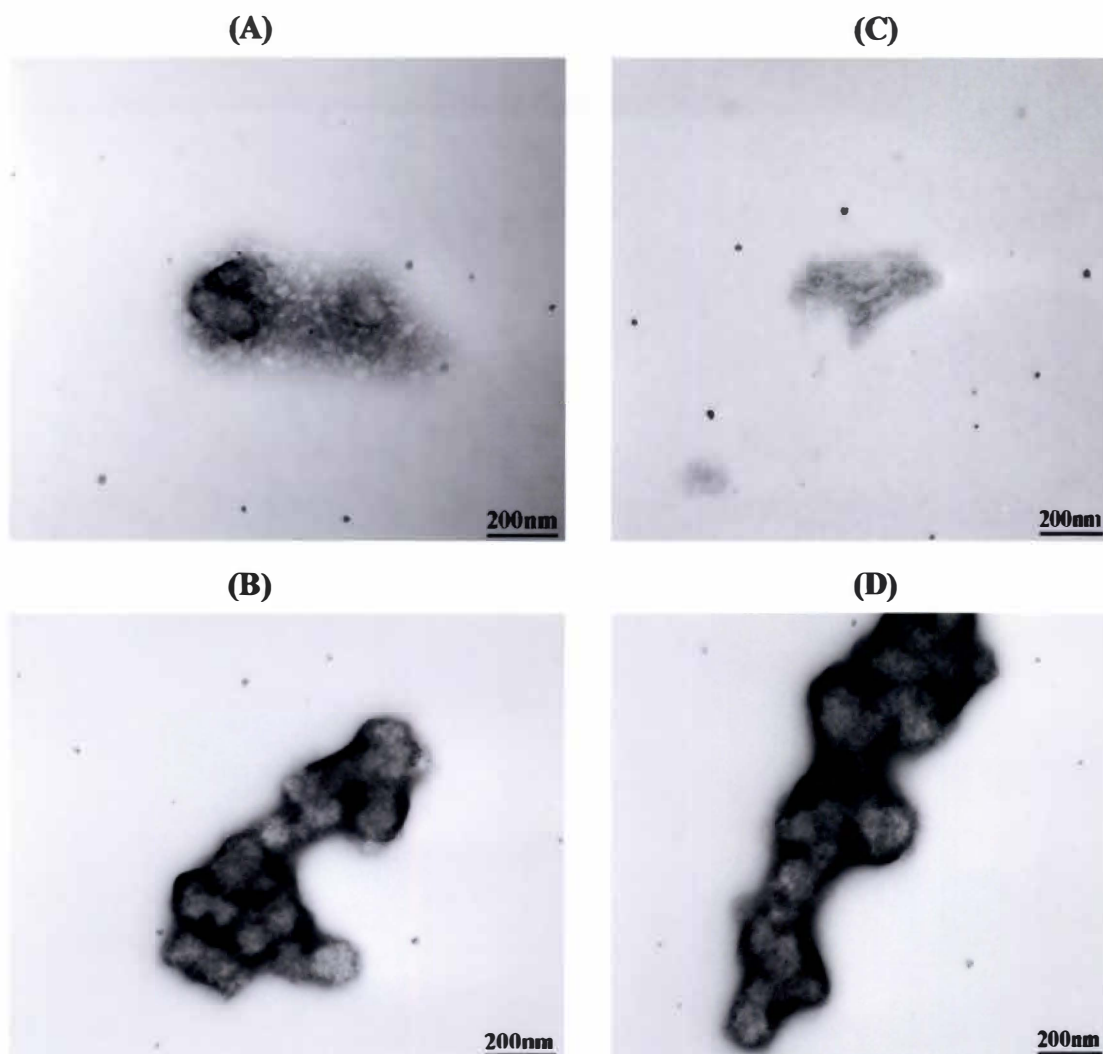


Figure 23 Aβ aggregates grown in presence of 100 μM calmidazolium after 2 days.

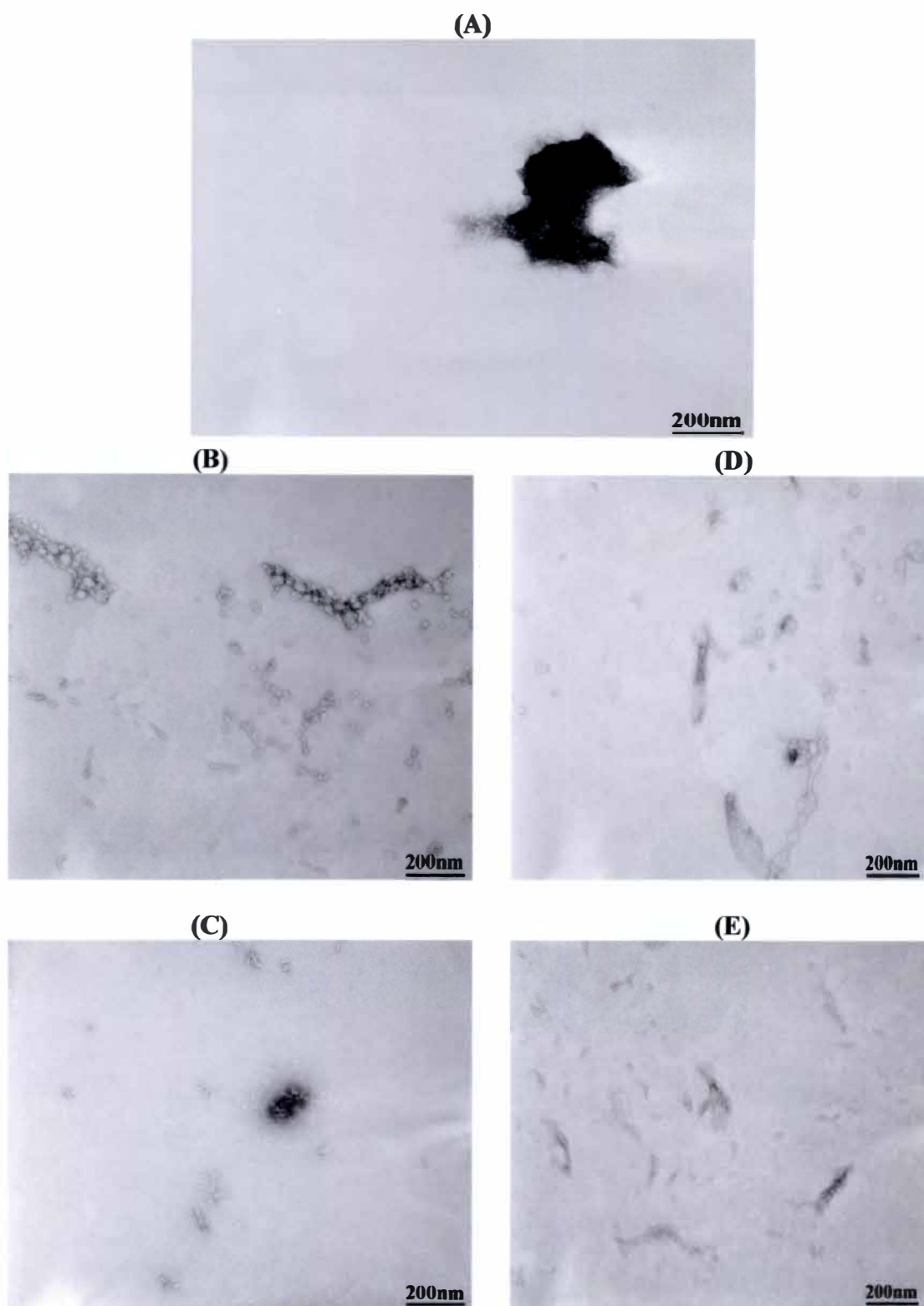


Figure 24 A β aggregates grown in presence of 10 μ M calmidazolium after 2 days.

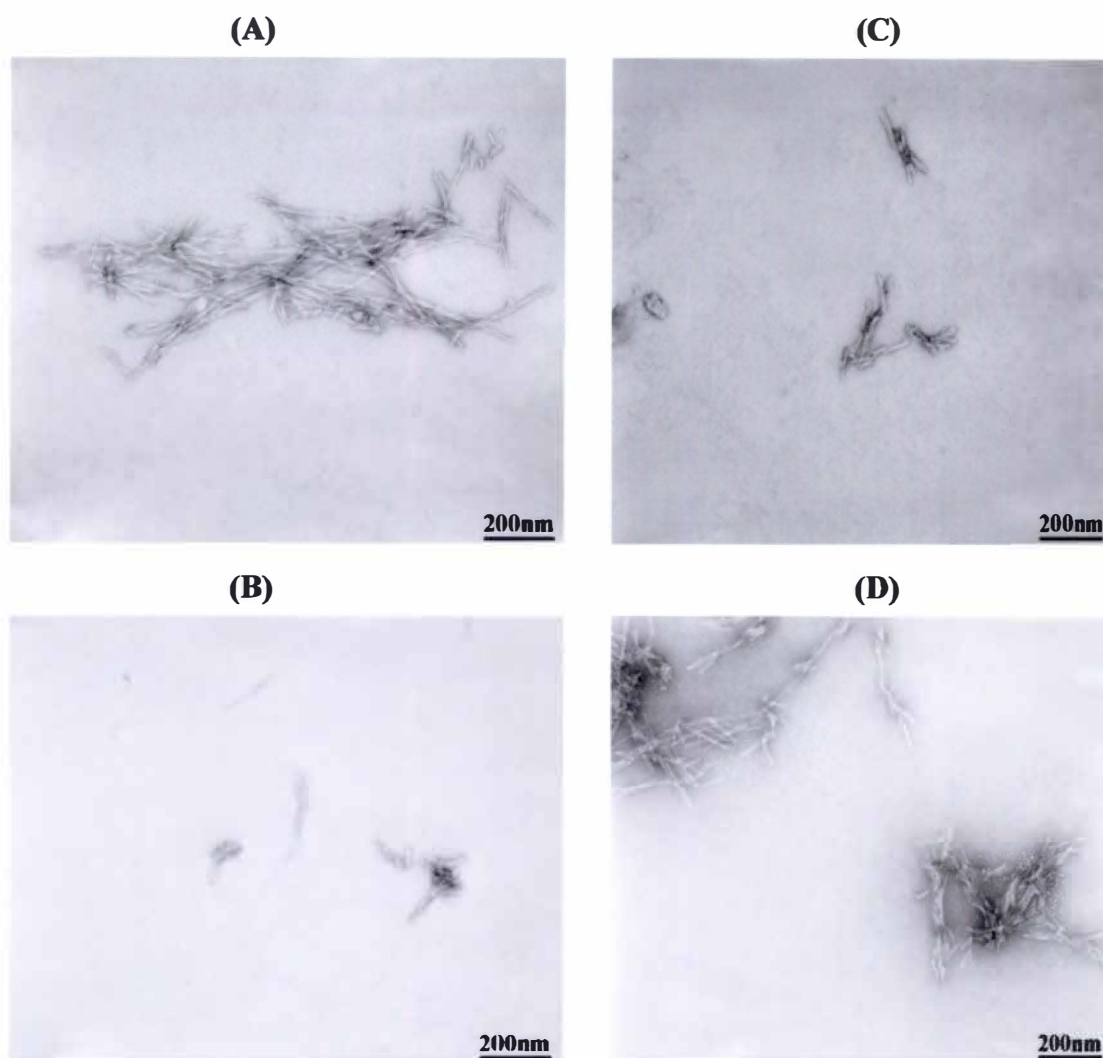


Figure 25 Aβ aggregates grown in presence of 1 μM calmidazolium after 2 days.

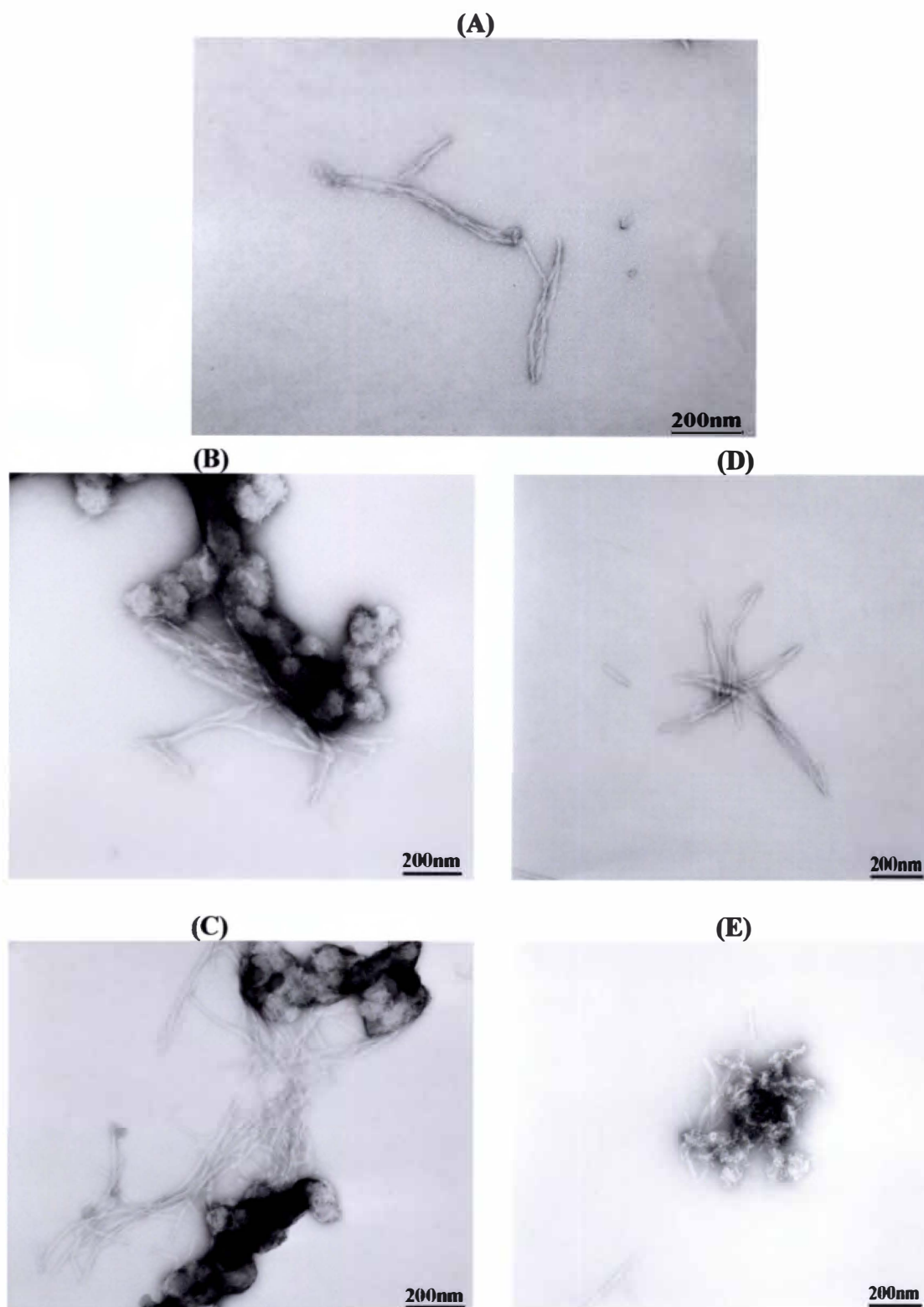


Figure 26 A β aggregates grown in presence of 100 μ M calmidazolium after 14 days.

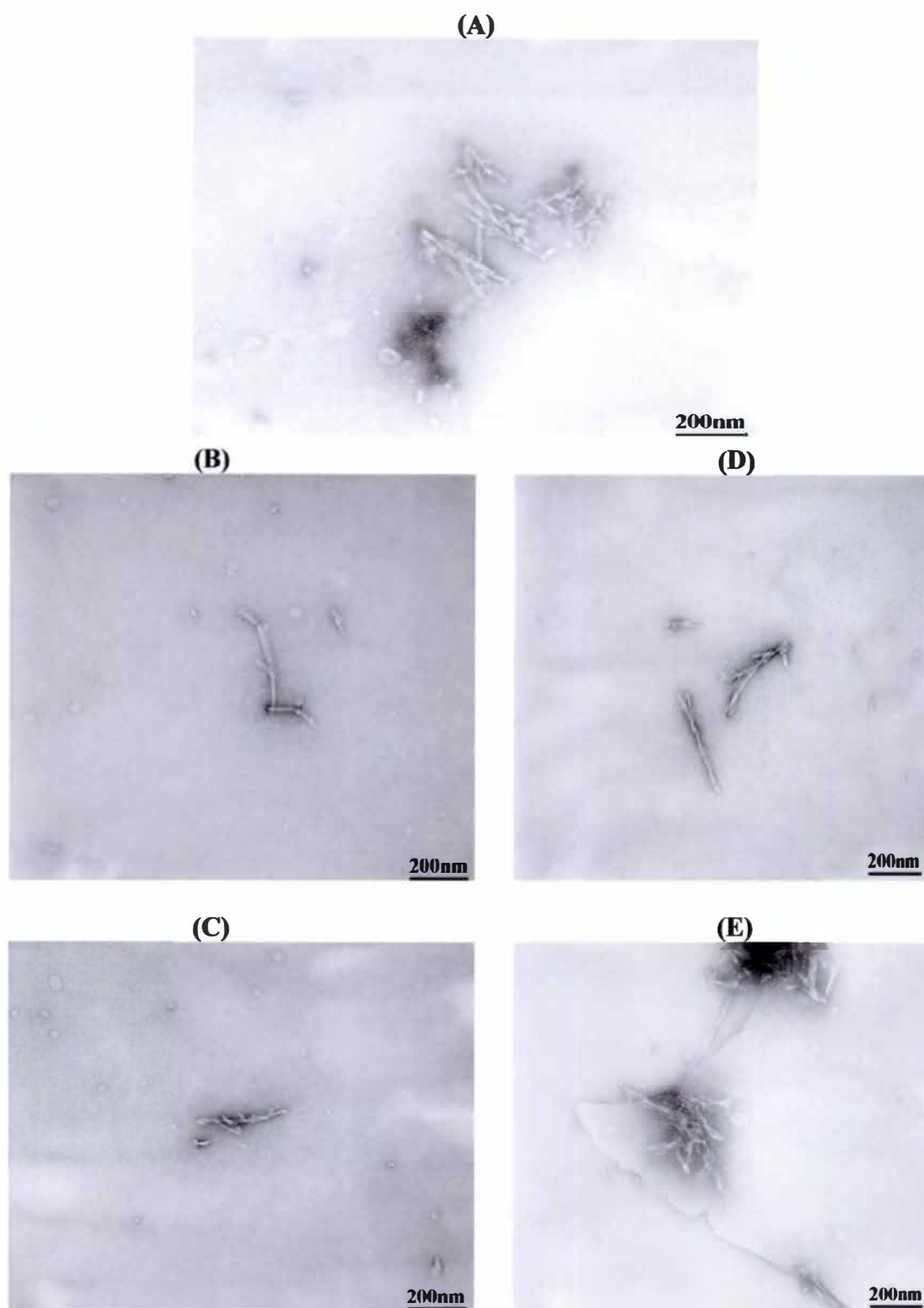


Figure 27 Aβ aggregates grown in presence of 10 μM calmidazolium after 14 days.

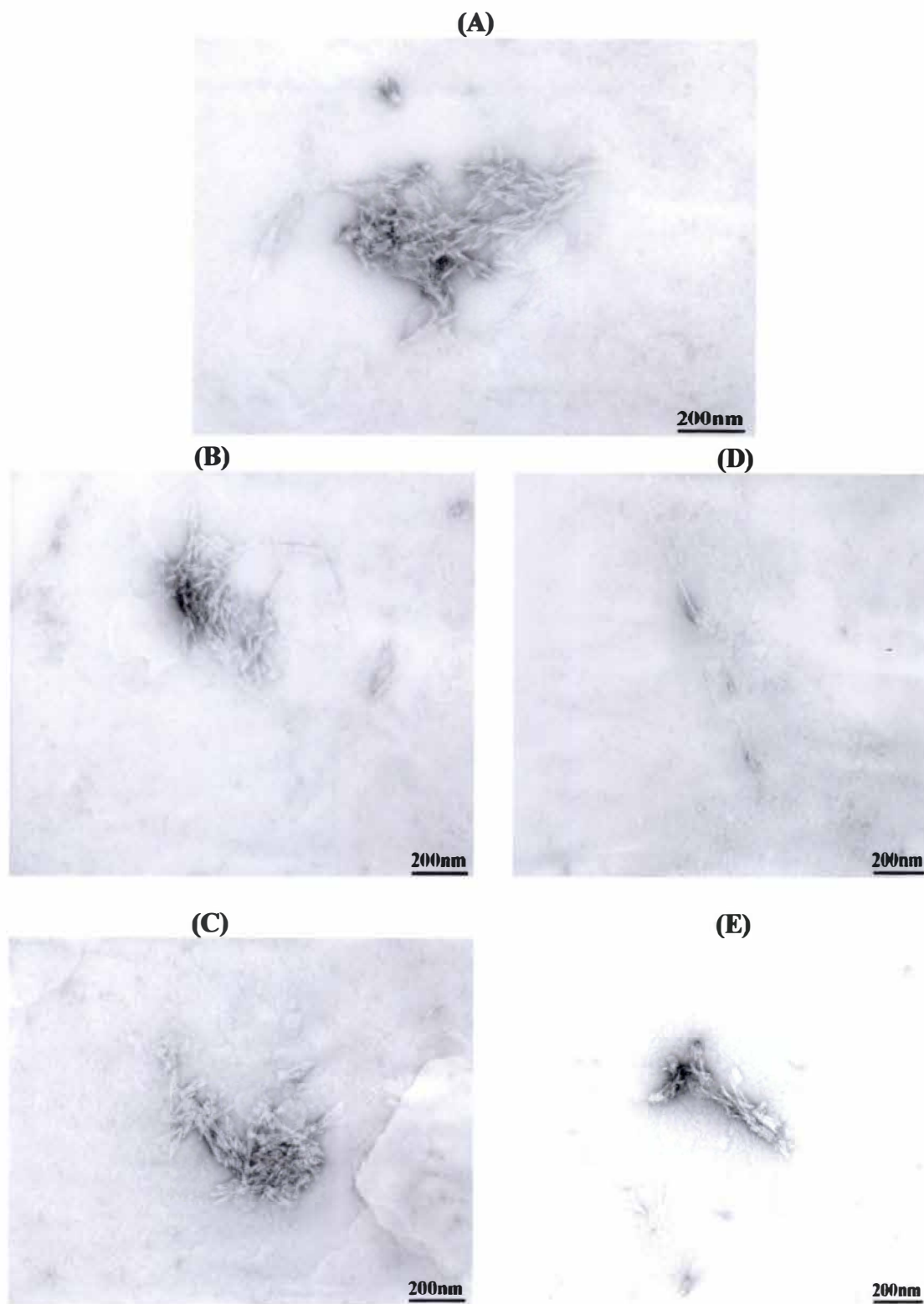


Figure 28 Aβ aggregates grown in presence of 1 μM calmidazolium after 14 days.

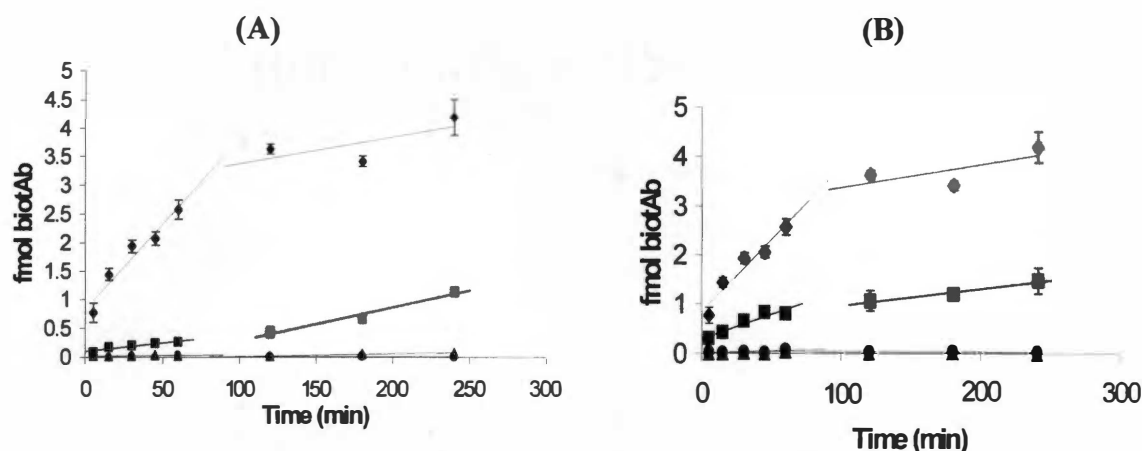


Figure 29 Seeding ability of A β aggregates grown in the presence of calmidazolium chloride. Aggregates were collected as described in Materials and Methods. **A** – Kinetic time course of A β deposition in microplate wells coated with A β aggregates grown in the presence of 100 μ M calmidazolium. **B** - Kinetic time course of A β deposition in microplate wells coated with A β aggregates grown in the presence of 10 μ M calmidazolium. The microplate assay was conducted as described in Materials and Methods. Data points shown represent aggregates grown in the presence of calmidazolium for 2 days (▲) and 14 days (■). Results are also shown for microplate wells coated with control fibrils grown in the absence of calmidazolium for 2 days (●) and 14 days (◆). Microplate wells coated with A β aggregates grown for only two days in both the presence (▲) and absence (●) of calmidazolium showed no ability to seed A β deposition in the microplate assay, and both sets of data are buried in the baseline of the figure. Results shown for microplate wells coated with control fibrils grown in the absence of calmidazolium for 2 days (●) and 14 days (◆) are the same in both **A** and **B**.

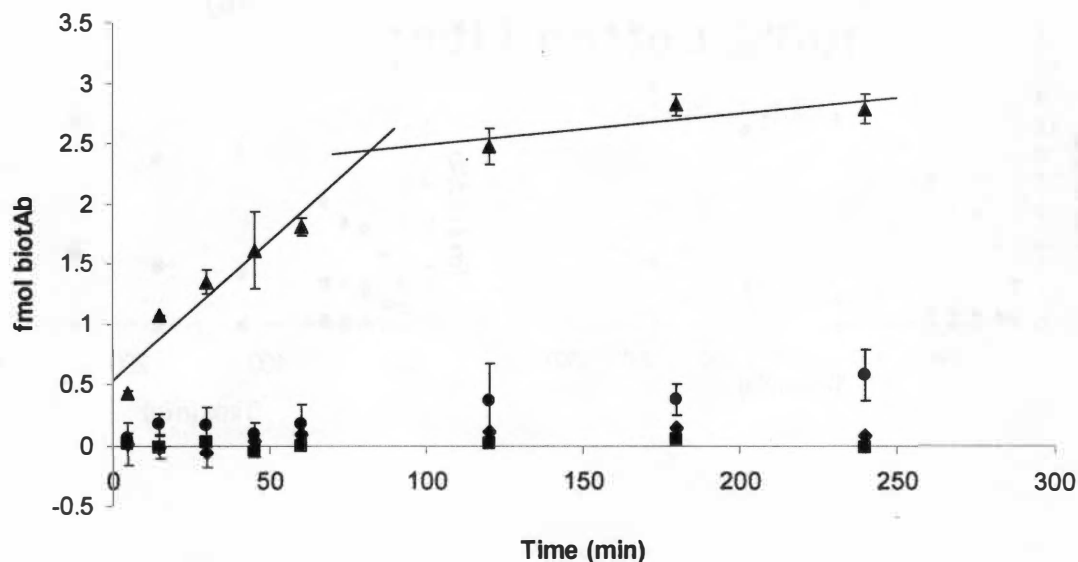


Figure 30 Seeding ability of A β /calmidazolium aggregates deposited in the microplate assay. Prior to conducting the microplate assay (as described in Materials and Methods), gelatin blocked microplate wells were incubated at 37°C for 1 hour with either 10 nM A β (non-biotinylated) plus 100 μ M calmidazolium chloride (♦), only 10 nM A β (non-biotinylated) (■), or only 100 μ M calmidazolium chloride (●) washed with buffer, and conducting the microplate assay as described in Materials and Methods. The standard microplate assay was also conducted in wells coated with 100 ng of fibrils (▲) (as described in Materials and Methods). No appreciable deposition of biotinyl-A β can be seen in any of the pre-incubated wells.

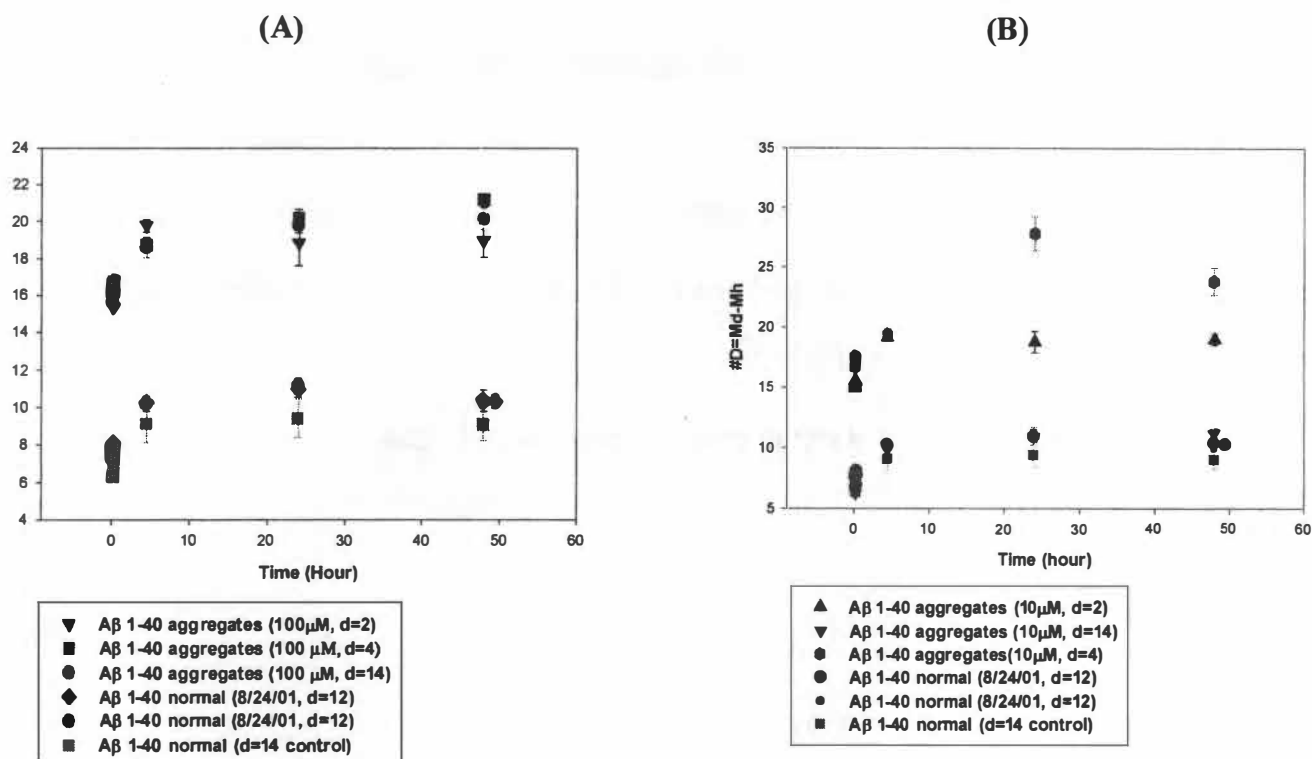


Figure 31 Deuterium content vs. time for deuterated A β aggregates. Data points represent the extent of deuteriation of A β aggregates grown in the presence of 100 μ M calmidazolium (A) and 10 μ M calmidazolium (B) for 2 days, 4 days, and 14 days. The same set of control data points is shown on each graph, representing A β fibrils grown using the same A β stock solution as the calmidazolium aggregates (A β 1-40 normal(d=14 control)) and hydrogen/deuterium exchange data obtained previously for wildtype A β (1–40) fibrils (A β 1-40 normal (8/24/01, d=12)). All aggregates were collected and washed as described in Materials and Methods. Deuteriation of A β aggregates grown in the presence of calmidazolium after 4 days is about as great as that of A β protofibrils collected after 2 days ($\approx$ 20 deuteriums) (I. Kheterpal and H. Lashuel, unpublished). After 14 days, 10 μ M calmidazolium A β aggregates show the same amount of deuteriation as A β fibrils ($\approx$ 10 deuteriums) while 100 μ M calmidazolium A β aggregates remain open to hydrogen/deuterium exchange ($\approx$ 20 deuteriums). (figure courtesy of I. Kheterpal, unpublished).

Vita

Matthew Sega, like most children, was born at a very young age. He was raised in Oak Ridge, TN where he attended St. Mary's School until the eighth grade. He attended Jefferson Junior High School in the ninth grade, and graduated from Oak Ridge High School in 1993. He moved on to the University of Tennessee, Knoxville, where he earned a B.S. in chemistry in 1998. He is anticipating a M.S. degree in Life Science in 2003, after which he plans to enroll in the doctor of pharmacy program at Auburn University. His interests are quite varied, and are too numerous to list here.

