

Inhibition of amyloid fibril formation of β -amyloid peptides via the amphiphilic surfactants

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Abstract

β -amyloid peptide ($A\beta$) is the major proteinaceous constituent of senile plaques in Alzheimer's disease and is believed to be responsible for the neurodegeneration process associated with the disease. While the actual size of the aggregated species responsible for $A\beta$ neurotoxicity and fibrillogenesis mechanism(s) remain unknown, retardation of $A\beta$ aggregation still holds assurance as an effective strategy in reducing $A\beta$ -elicited toxicity. The research presented here is aimed at examining the inhibitory effect of two amphiphilic surfactants, di-C6-PC and di-C7-PC, on the *in vitro* fibrillogenesis process of $A\beta(1-40)$ peptides at physiological pH (pH 7.2). Using ThT-induced fluorescence, turbidity, Congo red binding, and circular dichroism spectroscopy studies, our research demonstrated that the inhibition of $A\beta(1-40)$ fibril formation was di-C6-PC and di-C7-PC concentration-dependent. The best inhibitory action on fibril formation was observed when $A\beta$ was incubated with di-C7-PC at 100 μ M over time. We believe that the outcome from this work will aid in the development and/or design of potential inhibitory agents against amyloid formation associated with Alzheimer's and other amyloid diseases.

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1. Introduction

Alzheimer's disease (AD) is the leading cause of dementia in the aging population, affecting approximately 15 million people worldwide annually. The incidence rises from 0.5% per year at age 65 to 8% per year at age 85 years or above [1,2]. Although AD can be diagnosed via the evaluation of symptoms, it is not confirmed until a post-mortem examination of the brain tissue has been conducted. While Alzheimer's disease and other neurodegenerative diseases have been the center of intense research efforts, effective treatments are still unavailable. The defining pathological feature of Alzheimer's disease observed in an autopsy is the formation and the progressive deposition of insoluble amyloid fibrils within the cerebral cortex [3].

The β -amyloid peptide ($A\beta$), consisting of 39–43 amino acid chain, is a proteolytic product of a much larger amyloid precursor protein (APP). It is the major protein constituent of the senile plaques [4]. $A\beta$ with the molecular weight of 4.3 kDa self-assembles into a larger unbranched fibrillar structure of several microns in length. Dramatic neuron loss and/or neuron deterioration are normally observed in the vicinity of the senile plaques.

It is widely, but not universally, believed that the extracellular aggregation of $A\beta$ into fibrils in AD is a critical event in the neurodegeneration observed during the disease process and this $A\beta$ peptide, when aggregated, may serve as a significant contributing factor in the onset or progression of AD. Mounting evidence originating from *in vitro* toxicity studies with synthetic $A\beta$ peptides has shown that $A\beta$, in an aggregated state (fibril, protofibril, low molecular weight oligomer, or diffusible, non-fibrillar ligand), is toxic to neurons in cultures [5–9].

Extensive efforts have been directed toward seeking or developing anti-amyloidogenic or anti-aggregating agents as

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potential strategies to combat AD [10]. A variety of molecules/compounds have been reported to retard the formation of fibrillar or aggregated species of β -amyloid both in vitro and in vivo. Evidence showed that various molecules/drugs, such as nicotine, nordihydroguaiaretic acid (NDGA), rosemarinic acid (RA), rifampicin (RIF), tannic acid, curcumin, aspirin, melatonin, and alpha-crystallin Hsp20, slowed or inhibited fibrillogenesis of β -amyloid peptides [10–20]. In addition, several synthesized compounds as well as surfactants [21–23] were found to delay or prevent fibril formation.

In the present work, via turbidity measurement, fluorescence spectroscopy with ThT, circular dichroism spectroscopy, and Congo red binding assay, we investigated the anti-amyloidogenic effects of the two amphiphilic surfactants, 1,2-dihexanoyl-*sn*-glycero-3-phosphocholine (di-C6-PC) and 1,2-diheptanoyl-*sn*-glycero-3-phosphocholine (di-C7-PC), on the in vitro fibrillogenesis of β -amyloid peptides at physiological pH (pH 7.2). These two surfactants were shown to possess anti-aggregating and anti-amyloidogenic activities which were found to be dependent upon incubation time and surfactant concentration. In addition, our results demonstrated that surfactant di-C7-PC at 100 μ M strongly interfered with the fibrillization of $A\beta(1-40)$ peptides on Day 6 of incubation. It is our belief that the outcome from this report will shed light on the development and/or design of effective inhibitory agents against amyloid formation implicated in Alzheimer's and other amyloid diseases.

2. Materials and methods

2.1. Materials

Synthetic full-length $A\beta(1-40)$ peptides were purchased from Biosource International (Camarillo, CA, USA). All other chemicals, unless otherwise specified, were obtained from Sigma (St. Louis, MO, USA).

2.2. $A\beta$ peptide sample preparation

Stock solutions of 10 mg/mL were prepared by dissolving the lyophilized $A\beta(1-40)$ peptides in 0.1% (v/v) trifluoroacetic acid (TFA) in deionized water. After incubating for 1.5 h at 25 °C, the peptide stock solutions were diluted in phosphate buffered saline (PBS, 13.67 mM NaCl, 2.68 mM KCl, 10 mM Na_2HPO_4 , 1.76 mM KH_2PO_4 , pH 7.2) with 0.01% (w/v) sodium azide to the concentration (50 μ M) used in experiments. These final $A\beta$ sample solutions were rotated at 60 rpm at 25 °C during the course of aggregation process.

2.3. Spectroscopic aggregation analysis

Absorbance at 405 nm was monitored at different times. 300 μ L of $A\beta(1-40)$ samples taken at different times were

added to a well of 96-well plate. The absorbance was measured using a μ Quant Microplate reader (Bio-Tek Instruments, VT, USA). In order to well suspend the fibrillar species, the $A\beta$ sample solutions were mixed by vortexing prior to each absorbance measurement.

2.4. Thioflavin T fluorescence (ThT) assay

40 μ L of $A\beta(1-40)$ samples taken at different times was mixed with 960 μ L of 10 μ M thioflavine T (ThT) in PBS. ThT fluorescence intensity measurements were performed by exciting samples at 437 nm and measuring emission intensities at 485 nm using a F-2500 Fluorescence Spectrophotometer (Hitachi, Japan).

2.5. Congo red binding assay

Congo red binding was measured as described [24], with minor modification for use with a microplate reader. 25 μ L of samples was mixed with 225 μ L of 20 μ M Congo red in PBS, and incubated at room temperature for at least 30 min prior to absorbance measurement. Absorbance measurement was conducted using a μ Quant Microplate reader (Bio-Tek Instruments, VT, USA) at 25 °C. The concentration of the $A\beta$ fibrillar species in peptide solutions was determined using the following equation:

$$[\text{fibrillar } A\beta] = A^{540}/4780 - A^{405}/6830 - A_{CR}^{405}/8620$$

where [fibrillar $A\beta$] is the concentration of $A\beta$ fibril, A_{CR}^{405} is the absorbance of Congo red alone at the wavelength of 405 nm, and A^{540} and A^{405} are the absorbances of peptide sample and Congo red at the wavelengths of 540 nm and 405 nm, respectively [24]. The relative fibril formation was determined as the ratio of sample fibril concentration to pure $A\beta$ fibril concentration.

2.6. Circular dichroism spectroscopy

Circular dichroism (CD) spectra of the $A\beta(1-40)$ peptides were recorded on a JASCO J-715 (150-S Type) spectrometer (Sunway Scientific Corporation) at 25 °C using a bandwidth of 1.0 nm, a step interval of 0.1 nm, a scanning speed of 50 nm/min, a resolution of 0.1 nm, and an averaging time of 2 s. A 0.1 cm quartz cell was used for far-UV (190–260 nm) measurements. Three scans each of duplicate samples were measured and averaged. Control buffer scans were run in duplicate, averaged, and then subtracted from the sample spectra. In order to well suspend the fibrillar species, the $A\beta$ sample solutions were mixed by vortexing or pipetting prior to each CD measurement. The results of CD measurement were plotted as ellipticity (millidegree) vs. wavelength (nm). The secondary structure contents (α -helix, β -sheet, turn, and random coil) of the $A\beta$ peptide samples were estimated from the CD spectra using the CDPro software [25,26].

2.7. Statistical analysis

All data represent means \pm standard deviation (S.D.) for n independent determinations. Significance of results was determined with a Student's t -test on n independent measurements, where n is specified in the figure legend. Unless otherwise indicated, significance was taken as $P < 0.05$.

3. Results

3.1. Spectroscopic aggregation/turbidity studies

The effects of two surfactants, 1,2-dihexanoyl-*sn*-glycero-3-phosphocholine (di-C6-PC) and 1,2-diheptanoyl-*sn*-glycero-3-phosphocholine (di-C7-PC), on A β (1–40) aggregation were examined via turbidity measurements. The absorbance/or turbidity of the A β sample solution measured at 405 nm was used as an indication of the degree of aggregation. As depicted in Fig. 1, the absorbance of A β (1–40) peptide alone increased with the progression of aggregation. Except for di-C7-PC at 100 μ M, no inhibitory action from the surfactants was seen at all concentration levels on Day 3 of A β aggregation. However, on either Day 6 or 9 of incubation, the presence of both surfactants led to a

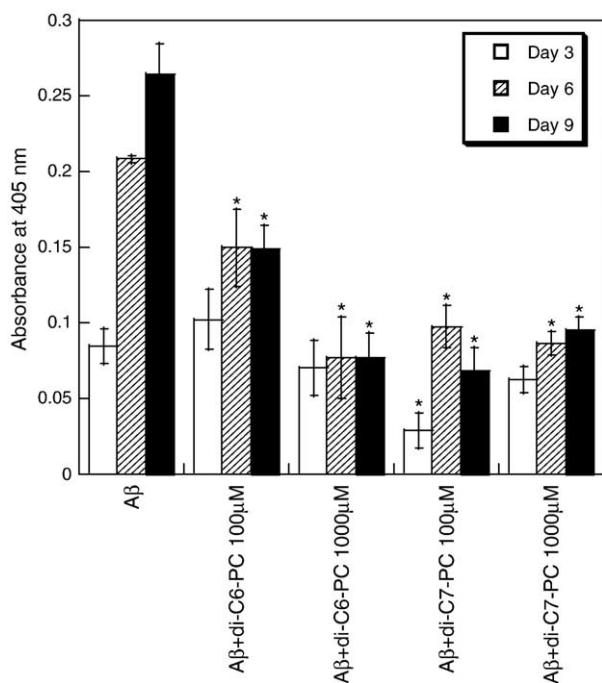


Fig. 1. Effect of amphiphilic surfactants on the aggregation kinetics of A β (1–40) peptides. The extent of aggregation was measured via turbidity as a function of elapsed time of aggregation. A β (1–40) peptides were first dissolved in 0.1% (v/v) TFA and then diluted with phosphate buffered saline (PBS). Aged A β peptide samples taken on Days 3, 6, or 9 (A β fibrils) were then tested for aggregation formation. Data represent the mean turbidity measurement of at least 6 independent experiments ($n \geq 6$). Error bars represent the standard deviation (S.D.) of the turbidity measurement.

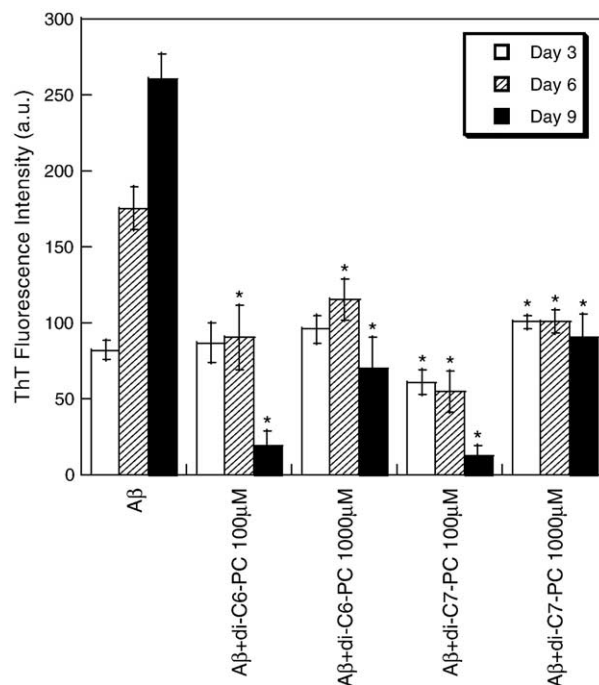


Fig. 2. Effect of amphiphilic surfactants on the kinetics of the amyloid fibril formation of A β (1–40) peptides. The extent of fibril formation was measured via ThT fluorescence as a function of elapsed time of fibrillogenesis. A β (1–40) peptides were first dissolved in 0.1% TFA and then diluted with PBS. Aged A β peptide samples taken on Days 3, 6, or 9 (A β fibrils) were then tested for amyloid fibril formation. Data represent the mean ThT fluorescence measurement of at least 6 independent experiments ($n \geq 6$). Error bars represent the standard deviation (S.D.) of the fluorescence measurement.

significant decrease in the extent of A β aggregation as compared to A β (1–40) peptide alone ($P < 0.05$) (Fig. 1).

3.2. Fluorometric studies

To explore if di-C6-PC and di-C7-PC exerted an inhibitory action on the formation of A β (1–40) fibrils, we monitored the changes of ThT fluorescence intensity of A β (1–40) with and without surfactants as a function of incubation time. ThT is believed to rapidly interact with amyloid fibrils in a specific manner, and the sequence of proteins has negligible effect on the binding between ThT and protein molecules. An increase in ThT fluorescence intensity has been reported as an important indicator of the presence of amyloid fibril [27]. As can be seen in Fig. 2, compared with 6.2 units on Day 1, the ThT fluorescence intensities of samples with A β (1–40) alone on Days 3, 6, and 9 were observed to be around 82, 175, and 260 units, respectively, indicating an enhancement in the amount of fibril formation with increasing incubation time.

The kinetics of disaggregation of A β (1–40) fibrils by these two compounds are also shown in Fig. 2. Our results revealed that the reduction of A β (1–40) fibrillogenesis is dependent on the concentration of surfactants. The fluorescence intensity observed was not significantly different

between the A β samples with and without surfactants on Day 3 of incubation ($P>0.5$). However, on Day 6 of incubation, A β (1–40) peptides with di-C6-PC and di-C7-PC at both concentrations (100 μ M and 1000 μ M) fluoresced with much smaller intensities relative to that of A β (1–40) peptide alone ($P<0.05$). For example, on Day 9, the percentage reductions of ThT fluorescence by di-C6-PC at 100 μ M and 1000 μ M and di-C7-PC at 100 μ M and 1000 μ M were approximately 93%, 73%, 95%, and 65%, respectively. The presence of ThT during the course of fibril formation was found not to interfere with the formation of A β fibrils. Regardless of the surfactants used, no detectable absorption was observed at the wavelength of ThT excitation.

3.3. Congo red binding studies

The effect of di-C6-PC and di-C7-PC on A β peptide fibril formation, determined by Congo red binding assay, was monitored as a function of incubation time. Similar to the turbidity and ThT fluorescence results, the attenuation of A β (1–40) fibril formation due to the surfactants appears to be a function of incubation time and surfactant concentration. In Fig. 3, the concentrations of pure A β

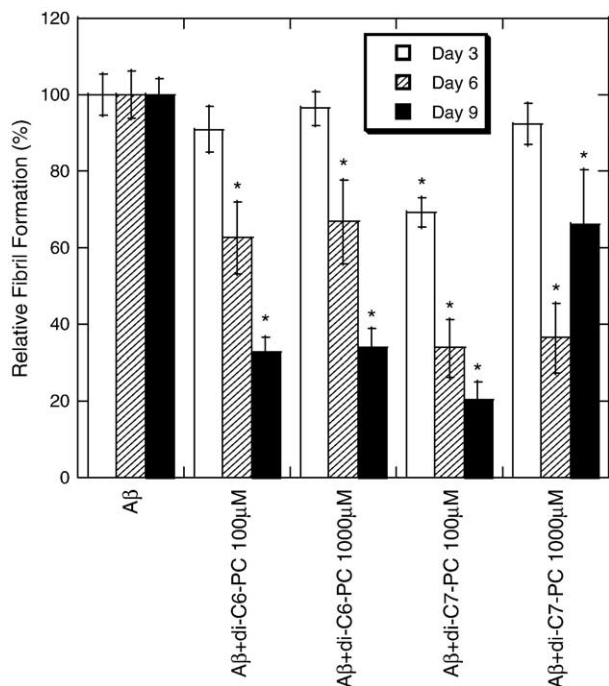


Fig. 3. Effect of amphiphilic surfactants on the kinetics of the amyloid fibril formation of A β (1–40) peptides. Relative fibril formation is estimated via Congo red binding as a function of elapsed time of fibrillogenesis. Relative fibril formation is reported as the ratio of sample fibril concentration to pure A β fibril concentration. A β (1–40) peptides were first dissolved in 0.1% (v/v) TFA and then diluted with PBS. Aged A β peptide samples taken on Days 3, 6, or 9 (A β fibrils) were then tested for amyloid fibril formation. Data represent the mean Congo red binding measurement of at least 6 independent experiments ($n \geq 6$). Error bars represent the standard deviation (S.D.) of the fluorescence measurement.

fibrils on Days 3, 6, and 9 were taken as 100% of relative fibril formation. As we can see in the figure, except for di-C7-PC at 100 μ M, no prevention of fibril was observed in all other conditions on Day 3. However, both surfactants at either a concentration of 100 μ M or 1000 μ M, when added to A β prior to fibril formation, had profound anti-amyloidogenic activity on A β (1–40) fibril formation on Day 6 of incubation (37.4% and 33.2% inhibition for di-C6-PC at 100 μ M and 1000 μ M, respectively; 66.2% and 63.6% inhibition for di-C7-PC at 100 μ M and 1000 μ M, respectively). Furthermore, di-C7-PC at the concentration of 100 μ M presented the optimal inhibitory potency relative to the other three conditions (see Fig. 3). Surfactants by themselves did not alter the Congo red binding absorbance.

3.4. Circular dichroism absorption spectroscopy studies

To elucidate the structural details of the interactions between A β (1–40) and the two surfactants, CD absorption spectroscopy was utilized as a means to investigate the structural transition of the A β sample, in particular the secondary structure. As can be seen in Fig. 4A, the CD spectra of aged or aggregated A β (1–40) alone exhibited a characteristic pattern of β -sheet conformation with absorption minimum around 218 nm. However, the treatment of di-C6-PC or di-C7-PC to A β (1–40) peptide permitted different structural transitions compared with those observed when A β was incubated alone, resulting in a striking alteration in the relative proportion of solution structure. In addition, the changes in CD signal were observed to follow a concentration-dependent fashion for A β peptide solutions in the presence of surfactants.

Quantitatively, differences in the secondary structure, in particular the β -sheet conformation, induced by a range of concentrations of di-C6-PC or di-C7-PC surfactant can be demonstrated by the variation of CD ellipticity measured at 218 nm (see Fig. 4B). For example, on Day 9 of incubation, our data suggested that either surfactant at both concentrations of 100 and 1000 μ M caused a decrease in the CD ellipticity measured at 218 nm, indicating their preventive effect on A β (1–40) from adopting a β -sheet conformation. In addition, in order to retrieve better quantitative information regarding the structural transition, the CD spectra that we obtained were further de-convoluted using the CDPro software. As expected, the β -sheet content of pure A β (1–40) increased over time from 58% on Day 3 to ~60% after Day 6 at 25 $^{\circ}$ C (see Table 1). However, the fraction of β -sheet in the A β peptide solution was significantly diminished while random conformation was markedly elevated by the addition of di-C6-PC or di-C7-PC on Day 6 at 25 $^{\circ}$ C. In agreement with our previous results, di-C7-PC at 100 μ M exerted the best inhibitory potency against the formation of β -sheet structures in comparison to all other conditions. The amount of β -sheet within the A β peptide

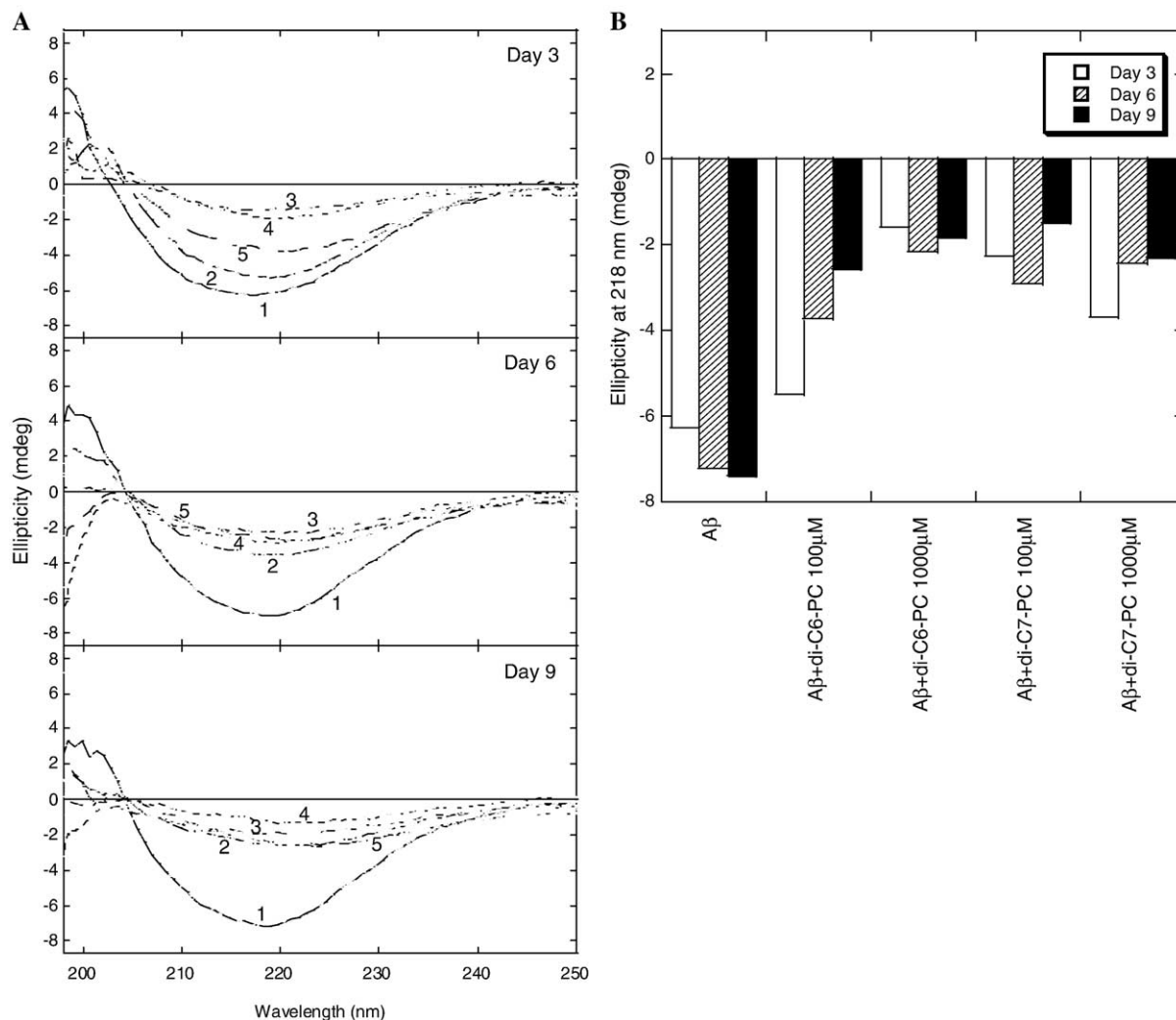


Fig. 4. The influence of amphiphilic surfactants on the secondary structure transitions in the A β (1–40) peptide solution studied by circular dichroism. (A) Representative CD spectra of 50 μ M A β (1–40) peptide alone (line 1) or incubated with 100 μ M di-C6-PC (line 2), 1000 μ M di-C6-PC (line 3), 100 μ M di-C7-PC (line 4), and 1000 μ M di-C7-PC (line 5). (B) The CD ellipticity at 218 nm of 50 μ M A β (1–40) peptide solution alone or incubated with di-C6-PC or di-C7-PC as estimated from CD spectra.

plus di-C7-PC decreased with time and reached 8.8% on Day 6 of incubation. This percentage continued to drop to 3.8% on Day 9 of incubation (see Table 1).

4. Discussion

More than sixteen different proteins including A β have the potential to fold abnormally and form amyloid fibrils, thus leading to their accompanying pathologies [28–32]. Numerous studies under different approaches (in vitro, genetic, and transgenic animal) have indicated that monomeric A β has to aggregate to its polymeric or fibrillar form before it is toxic to neurons in cultures [33]. Such behavior suggests that the A β aggregation process in vivo is responsible for initiating a cascade of physiological events that are pivotal for the progression of AD. As a result, the inhibition of aggregation or fibrillogenesis of

A β is considered a promising therapeutic approach to battle AD.

Based on the nucleation-dependent polymerization model, several molecules/compounds, for instance, aspirin, basement membrane components, curcumin, melatonin, nicotine, nordihydroguaiaretic acid, rifampicin, rosemarinic acid, and tannic acid, have been proposed as potential in vitro or in vivo anti-amyloidogenic inhibitors that retard or inhibit the formation of A β fibrillar species [10–19,34,35]. In addition, a few di- and tri-substituted aromatic compounds [17] and amphipathic molecule, including hexadecyl-*N*-methylpiperidinium bromide [36], have been reported to exhibit anti-amyloidogenic actions on both toxic oligomeric and fibrillar A β species.

Methods using shorter peptide fragments have been employed for the prevention of A β fibrillogenesis [37–39]. These shorter peptide fragments, consisting of pentapeptides, have been shown to disrupt A β fibril formation. Due

Table 1

Estimates of the secondary structural components of A β (1–40) peptide in the presence of amphiphilic surfactants as obtained from CD spectral analyses

	α -Helix (%)	β -Sheet (%)	Turn (%)	Random (%)
<i>Day 3</i>				
A β	11.4	58.0	15.3	15.3
A β +di-C6-PC 100 μ M	20.9	17.7	29.6	31.8
A β +di-C6-PC 1000 μ M	6.9	43.6	19.5	30.0
A β +di-C7-PC 100 μ M	2.6	49.0	22.9	25.5
A β +di-C7-PC 1000 μ M	13.6	46.4	15.4	24.6
<i>Day 6</i>				
A β	10.2	60.0	15.5	14.3
A β +di-C6-PC 100 μ M	0.0	11.0	28.3	60.7
A β +di-C6-PC 1000 μ M	0.0	10.9	28.4	60.7
A β +di-C7-PC 100 μ M	3.8	8.8	2.0	85.4
A β +di-C7-PC 1000 μ M	31.8	17.7	20.9	29.6
<i>Day 9</i>				
A β	9.4	62.2	15.3	13.1
A β +di-C6-PC 100 μ M	0.0	10.7	28.4	60.9
A β +di-C6-PC 1000 μ M	0.0	10.3	28.4	61.3
A β +di-C7-PC 100 μ M	4.0	3.8	2.1	90.1
A β +di-C7-PC 1000 μ M	56.3	19.1	4.3	20.3

to their residues mimicking the segment(s) critical for A β fibril formation, these pentapeptides are able to interact with A β molecule and thus change the structure that A β adopts.

A few reports have been focused on the interaction(s) between A β species or its derivatives and surfactant molecules. Three micelle systems, the negatively charged utilized sodium dodecyl sulfate, the neutral zwitterionic dodecylphosphocholine, and the positively charged dodecyltrimethylammonium chloride, in heterogeneous amphiphilic environment have been utilized to interact with A β peptide fragment (A β (1–28)) [23]. Their results showed that the promotion and the stabilization of α -helix secondary structure were highly correlated with the availability of the surfactant's charged surface, indicating the significant role of electrostatic forces in the interaction between A β and the lipid/water interface of detergents. Lomakin et al. employed the quasi-elastic light scattering technique to quantitatively monitor the kinetics of A β aggregation process, which is composed of nucleation and growth steps, and carefully characterize the size of A β aggregates [21]. Upon the addition of an amphiphilic surfactant *n*-dodecylhexaoxyethylene glycol monoether in a concentration-dependent fashion, a decrease in the rate of aggregation was also observed in their study [21].

Via atomic force microscopy and dynamic light scattering spectroscopy, the addition of di-C6-PC to 125 μ M A β (1–40) under the acidic condition in 0.1 M HCl leads to a concentration-dependent reduction in the length, but not the diameter, of A β fibrils [21–23]. However, no evidence has indicated the anti-amyloidogenic or anti-aggregating activity of the amphiphilic surfactants in physiological condition.

As seen in Figs. 1–3, our experiments showed that di-C6-PC and di-C7-PC inhibited A β fibril formation or A β aggregation at physiological pH, as indicated by the reductions in turbidity, ThT fluorescence intensity, and Congo red binding. The inhibitory actions of di-C6-PC and di-C7-PC were both time and concentration dependent. Our results demonstrated that the inhibitory effects of both surfactants increased tremendously with longer incubation time. Moreover, judging from all the results of our spectroscopic analyses, di-C7-PC surfactant at 100 μ M more potently suppressed fibril formation on Day 6 of A β polymerization.

A β fibril formation has been correlated with secondary structure transitioning from disordered random structure to ordered β -sheet-rich conformation [21,40]. This β -sheet structure has been found to be associated with insolubility and protease resistance [41,42]. Here, assessed by CD absorption spectroscopy, we show in Fig. 4 and Table 1 that these two amphiphilic surfactants could prevent A β from adopting a β -sheet-rich conformation.

In conclusion, this work examines the inhibitory action of amphiphilic surfactants, di-C6-PC and di-C7-PC, on in vitro A β fibrillogenesis via ThT fluorescence enhancement, turbidity measurement, Congo red binding experiments, and circular dichroism spectroscopy. Our findings have demonstrated that di-C6-PC and di-C7-PC hampered β -sheet formation and aggregation, characteristics of A β (1–40) amyloid fibril formation, in a concentration-dependent fashion. Further verification of the exact mechanism of A β –surfactant interaction is still under investigation. However, we believe that the outcome from this work will enable us not only to comprehend the mechanism(s) of amyloid protein self-assembly but also to aid in designing potential targets for molecular therapeutics in the prevention or retardation of amyloid formation implicated in Alzheimer's and other amyloid diseases.

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