*МОЛЕКУЛЯРНА БІОФІЗИКА*

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# PHOSPHOLIPIDS AS INHIBITORS OF AMYLOID FIBRIL FORMATION

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Amyloid fibrils are the protein aggregates, whose formation is involved in the pathogenesis of Alzheimer's disease, systemic amyloidosis, etc. Since there is no effective ways to treat these diseases, developing the new anti-amyloid drugs is of great importance. In this study a series of phospholipids have been tested for their ability to inhibit lysozyme and insulin amyloid fibril formation at acidic or neutral pH and elevated temperature. The lag time, elongation rate and fibrillization extent were estimated using Thioflavin T fluorescence assay. It is found that the oxidized and charged phospholipids, included into the liposomes, were the most effective inhibitors of the protein fibrillization. By comparing the magnitude and direction of the lipid effect in different lipid-protein systems it was concluded that the reduction of the amyloid fibril formation is governed by hydrophobic and specific liposome-protein interactions. It is hypothesized that the presence of the surface formed by the lipid polar heads is critical for reducing the protein fibrillization extent.
**KEY WORDS:** amyloid fibrils of lysozyme/insulin, inhibition, nucleation, oxidized phospholipids, Thioflavin T.

## ФОСФОЛІПІДИ ЯК ІНГІБІТОРИ ПРОЦЕСУ УТВОРЕННЯ АМІЛОЇДНИХ ФІБРИЛ

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Амілоїдні фібрили – це білкові агрегати, формування яких пов'язане з патогенезом хвороби Альцгеймера, системного амілоїдозу, тощо. Через відсутність ефективних методів лікування цих захворювань, розробка нових анти-амілоїдних ліків є дуже важливою. У цій роботі серія фосфоліпідів була протестована щодо їх здатності інгібувати формування амілоїдних фібрил лізоциму та інсуліну за умов кислого чи нейтрального pH та підвищеної температури. Лаг-період, швидкість росту та рівень фібрилізації оцінювали, вимірюючи флуоресценцію тіофлавіну Т. Встановлено, що окислені та заряджені фосфоліпіди, що входили до складу ліпосом, були найбільш ефективними інгібіторами фібрилізації білка. При порівнянні величини та напряму ефекту ліпідів у різних ліпід-білкових системах зроблено висновок про те, що зниження ступеня формування фібрил регулюється гідрофобними та специфічними взаємодіями між ліпосомами та білками. Висловлено припущення, що наявність поверхні, сформованої полярними головками ліпідів, є критичною для зниження рівня фібрилізації білка.
**КЛЮЧОВІ СЛОВА:** амілоїдні фібрили лізоциму/інсуліну, інгібування, нуклеація, окислені фосфоліпіди, тіофлавін Т.

## ФОСФОЛИПИДЫ КАК ИНГИБИТОРЫ ПРОЦЕССА ОБРАЗОВАНИЯ АМИЛОИДНЫХ ФИБРИЛЛ

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Амилоидные фибриллы – это белковые агрегаты, формирование которых связано с патогенезом заболевания Альцгеймера, системного амилоидоза, и т.д. Из-за отсутствия эффективных методов лечения этих заболеваний, разработка новых анти-амилоидных лекарств является очень важной. В этой работе серия фосфолипидов была протестирована относительно их способности ингибировать формирование амилоидных фибрилл лизоцима и инсулина в условиях кислого или нейтрального pH и повышенной температуры. Лаг-период, скорость роста и уровень фибриллизации оценивали, измеряя флуоресценцию тиофлавина Т. Обнаружено, что окисленные и заряженные фосфолипиды, входящие в состав липосом, были наиболее эффективными

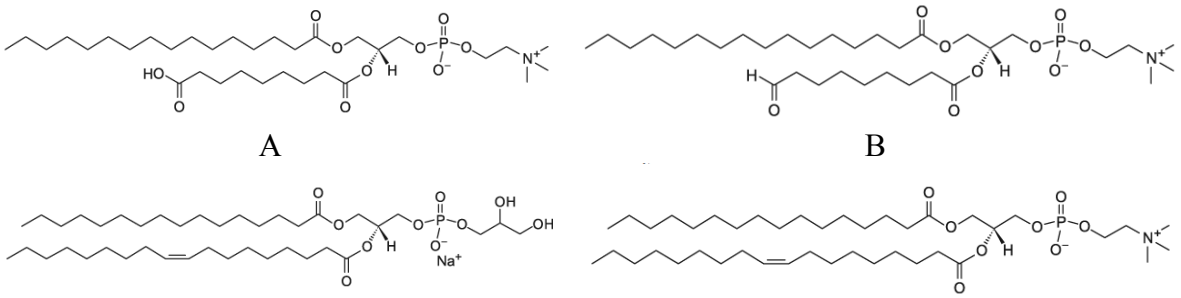
ингибиторами фибриллизации белка. При сравнении величины и направления эффекта липидов в разных липид-белковых системах сделан вывод о том, что снижение степени формирования фибрилл регулируется гидрофобными и специфическими взаимодействиями между липосомами и белками. Высказано предположение, что наличие поверхности, сформированной полярными головками липидов, является критическим для снижения уровня фибриллизации белка.
**КЛЮЧЕВЫЕ СЛОВА:** амилоидные фибриллы лизоцима/инсулина, ингибирование, нуклеация, окисленные фосфолипиды, тиофлавин Т.

Amyloid fibrils are protein aggregates with a core cross-β structure implicated into the etiology of Alzheimer's, Parkinson's, Huntington's diseases, etc. [1, 2]. The treatment of these human disorders has become an urgent biomedical problem. In recent years, two main strategies for inhibition of the protein fibrillization have been proposed, viz.: i) developing inhibitors of β- and γ-secretases that prevent Aβ-peptide cleavage from the amyloid precursor protein; ii) inhibition or reversing protein aggregation itself by the ligands strongly associating with the core regions of the amyloidogenic proteins [3]. The latter is more reasonable, because protein aggregation into amyloid fibrils is not associated with certain biological functions. Despite the presence of some therapy approaches, the majority of drugs possess high toxicity and can only slow the disease progression [4]. Therefore, much attention is paid to development of biocompatible and effective inhibitors of the fibrillogenesis. In particular, natural polyphenols, possessing antioxidant properties, appeared to be superior inhibitors of Aβ42 and α-synuclein fibrillization [5, 6], while deoxydoxorubicin and tetracyclines induced disruption of transthyretin amyloid fibrils with the production of nontoxic species [7]. Notably, small molecules seem to inhibit protein fibrillization via stacking between β-strands and preventing β-sheet elongation [8] or by creating protein-resistive coatings on the fibril surface [9]. Of these, very interesting objects to be tested for their applicability as biocompatible anti-amyloid drugs are surfactants and phospholipids. Indeed, ionic surfactants, sodium dodecyl sulfate and cetyltrimethylammonium bromide, exerted inhibiting effects on lysozyme fibrillization due to the strong protein-surfactant electrostatic complex formation, while hydrophobic interactions raised the lag time of amyloid fibril formation [10]. Furthermore, amphiphilic 1,2-diheptanoyl-sn-glycero-3-phosphocholine reduced the extent of insulin fibril formation [11]. However, lipids have also been frequently reported to promote amyloid nucleation, because the protein molecules accumulated on the bilayer could adopt specific aggregation-competent conformation and orientation [12, 13]. For instance, the oxidized phospholipids involved in the oxidative stress, have been reported to accelerate Aβ42 and gelsolin fibrillization [14]. In order to solve the above discrepancies, the present study was aimed at testing the ability of the phospholipids to reduce insulin and lysozyme amyloid fibril formation. Specifically, our goal was twofold: i) to analyze the kinetic parameters for the protein aggregation in the presence and absence of lipids using fluorescent amyloid marker Thioflavin T; ii) to evaluate the inhibiting effects of lipid dispersions and vesicles on the extent of amyloid fibril formation.

## MATERIALS AND METHODS

Insulin from bovine pancreas and hen egg white lysozyme were purchased from Sigma. Thioflavin T (ThT) concentration was determined using the extinction coefficient $\varepsilon_{412}$=23800 $M^{-1}cm^{-1}$. 1-palmitoyl-2-azelaoyl-sn-glycero-3-phosphocholine (PazePC) (Fig. 1A), 1-palmitoyl-2-(9′-oxononanoyl)-sn-glycero-3-phosphocholine (PoxnoPC) (Fig. 1B), 1-hexadecanoyl-2-(9Z-octadecenoyl)-sn-glycero-3-phospho-(1′-rac-glycerol) (PG) and 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine (PC) lipids were from Avanti Polar Lipids. Lipid chloroform solutions were dried under a stream of nitrogen, followed by their hydration in buffer. Next, PazePC and PoxnoPC samples were placed in a bath sonicator for 1 h to obtain optically clear lipid dispersions. Liposomes of the following composition: PC:PazePC

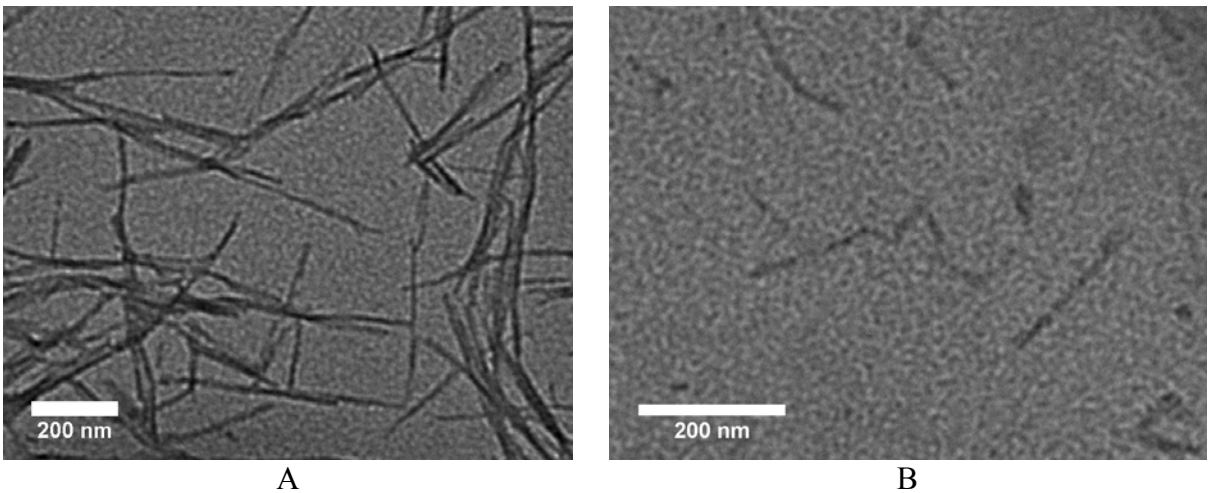
(4:1, mol:mol); PC:PoxnoPC (4:1, mol:mol); PC:PG (4:1, mol:mol); PC, below referred to as $PazePC_{20}$, $PoxnoPC_{20}$, $PG_{20}$ and PC, respectively, were prepared by the extrusion technique.



A B C D

Fig. 1. Structure of the oxidized – PazePC (A), PoxnoPC (B), and nonoxidized phospholipids – PG (C), PC (D)

Amyloid fibril formation in control samples was initiated at varying pH (2 or 7.4), lipid concentration (0, 1.6 and 16.4 μM) under constant stirring at 60 °C. Amyloid nature of the obtained aggregates was confirmed by TEM (Fig. 2). As seen in Fig. 2, protein aggregates are rod-like, unbranched and about 0.2–1.5 μm in length and 10–30 nm in width that is typical for amyloid fibrils [15]. Quantitative characteristics of the fibrillization process were obtained by approximation of the time ($t$) dependence of the ThT fluorescence intensity at 480 nm ($F$) with the sigmoidal curve, yielding the lag time, apparent rate constant for the fibril growth ($k$) and maximal fluorescence of the dye after the saturation has been reached ($F_{max}$) [16]. Steady-state fluorescence measurements were performed using Varian Cary Eclipse (Varian Instruments, Walnut Creek, CA) spectrofluorimeter. ThT fluorescence was excited at 430 nm.




A B

Fig. 2. TEM images of lysozyme fibrils formed at pH 2, 60 °C (A) and insulin fibrils obtained at pH 7.4, 60 °C (B)

## RESULTS AND DISCUSSION

As seen in Fig. 3A-C, ThT fluorescence in the presence of $PazePC_{20}$, $PoxnoPC_{20}$, $PG_{20}$ and PC vesicles is reduced as compared to that in control samples. Specifically, the $F_{max}$ values were 19 (for insulin fibrils grown in the presence of $PazePC_{20}$, pH 2, 60 °C) – 48% (for insulin fibrils grown in the presence of $PG_{20}$, pH 2, 60 °C) lower for the fibrils grown in the presence of lipids (Table 1). The observed effects can be attributed to decrease in the extent of lysozyme and insulin fibrillization. This interpretation is corroborated by the fact that fibril

morphology was similar for the lipid-modulated and control samples, being suggestive of similar binding sites for ThT [16].

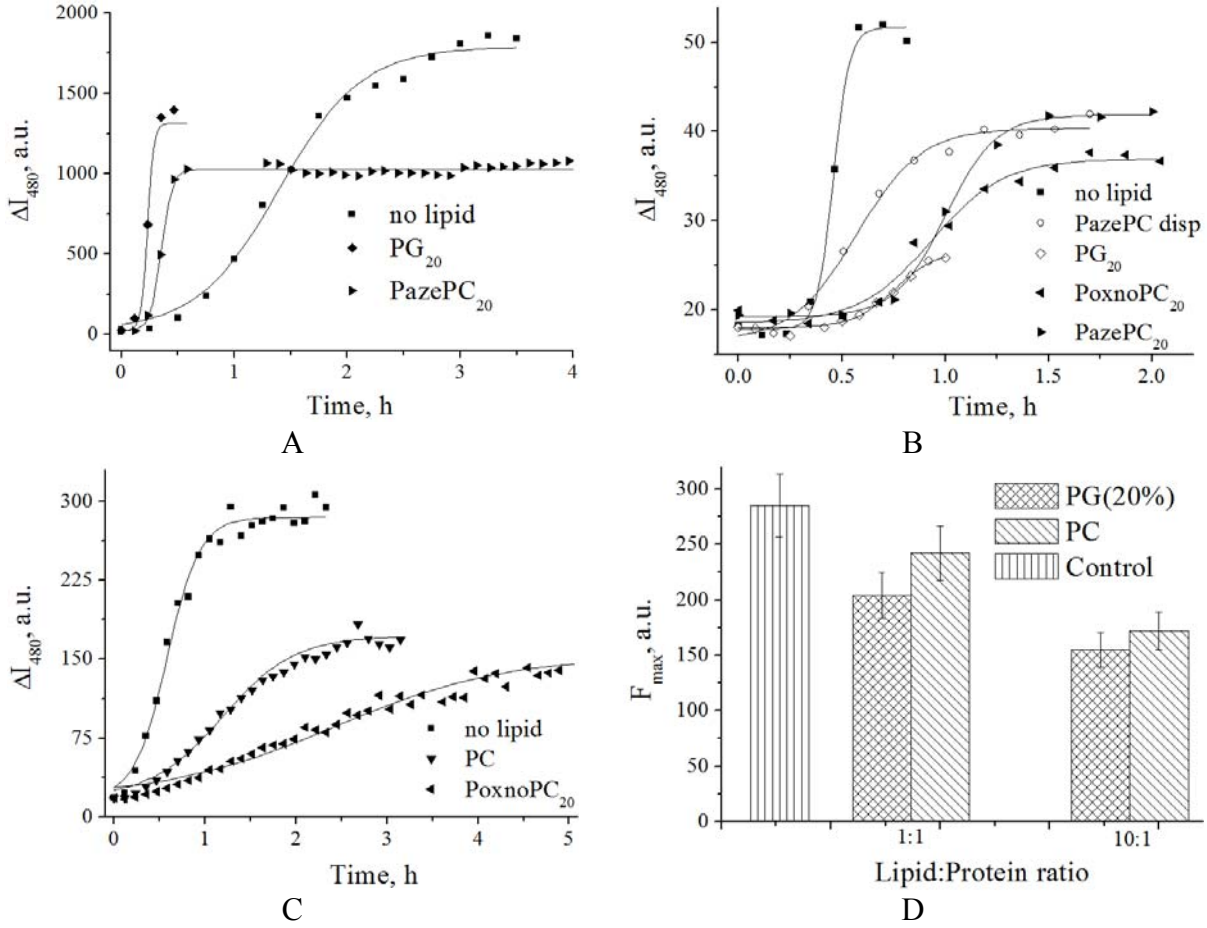



Fig. 3. Kinetics of lysozyme (A) and insulin (B, C) fibrillization at pH 2, 60 °C (A, B), and pH 7.4, 60 °C (C), and dependence of insulin fibrillization rate on lipid-to-protein molar ratio. Lysozyme (insulin), lipid and ThT concentrations were 16.4 (1.6), 16.4 and 14 μM, respectively

Analysis of the results obtained have shown that: i) lipid vesicles inhibited the amyloid fibril formation more effectively than lipid dispersions; ii) $PazePC_{20}$ and $PoxnoPC_{20}$ were the most remarkable species, which led to a significant attenuation of lysozyme and insulin fibril growth, respectively; iii) $PG_{20}$ and PC reduced insulin fibrillization at acidic and neutral pH, respectively; iv) inhibition of lysozyme fibril formation was accompanied by the reduction of the lag time and increase of the elongation rate, while the opposite effect was observed for insulin. Interestingly, more substantial inhibition of insulin fibrillization was revealed in the presence of liposomes as compared to that caused by the amphiphylic surfactants above the CMC [11]. Despite the fact that hydrophobic interactions are the key driving force for insulin fibrillization, the more pronounced effect of the lipid vesicles on the protein aggregation is due to the hydrophobic effects and lipid structural specificity. For example, higher amount of the exposed hydrophobic patches of insulin at pH 2 led to the faster fibril elongation than at pH 7.4, while lipid-protein hydrophobic interactions attenuated this process (Table 1).

In turn, at pH 7.4, inhibiting effect could be evoked by specific lipid-protein interactions: e.g., protein monomers could be embedded into the $PoxnoPC_{20}$ domains of the lipid vesicles due to the Shiff base formation, stabilizing an aggregation-resistant protein conformation. Furthermore, liposomes could induce a decrease of the nucleation phase of insulin fibrillization due to the presence of the charged surfaces, inhibiting protein-protein

interactions [10]. In turn, ionic surfactants reduced the lag phase of lysozyme fibrillization at high concentrations where surfactant-protein hydrophobic interactions are predominant, due to complete charge neutralization [10]. The opposite effects of the ionic surfactants and $PG_{20}$ on lysozyme and insulin lag phases and aggregation rates could result from the stabilization of the lysozyme aggregation-prone conformation. Interestingly, the decrease of the insulin fibrillization extent by 25–40% was observed at the increased lipid-to-protein molar ratio (Fig.1 D) due to the more pronounced protein-lipid hydrophobic and specific interactions.

Table 1

Kinetic parameters of lysozyme and insulin fibrillization in the presence of phospholipids

| Protein | Lysozyme (pH 2, 60 ˚C) | | | Insulin (pH 2, 60 ˚C) | | | Insulin (pH 7.4, 60 ˚C) | | |
|---|---|---|---|---|---|---|---|---|---|
| Parameters | $k$, $h^{-1}$ | lag time, h | $F_{max}$, a.u. | $k$, $h^{-1}$ | lag time, h | $F_{max}$, a.u. | $k$, $h^{-1}$ | lag time, h | $F_{max}$, a.u. |
| Control | 2.7 | 0.7 | 1788 | 26 | 0.4 | 52 | 5.5 | 0.2 | 285 |
| $PG_{20}$ | 35 | 0.2 | 1315 | 10 | 0.6 | 27 | 5.3 | 0.5 | 293 |
| PC | 6.3 | 0.2 | 2500 | 18 | 1.4 | 77 | 2.5 | 0.4 | 172 |
| $PazePC_{20}$ | 21.5 | 0.3 | 1029 | 8 | 0.8 | 42 | 4.4 | 0.3 | 303 |
| $PoxnoPC_{20}$ | 3 | 0.2 | 1750 | 6 | 0.6 | 37 | 1.1 | 0.5 | 153 |
| PoxnoPC dispersions | 1.1 | 0.4 | 4935 | 7 | 0.2 | 50 | 1.9 | 0.5 | 334 |
| PazePC dispersions | 3.5 | 0.7 | 5331 | 6 | 0.3 | 40 | 2.9 | 0.5 | 457 |

It should be noted that a number of lipids exerted triggering effect on amyloid formation that is in accordance with the data reported elsewhere (Table 1) [13, 14]. Furthermore, their inhibiting effects were modulated by the environmental conditions: e.g. $PG_{20}$ reduced insulin fibrillization at pH 2, but had no effect at pH 7.4. The above results confirm the hypothesis emphasizing a significant role of the lipid-protein hydrophobic interactions in the inhibition of fibrillogenesis. Furthermore, specific lipid-protein interactions modulate the influence of vesicles on lysozyme and insulin aggregation: e.g., $PoxnoPC_{20}$ induced the decrease of insulin fibrillization extent at pH 2, while lysozyme aggregation extent did not change as compared to the control sample (Table 1). Notably, lysozyme fibrillization was not studied at pH 7.4, because this protein usually forms amorphous aggregates at neutral pH and high temperature [17]. Interestingly, lipid vesicles appeared to be the most reliable form of the potential anti-amyloid drugs, as compared to the dispersions of the oxidized phospholipids (Table 1). This finding highlights the important role of the polar surfaces of the lipids in the reduction of protein fibrillization extent. Similarly, non-ionic surfactants above the critical micelle concentration inhibited lysozyme aggregation due to the hydrophobic interactions between their hydrophobic tails and protein hydrophobic surfaces, while the surfaces of the surfactants reduced the interaction between the individual protein chains [10].

In conclusion, liposomes composed of the phosphatidylcholine and its mixtures with charged or oxidized phospholipids reduced lysozyme and/or insulin fibrillization at pH 2 and/or 7.4. The direction of the lipid effect was governed by the protein amino-acid sequence, lipid type and pH, pointing to the important role of lipid-protein hydrophobic and specific interactions in the inhibition of the fibrillization process. Furthermore, the potential of phospholipids as prospective anti-amyloid drugs could be tested *in vivo*, as well, because they are nontoxic to biological systems. This work was supported by the Ministry of Education and

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