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CONCERTED ACTION OF METALS AND MACROMOLECULAR CROWDING ON THE FIBRILLATION OF α -SYNUCLEIN

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Abstract

Certain metals lead to increased risk of Parkinson's disease (PD) and the aggregation of α -synuclein is implicated in the PD pathology. Although α -synuclein fibrillation has been extensively studied in dilute solutions *in vitro*, the intracellular environment is highly crowded. We are showing here that certain metals cause a significant acceleration of α -synuclein fibrillation in the presence of high concentrations of various macromolecules mostly through decreasing the fibrillation lagtime. The faster fibrillation in crowded environments in the presence of heavy metals suggests a simple molecular basis for the observed elevated risk of PD due to exposure to metals.

Keywords

Parkinson's disease; α -synuclein; crowding; fibrillation; aggregation; metals

Introduction

α -Synuclein is a small (14 kDa), abundant, and highly conserved presynaptic protein [1-3]. Structurally, purified α -synuclein is a typical natively unfolded protein [4,5], aggregation of which is implicated in the formation of inclusions in the brain, Lewy bodies and Lewy neurites, that are characteristic of several neurodegenerative disorders including Parkinson's disease (PD) [6-8]. The cause of PD is unknown, but considerable evidence suggests a multifactorial etiology, involving genetic susceptibility and environmental factors. In fact, recent work has shown that, except in extremely rare cases, there appears to be no direct genetic basis for PD [9]. However, several studies have implicated environmental factors, especially pesticides and metals [10-17]. Several metals have been shown to induce conformational changes in α -synuclein and accelerate the rate of its fibrillation *in vitro* [18-20]. In addition, elevated levels of some metals are found in the brains of PD patients [21-23].

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Dedication: This work is dedicated to the memory of Anthony L. Fink, who passed away on March 02, 2008. He was a brilliant scientist, outstanding teacher and a very good friend.

Usually, the *in vitro* studies on protein aggregation are performed under relatively ideal thermodynamic conditions; i.e., low protein and moderate salt concentrations. However, the inside of a cell is a very crowded environment, which is poorly modeled by dilute solutions. In fact, the concentration of macromolecules, including proteins, nucleic acids, and carbohydrates, within a cell can be as high as 400 g/L [24,25]. These intracellular solutes occupy as much as 20-30% of the total cellular volume [24,26-28], creating a crowded medium, with considerably restricted amounts of free water, but where, in general, no individual macromolecular species is present at high concentration [28,29]. Since the volume occupied by these macromolecular solutes is unavailable to other molecules, this gives rise to excluded volume effects [25,30], which may have a large influence on the behavior of biological macromolecules [31-34], and protein-protein interactions [31,35]. Suggestion that volume exclusion in physiological media could modulate both the rate and the extent of amyloid formation *in vivo* [29,36] has recently been confirmed for the *in vitro* fibrillation of human apolipoprotein C-II [37], α -synuclein [38-40], and β -synuclein [41].

The addition of high concentrations of different polymers (proteins, polysaccharides and polyethyleneglycols) dramatically accelerated α -synuclein fibrillation *in vitro*, with the magnitude of the accelerating effect being strongly dependent on the nature of the polymer, its length and concentration [38]. Previously, we have also shown that various metals are able to induce partial folding of α -synuclein, giving rise to the accelerated fibrillation *in vitro* [18-20]. The addition of relatively high (mM) concentrations of calcium did not induce partial folding of α -synuclein, and had no effect on its fibrillation [20], however Zn^{2+} [20], Pb^{2+} and Hg^{2+} [18] were very effective both in inducing structural changes and enhancing fibrillation of α -synuclein *in vitro* [18,20].

In the present study we analyzed the potential role of metals in the fibrillation of human α -synuclein in crowded environments, and show that metals can significantly accelerate the fibrillation of α -synuclein under crowded conditions.

Materials and methods

Materials

Thioflavin T (ThT), polyethylene glycols PEG-200, PEG-3350 and PEG-10000 (with average molecular masses of 200, 3,350 and 10,000 Da, respectively), and Ficoll-400 (with an average molecular mass of 400,000 Da) were obtained from Sigma, St. Louis, MO. $CaCl_2$, $ZnSO_4$, $Pb(NO_3)_2$ and $Hg(CH_3COO)_2$ were from Mallinckrodt (Mallinckrodt Chemical Works, St. Louis). All other chemicals were of analytical grade from Fisher Chemicals.

Expression and purification of recombinant α -synuclein

The expression (in *E. coli*) and purification of α -synuclein were as described previously [42]. α -Synuclein concentration was determined spectrophotometrically using the extinction coefficient $\epsilon_{276\text{ nm}}=0.401$.

Fibril formation assay

Assay solutions contained 15 μ M ThT, α -synuclein at a concentration of 35 μ M (0.5 mg/ml) in 20 mM Tris-HCl, 0.1 M NaCl, pH 7.5, and the desired concentrations of the crowding agent. ThT assays were performed with 96-well plates and analyzed as previously described [43].

Electron Microscopy

Transmission electron micrographs were collected using a JEOL JEM-100B microscope operating with an accelerating voltage of 80 kV. Typical nominal magnifications were 75,000.

Samples were deposited on Formvar-coated 300 mesh copper grids and negatively stained with 1% aqueous uranyl acetate.

Results

The effects of molecular crowding may be modeled *in vitro* via the use of concentrated solutions of a model macromolecular crowding agent such as polyethylene glycols, Ficolls or inert protein. The effects of certain metals on the fibrillation of α -synuclein in crowded milieu were monitored by Thioflavin T fluorescence and EM images.

Figure (1) compares the effects of Ca^{2+} (**A-E**) and Zn^{2+} (**a-e**) (both at a concentration of 500 μM) on the fibrillation of α -synuclein in the presence of high concentrations (100 mg/ml) of various crowding agents: PEG-200 (**A, a**), PEG-3350 (**B, b**), PEG-10000 (**C, c**), Ficoll-400 (**D, d**) and BSA (**E, e**). The data show that both PEG 200 and 3350 caused significant acceleration in the rate of fibrillation, mostly through decreasing the lagtime; i.e., increasing the rate of the nucleation stage of the aggregation process. Interestingly, the rate acceleration was somewhat less with PEG 10000, probably reflecting some offsetting effects due to the increased viscosity. BSA had similar effects as the polyethylene glycols, except the acceleration of nucleation was less pronounced. On the other hand, with Ficoll although the lagtime was similar to that with the other crowding agents, the rate of fibril growth/elongation was markedly decreased relative to that in the absence of the crowding agent.

In agreement with the results of previous studies, Ca^{2+} in dilute solution had no effect on the rate of fibrillation of α -synuclein whereas the presence of Zn^{2+} caused significant acceleration (compare black and open circles in Figure (1A) and (1a)). However, in the presence of the macromolecular crowding agents, both metals were able to further accelerate the process of fibril formation. This additional accelerating effect was more pronounced for Zn^{2+} (see Figure (1)). Thus, metals accelerate α -synuclein fibrillation in the presence of the fibril-promoting effects of macromolecular crowding agents, even for Ca^{2+} , which did not show detectable effects on the rate of fibrillation of α -synuclein in the absence of the crowding agents.

Figure (2) further summarizes the results on the effect of various factors on the α -synuclein fibrillation lagtime and show that the addition of such metals as Ca^{2+} , Zn^{2+} , Pb^{2+} and Hg^{2+} to the crowded α -synuclein solutions noticeably shortened the fibrillation lagtimes.

Figure (3) confirms the metal-enhanced fibrillation of α -synuclein in crowded environments. This figure represents the results of two sets of experiments, where fibril formation of α -synuclein in the presence of different metals and crowding agents was additionally modulated by the presence (**A-D**) or absence (**a-d**) of a Teflon bead. Figure (3A) and (3B) shows that in agreement with earlier studies the addition of different metals (Zn^{2+} , Pb^{2+} , or Hg^{2+} , Figure (3A)) or different crowding agents (PEG-3350 or Ficoll-400, Figure (3B)) alone leads to significant acceleration of α -synuclein fibrillation. The metals further accelerated the fibrillation in the presence of high concentrations of PEG-3350 (Figure (3C)) or Ficoll-400 (Figure (3D)).

The fibrillation of α -synuclein, as well as most, if not all, other proteins, is dramatically accelerated due to agitation of the protein-containing solutions. For example, with α -synuclein fibrillation in the absence of agitation requires higher protein concentrations and still takes several weeks. The presence of the Teflon bead in the wells of the 96-well plate significantly increases the agitation efficiency, compared to shaking alone, and also increases the hydrophobic surface area. Figures (3a-3d) illustrate that the kinetics of α -synuclein fibril formation are significantly decreased in the absence of the Teflon bead, with all other experimental conditions remaining the same. In fact, Figures (3a) and (3b) show that in the absence of metals or macromolecular crowding agents, α -synuclein does not fibrillate within

the time period of the experiment, 300 hrs. However, in the presence of the macromolecular crowding agents fibrillation of α -synuclein occurred in the absence of the beads (see Figure (3b)). For example, for PEG 3350 the lagtime is only about four-times longer (slower) than in the presence of the Teflon bead.

The metals themselves showed stronger acceleration of α -synuclein fibrillation in dilute solution in the absence of the beads, e. g. Figure (3a), which shows that in the absence of the beads Zn^{2+} and Pb^{2+} accelerated fibrillation.

Most significantly, however, in the presence of 100 mg/ml of PEG-3350 (Figure (3c)) or Ficoll-400 (Figure (3d)) all three metals caused an additional acceleration of α -synuclein fibrillation over that seen with just the crowding agent. For each metal, Zn^{2+} , Hg^{2+} and Pb^{2+} , both the rate of nucleation and fibril growth/elongation were increased.

Transmission electron microscopy images of α -synuclein fibrils grown under crowding conditions were examined. In general, the addition of the crowding agent had little effect on the morphology of the fibrils grown under similar conditions in the absence of the crowding agent, except for somewhat more clumping of fibrils. However, significant differences in appearance of the fibrils were observed in the presence of some of the metals. For example, in the presence of lead and zinc the fibrils were short and clumped laterally, and appeared more like crystallites (Figure (4)), both in the absence and presence of PEG 3350, and with and without the Teflon bead. On the other hand in the presence of Ficoll, the fibrils tended to be longer and less clumped (Figure (4)).

Discussion

Epidemiological studies have shown that exposure to a variety of metals, including copper, lead, iron, manganese and aluminum, are associated with elevated risk of PD [12-14,22,44, 45]. A correlation with increased incidence of PD has also been reported for individuals with mercury amalgam fillings [15] and in welders [46,47]. There are several possible ways in which toxic metal exposure could lead to increased risk of PD. Perhaps the simplest would be a direct effect of the metal on the aggregation of α -synuclein, which is believed to be the critical process in the development of the disease. The present investigation was undertaken to determine if metals would accelerate α -synuclein fibrillation under the crowded conditions present in a cell.

There are a number of simple corollaries stemming from the effect of macromolecular crowding on the underlying thermodynamics of proteins. Excluded volume effects will favor the most compact states, e.g. associated states, since oligomers will have less excluded volume than the corresponding amount of individual units. On the other hand increased viscosity effects will result in decreased diffusion rates and will lead to decreased kinetics of diffusion-controlled reactions, as in aggregation. Decreased water activity (also reflected in the excluded volume free energy) will also decrease protein solubility, favoring self-association. These predictions are borne out by our data.

Our results demonstrate that molecular crowding causes substantial acceleration of α -synuclein fibrillation *in vitro*, and that in the presence of certain metals, there was further acceleration. We have previously shown that the accelerating effect of a given macromolecular crowding agent on α -synuclein fibrillation is proportional to the concentration of polymer and to its length, up to some limiting value, at which point the rates begin to decline, probably due to viscosity effects [38]. The present studies were carried out with macromolecular crowding agent concentrations of 100 mg/ml, a value somewhat lower than that found for proteins in typical eukaryotic cells (\sim 150 mg/ml). It is not surprising that the different macromolecular crowding agents used in these experiments had somewhat different effects of the kinetics of

α -synuclein fibrillation, given that the properties of the crowded solutions differed significantly, both with respect to the size and molal volumes of the crowding agents, as well as such physical parameters as viscosity and chemical activity.

The net effect of a given factor, such as a metal ion, for example, on the kinetics of aggregation in a crowded environment is likely to be dependent on the same underlying molecular basis for the effect in dilute solution. For example, if metal ions accelerate aggregation due to electrostatic effects in which they bind to negatively charged side-chains on the aggregating protein, then the effect in crowded conditions will be determined by how the crowding affects the ion binding. This in turn will also depend on the nature of the crowding agent (charged versus neutral) and also other ions that are present (e.g. salts). Because of their small size, metal ions are unlikely to be directly affected by macromolecular crowding; however, the decreased activity of the solution will potentially affect their reactivity. The presence of the crowding conditions will potentially affect the state of the protein, and thus could result in differences in the interaction of the metal with the protein under crowding conditions. This appears to be the case with α -synuclein, and suggests also that there might be specific metal-binding sites in the protein. Support for specific metal binding sites comes from the substantially different effects of zinc and calcium, the former causes large increases in the rate of fibrillation, whereas the latter has only a minor effect under conditions of macromolecular crowding (Figures (1) and (2)).

We propose that the different extents of acceleration of α -synuclein fibrillation by the different crowding agents can be explained as follows. The effects of PEGs were greatest due to their greater excluded volume relative to BSA, which reflects the compact globular structure of BSA and the more expanded conformation of PEGs [38]. A similar situation holds for Ficoll which is more compact than comparable MW PEGs [38]. These excluded volume effects are manifested mostly on the nucleation step of the aggregation, and lead to the observed shorter lagtimes in the presence of the crowding agents.

The presence or absence of the Teflon bead was observed to make a very large difference in the rate of fibrillation, and to dramatically differentiate the different conditions on the rate of α -synuclein fibrillation. This finding suggests that the surface of the bead is important in the genesis of fibrillation, and, in fact, may act as a catalyst for fibrillation [38,48]. The faster fibrillation observed in the presence of the metals under crowding conditions in the absence of the beads, which can be considered to be more stringent conditions for fibrillation, is anticipated to be a better indicator of the “intrinsic” effect of the metal on fibrillation.

The most important finding of this study is the fact that the propensity of α -synuclein to form fibrils in crowded environments is further modulated by the addition of certain metals; for example, mercury increased the rate of nucleation by a factor of 2.5 (Figure (3c)). This means that the crowding agents and metals affect the structure/fibrillation of α -synuclein via different and independent molecular mechanisms. It has been assumed that the molecular crowding accelerates fibrillation due predominantly to excluded volume effects, which favor protein self-association, due to the effective increase in protein concentration [38]. In contrast, metals have been shown to bind directly to α -synuclein, and a model has been suggested in which the cations interact with α -synuclein to populate a partially folded conformation with a high propensity to aggregate [8, 18-20, 49]. α -Synuclein is rich in acidic residues and thus highly negatively charged at neutral pH. The resulting repulsive interactions represent the major factor leading to the natively unfolded conformation. The results with the metal ion-stimulated α -synuclein fibrillation and conformational changes indicate that the dominant effect of the metal ions is due to minimization of the Coulombic charge-charge repulsion [50].

Based on our previous studies of the effects of metals on α -synuclein fibrillation under non-crowding conditions, we anticipate that most transition metals, and those with high charge densities, such as aluminum, will have similar or greater accelerating effects under macromolecular crowding conditions to those observed here. At α -synuclein concentrations similar to those expected in the presynaptic region of neurons we find that low micromolar concentrations of metals bring about a significant increase in rate of fibrillation in the presence of macromolecular crowding agents (data not shown), suggesting that occupational metal exposure could directly accelerate α -synuclein fibrillation *in vivo*.

Our data suggest that macromolecular crowding not only enhances the probability of α -synuclein to form fibrils, but also modulates the direct interaction with metal ions — a possible factor in the pathogenesis of PD and other synucleinopathies. Thus, we show here, for the first time that an environmental risk factor for Parkinson's disease can trigger fibrillation of α -synuclein in the presence of high concentrations of a variety of macromolecules; i.e., under conditions analogous to those of the crowded interior of a living cell.

Our findings suggest that environmental contamination in the form of metals, especially heavy metals, could be a significant contributor to Parkinson's disease through direct effects on the aggregation of α -synuclein fibrillation. We believe that the effects of different metals that might be present would be additive, so that it would be the total neuronal load of these metals that would be important in accelerating α -synuclein aggregation. Our data are consistent with the limited number of epidemiological studies that show elevated risk of PD due to metal exposure, and suggest the need for additional epidemiological studies.

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List of abbreviation

PD, Parkinson's disease; ThT, Thioflavin T; PEG, polyethylene glycol.

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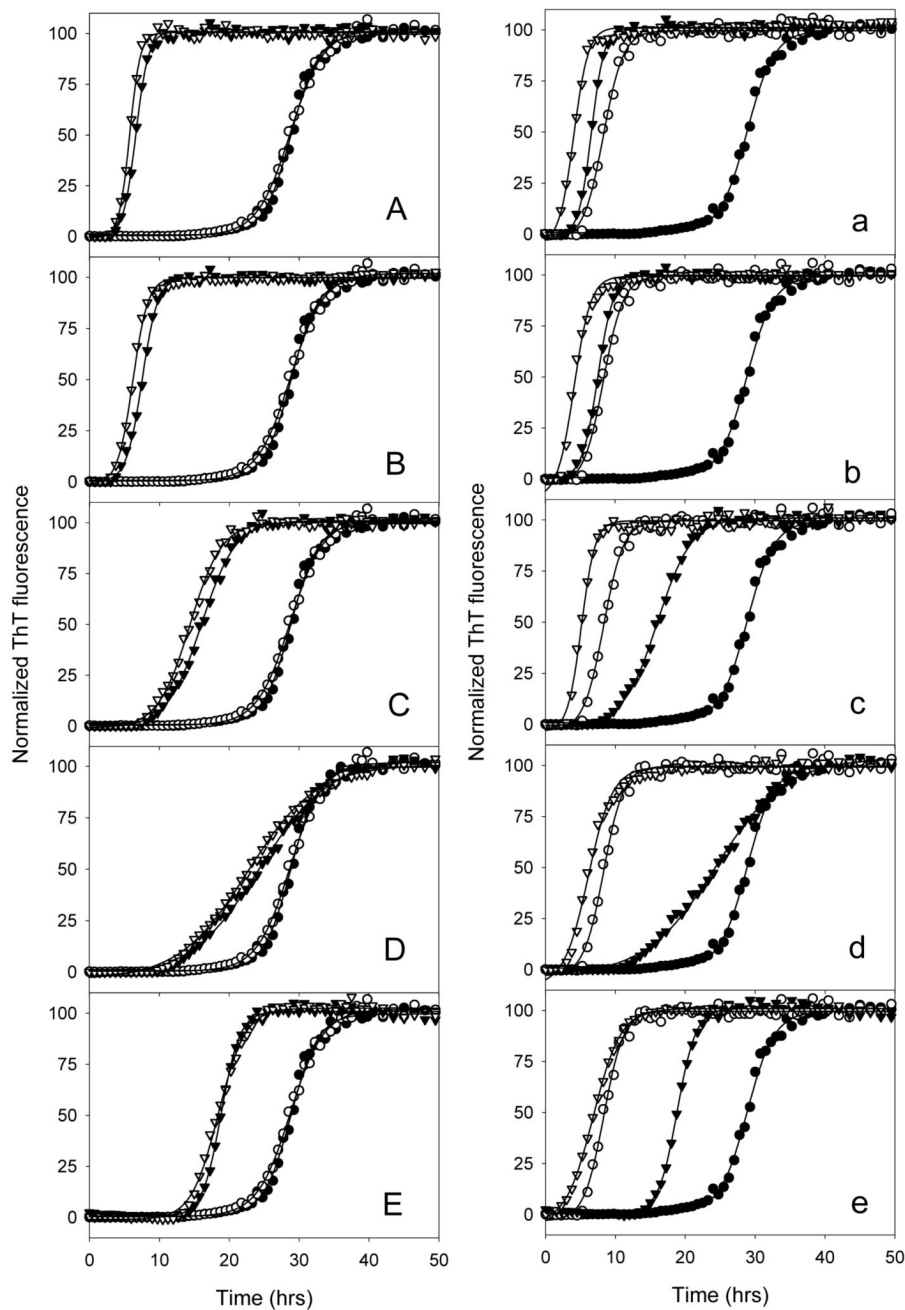


Figure 1.

Effect of Ca^{2+} and Zn^{2+} on fibrillation of human recombinant α -synuclein in the absence (circles) or presence of different crowding agents (triangles). Crowding agents are 100 mg/ml of: PEG-200 (A, a); PEG-3350 (B, b); PEG-10000 (C, c); Ficoll-400000 (D, d) and BSA (E, e). Measurements have been performed in the absence (black symbols) or presence (open symbols) of 500 μM Ca^{2+} (A-E) or Zn^{2+} (a-f). All experiments were performed in the presence of Teflon bead. α -Synuclein was kept at a concentration of 35 μM (0.5 mg/ml) in 20 mM Tris-HCl, 0.1 M NaCl, pH 7.5 throughout these experiments.

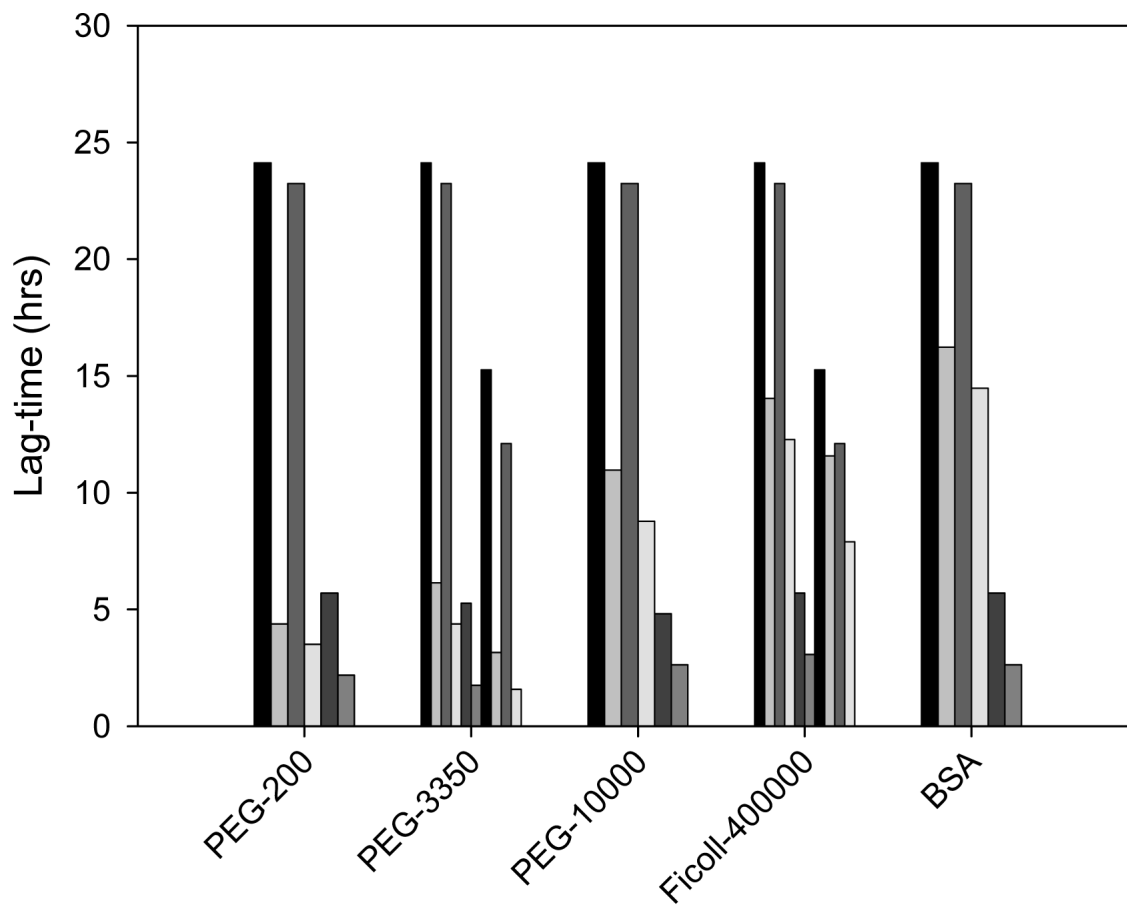


Figure 2.

Effect of metals and molecular crowding agents on the lag time of α -synuclein fibrillation in experiments in the presence of Teflon bead. From left to right bars in each group correspond to: control experiments (without metals and crowding agents); α -synuclein in the presence of corresponding crowder (100 mg/ml); α -synuclein in the presence of 500 μ M Ca^{2+} ; α -synuclein in the presence of 500 μ M Ca^{2+} and 100 mg/ml of the corresponding crowding agent; α -synuclein in the presence of 500 μ M Zn^{2+} ; α -synuclein in the presence of 500 μ M Zn^{2+} and 100 mg/ml of the corresponding crowding agent; α -synuclein in the presence of 500 μ M Pb^{2+} (for PEG-3350 and Ficoll-40000 experiments); α -synuclein in the presence of 500 μ M Pb^{2+} and 100 mg/ml of the corresponding crowding agent (for PEG-3350 and Ficoll-40000 experiments); α -synuclein in the presence of 500 μ M Hg^{2+} (for PEG-3350 and Ficoll-40000 experiments); α -synuclein in the presence of 500 μ M Hg^{2+} and 100 mg/ml of the corresponding crowding agent (for PEG-3350 and Ficoll-40000 experiments). Data for the effect of various crowders and Ca^{2+} and Zn^{2+} are derived from Figure 1, whereas data for the effect of various crowders and Pb^{2+} and Hg^{2+} are derived from Figure 3.

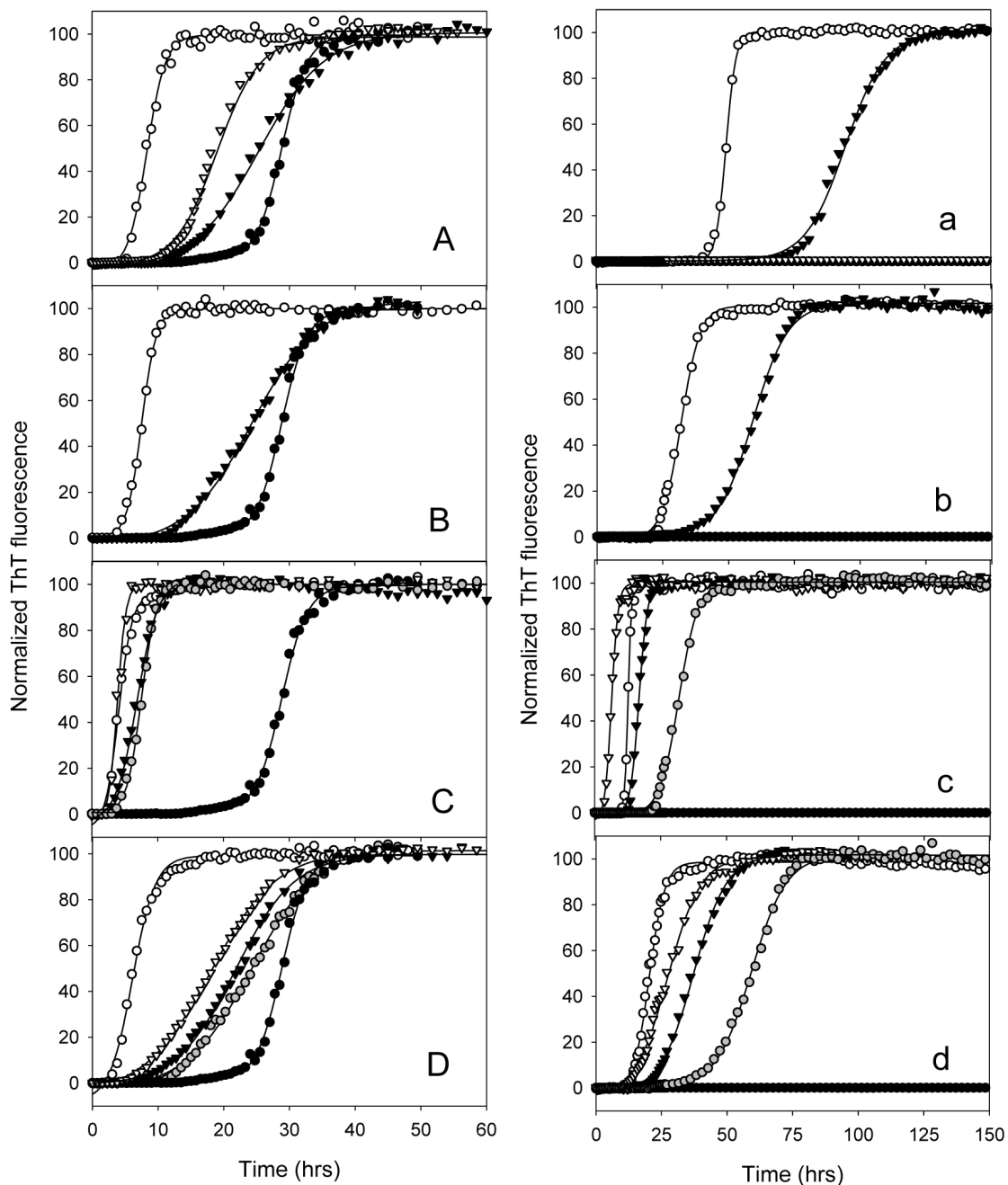


Figure 3.

Effect of metals on fibrillation of human α -synuclein in crowded environments in experiments performed in the presence (A-D) or absence of Teflon bead (a-d). **A, a.** Fibril-promoting effects of Zn^{2+} (open circles), Pb^{2+} (black triangles) and Hg^{2+} (open triangles). Metal concentration was 500 μ M. Black circles represent fibrillation of α -synuclein in control experiments without metal added. **B, b.** Fibril promoting effect of PEG-3350 (open circles), and Ficoll-400000 (black triangles), both in concentration of 100 mg/ml. Black circles represent fibrillation of α -synuclein in control experiments without crowding agents. **C, c.** Fibril-promoting effects of Zn^{2+} (open circles), Pb^{2+} (black triangles) and Hg^{2+} (open triangles) in the presence of PEG-3350 at a concentration of 100 mg/ml. Metal concentration was 500 μ M. Black circles

represent fibrillation of α -synuclein in control experiments. Gray circles represent fibrillation of α -synuclein in the absence of metals, but in the presence of PEG. **D, d.** Fibril-promoting effects of Zn^{2+} (open circles), Pb^{2+} (black triangles) and Hg^{2+} (open triangles) in the presence of 100 mg/ml of Ficoll-400000. Metal concentration was 500 μ M. Black circles represent fibrillation of α -synuclein in control experiments without any additives. Gray circles correspond to the fibrillation of α -synuclein in the absence of metals, but in the presence of Ficoll. α -Synuclein was kept at a concentration of 35 μ M (0.5 mg/ml) in 20 mM Tris-HCl, 0.1 M NaCl, pH 7.5 throughout these experiments.

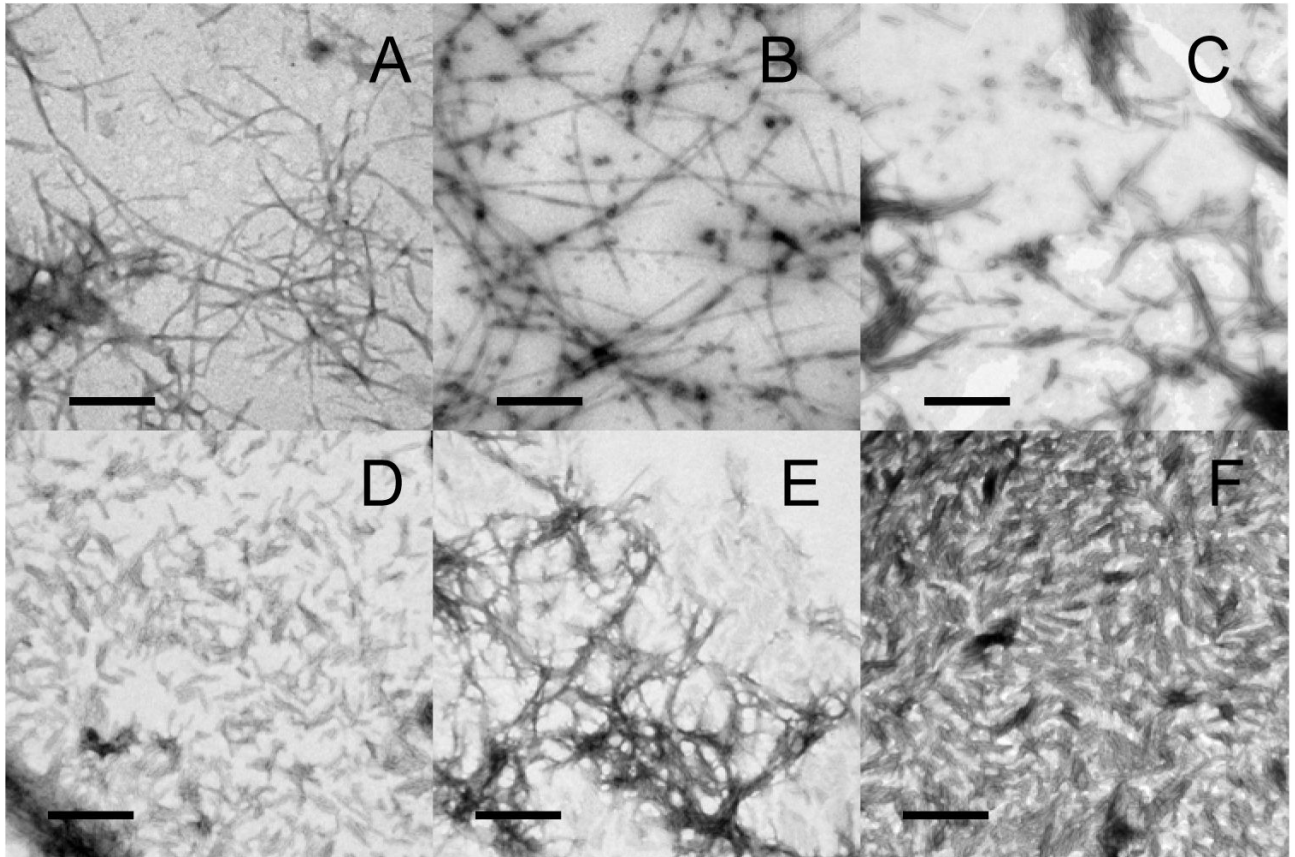


Figure 4. Negatively stained transmission electron micrographs of α -synuclein fibrils grown under various crowding conditions. A, Control (no crowding, no metals); B, Ficoll 400, no bead; C, Ficoll 400 with bead; D, lead, no crowding agent; E, PEG 3350; F, lead plus PEG 3350. Crowding agent concentrations were 100 mg/ml. Pb concentrations were 500 (M). Scale bar is 0.2 μ M.