

Polyoxometalates as Inhibitors of the Aggregation of Amyloid β Peptides Associated with Alzheimer's Disease**

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Alzheimer's disease (AD) is the most common form of dementia. It is characterized by cerebral extracellular amyloid plaques and intracellular neurofibrillary tangles.^[1] Although the molecular mechanisms of AD pathogenesis are not clearly understood owing to its complexity, recent advances have demonstrated that the polymerization of amyloid β -peptides (A β) into amyloid fibrils is crucial.^[2] Therefore, the development of A β inhibitors has received much attention. Peptides or peptide mimetics^[3–5] and small organic molecules^[6,7] are now the two main classes of A β inhibitors.

Since the discovery of the antitumor activity of cisplatin, many inorganic compounds have been used as important therapeutic drugs and diagnostic imaging agents.^[8] Worldwide sales of inorganic drugs are growing rapidly. However, few inorganic ligands have been reported that can inhibit A β amyloid formation.^[9,10] As important inorganic drug candidates, polyoxometalates (POMs) have shown promising antiviral and antitumor activities for more than a decade.^[11–16] On the basis of cell-culture assays, *in vitro* enzymatic activity, and molecular-modeling studies, POMs have regained considerable attention as a result of their remarkable effect against acquired immune deficiency syndrome (AIDS), as demonstrated by Hill, Pope, and co-workers.^[12,13] POMs can specifically bind to the basic fibroblast growth factor (bFGF) and induce dramatic conformational changes in the growth factor.^[16]

POMs are early-transition-metal–oxygen–anion clusters with incomparable structural versatility and interesting properties. The unique feature of POMs is that nearly every molecular property that can influence the recognition and reactivity of POMs with target biological macromolecules is changeable, including polarity, redox potential, surface charge

distribution, shape, and acidity.^[11d] Several research groups have reported detailed studies of the interaction between POMs and their target proteins.^[12–16] Given that the size and fairly rigid, cage-like structure of many POMs are similar, and in some cases nearly identical, to the size and structure of the water-solubilized fullerene derivatives reported to have fairly good anti-amyloid activity,^[10] POMs may also be potential A β inhibitors.

By using a high-throughput screening method based on the fluorescence of an A β -enhanced cyan fluorescent protein (ECFP) fusion expression system constructed in our laboratory and originally developed by Hecht and co-workers,^[17] we have identified four POMs capable of inhibiting A β aggregation. The highest inhibition was observed for $K_8[P_2CoW_{17}O_{61}]$ (see Figure S1 in the Supporting Information), a phosphotungstate with a Wells–Dawson structure (Figure 1A). POMs with a Keggin structure (Figure 1B) showed moderate to high inhibition of A β aggregation. Smaller POMs, such as $Na_5[IMo_6O_{24}]$ with an Anderson structure (Figure 1C), were inactive.

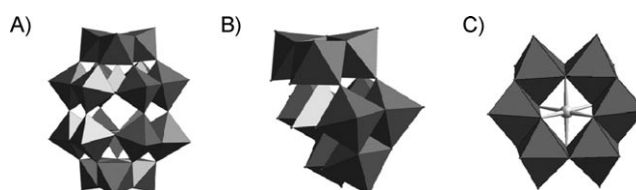


Figure 1. Structures of typical polyoxometalates: A) Wells–Dawson structure, $K_8[P_2CoW_{17}O_{61}]$; B) trivacant Keggin structure, $\alpha-Na_5H-[SiW_9O_{34}]$; C) Anderson structure, $Na_5[IMo_6O_{24}]$.

To verify the effect of POMs on the assembly of A β 1–40 into amyloid fibrils, we used an *in vitro* thioflavin T (ThT) fluorescence assay. ThT, an extrinsic fluorescent dye, is able to bind to amyloid fibrils; upon binding, its fluorescence intensity increases.^[18] Fibril formation was quantified by measuring the fluorescence intensity of ThT at 480 nm upon excitation at 444 nm. When fresh A β 1–40 alone was incubated at 37 °C, ThT fluorescence as a function of incubation time showed a sigmoidal shape (Figure 2A). This result is consistent with the nucleation-dependent polymerization model. However, in the presence of $K_8[P_2CoW_{17}O_{61}]$ (10 μ M or 100 μ M), ThT fluorescence did not increase, which indicated that A β amyloid formation was suppressed, in agreement with our fluorescence cell-based screening results. In the control experiment, there was no POM to influence the fluorescence of ThT (see Figure S2). Furthermore, A β 1–40 from the stock solution (without incubation) adopted the

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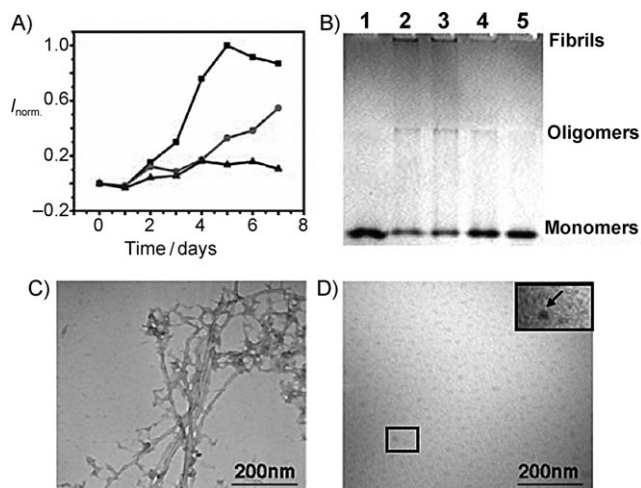


Figure 2. Inhibition of A β assembly by K₈[P₂CoW₁₇O₆₁]. A) Fibrillation kinetics of A β 1–40 as monitored by the development of thioflavin T binding in the absence of K₈[P₂CoW₁₇O₆₁] (■) or in the presence of K₈[P₂CoW₁₇O₆₁] in a 5:1 (●) or 1:2 (▲) A β 1–40/K₈[P₂CoW₁₇O₆₁] ratio. B) Determination of the dose-dependent effect of K₈[P₂CoW₁₇O₆₁] on the formation of A β fibrils by native PAGE. Samples were prepared as described in the Experimental Section with an increasing amount of K₈[P₂CoW₁₇O₆₁] and without K₈[P₂CoW₁₇O₆₁]: 1) control (A β 1–40 monomer), 2) A β 1–40, 3) 50:1 A β 1–40/K₈[P₂CoW₁₇O₆₁], 4) 5:1 A β 1–40/K₈[P₂CoW₁₇O₆₁], 5) 1:2 A β 1–40/K₈[P₂CoW₁₇O₆₁]. C) TEM micrograph of a control sample containing A β 1–40 (50 μ M) in 10 mM Tris buffer (pH 7.4) after incubation at 37 °C for 7 days. D) TEM micrograph of a sample containing A β 1–40 (50 μ M) and K₈[P₂CoW₁₇O₆₁] (1:2 molar ratio) in 10 mM Tris buffer (pH 7.4) after incubation at 37 °C for 7 days. The inset is a high-magnification image of spherical A β 1–40 oligomers.

monomeric form, as shown by native PAGE (Figure 2B, lane 1). After incubation for 7 days, the monomer band became weak, which suggested that A β 1–40 had aggregated into higher-order oligomers and fibrils (Figure 2B, lane 2). A β amyloid formation was inhibited by K₈[P₂CoW₁₇O₆₁], as shown by a stronger monomer band and weaker oligomer and fibril band in the native gel (Figure 2B, lanes 3–5).

We also investigated the effect of K₈[P₂CoW₁₇O₆₁] on the morphology of A β aggregates by negative-stain TEM.^[19] A 50 μ M solution of A β was incubated with and without K₈[P₂CoW₁₇O₆₁] in an amyloid-forming buffer (10 mM 2-amino-2-hydroxymethylpropane-1,3-diol (Tris), pH 7.4) at 37 °C for 7 days. A β formed long unbranched fibrils with diameters of about 10 nm: a typical structure for amyloid fibrils (Figure 2C). In contrast, in the presence of K₈[P₂CoW₁₇O₆₁] (100 μ M), spherical structures with heights of typically 3–4 nm were formed (Figure 2D). These results further support those found with ThT and indicate that K₈[P₂CoW₁₇O₆₁] can inhibit A β -fibril formation.

To better quantify the inhibitory effect of POMs and to attempt to correlate this effect with structural or compositional features, we determined the IC₅₀ value of a series of POMs in a ThT assay (see Figure S3). We studied a number of Dawson, Keggin, and Anderson structures to compare the effects of structure and charge (Table 1) and found that the inhibitory efficiency is related to the POM structure. The data obtained indicate that the inhibitory efficiency increases with

Table 1: Inhibitory efficiency of POMs with respect to A β fibrillization.

	Structure type	Charges	$K_a^{[a]}$ (M ⁻¹)	IC ₅₀ ^[b] (μ M)
Na ₅ [IMoO ₂₄]	Anderson	5	—	No inhibition
K ₇ [PTi ₂ W ₁₀ O ₄₀]	Keggin	7	4.65×10^5	39.04
α -Na ₉ H-[SiW ₉ O ₃₄]	Keggin	10	4.55×10^6	19.85
K ₈ [β -SiW ₁₁ O ₃₉]	Keggin	8	4.76×10^5	39.02
K ₈ [P ₂ CoW ₁₇ O ₆₁]	Dawson	8	2.08×10^6	16.68
H ₃ [PMo ₁₂ O ₄₀]	Keggin	3	$< 1 \times 10^5$	No inhibition

[a] Tyrosine-fluorescence titration data were used to estimate the binding constant by nonlinear least-squares fitting. [b] A ThT assay was used for determination of the IC₅₀ value.

the size of the administered POM. The small Anderson compound studied was inactive, larger Keggin compounds were moderately active, and the largest, Dawson compound was the best inhibitor. These results indicate that the size of the POM might play a key role in A β recognition and amyloid inhibition.

The four POMs of the Keggin type showed different inhibition abilities. To gain a better understanding of the inhibition activity of the Keggin-type POMs, we compared the binding affinity of the four POMs for A β . The fluorescence intensity of A β was more strongly quenched as the amount of the different POMs increased. The apparent binding constants yielded by a nonlinear least-squares fit^[20] (see Figure S4) were in agreement with the IC₅₀ values of the POMs against A β aggregation (Table 1). Therefore, we concluded that the inhibition ability of a given type of POM depended mainly on the binding affinity for A β . As the binding affinities of the POMs were in agreement with their surface charges (Table 1), we assumed that electrostatic effects play an important role in the interaction between A β and POMs. This hypothesis is further supported by the inhibitory effect of [NaP₅W₃₀O₁₁₀]¹⁴⁻ with a Preyssler structure, which shows a stronger inhibitory effect than that observed for the Dawson-type POM (Figure 3). These results further indicate that the inhibition selectivity of POMs might be related to size-specific electrostatic interactions between POMs and A β , although other interactions, such as hydrogen bonding, cannot be excluded.

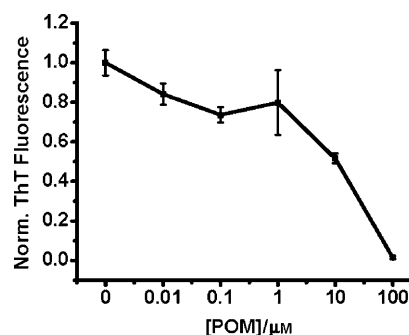


Figure 3. Dose-dependent inhibition of A β fibrillization by [NaP₅W₃₀O₁₁₀]¹⁴⁻, which has a Preyssler structure. The excitation wavelength was 444 nm, and the emission intensity at 480 nm was used for analysis.

Next, we carried out digestion experiments with trypsin to investigate how POMs bind to A β . Trypsin digestion cleaves exclusively C-terminal residues up to the first arginine or lysine residue. The results (see Figure S5A) showed that A β was almost completely degraded after 60 min, whereas proteolysis was prevented in the presence of K₈[P₂CoW₁₇O₆₁]. This protective effect was dose-dependent, and complete inhibition was observed at the molar ratio POM/A β = 2:1. Under similar conditions, in the presence of K₈[P₂CoW₁₇O₆₁], the trypsin-induced degradation of lysozyme was not affected (not shown). These results suggest that K₈[P₂CoW₁₇O₆₁] can interact with the A β monomer and protect it from trypsin-induced degradation, and that the positively charged arginine or lysine residues of A β play an important role in the binding of K₈[P₂CoW₁₇O₆₁] with A β . A salt effect on POM binding also showed that specific electrostatic interactions are important. With increasing ionic strength, the binding affinity^[20] decreased dramatically, as reflected by an increase in the intensity of tyrosine fluorescence (see Figure S5B).

On the basis of the above results, we further investigated the specific POM-binding site on A β . Hill and co-workers demonstrated that POMs can bind to a cationic pocket of lysine residues on the outer surface of the flaps that cover the active site.^[12] We reported previously that several classic POMs bind to the cationic pocket of basic fibroblast growth factor.^[16] These observations may suggest that the cationic cluster HHQK of A β could be the binding site for POMs. We first used competition assays to verify the putative POM-binding site on A β . The cationic cluster of A β is near the central hydrophobic area of the previously described binding site for 4,4'-bis(1-anilino-naphthalene 8-sulfonate) (bis-ANS).^[21,22] The fluorescence intensity of bis-ANS was strongly enhanced upon binding to A β . The addition of K₈[P₂CoW₁₇O₆₁] to the A β -bis-ANS complex resulted in a progressive decrease in the intensity of bis-ANS fluorescence (see Figure S6). This result suggests that the binding of K₈[P₂CoW₁₇O₆₁] to A β can influence the binding of bis-ANS to A β and implies that the binding site for POMs is near that for bis-ANS. Fluorescence-quenching data were fit with the Stern–Volmer equation,^[23] which yielded a quenching constant of $0.88 \times 10^6 \text{ M}^{-1}$.

We also used the shorter fragment A β 12–28 with the sequence VHHQKLVFFAEDVGSNK, which, like A β 1–40, can bind to bis-ANS.^[21] K₈[P₂CoW₁₇O₆₁] showed competitive binding to A β 12–28 with bis-ANS. The Stern–Volmer quenching constant was $0.90 \times 10^6 \text{ M}^{-1}$ (see Figure S7A), which is a similar value to that observed for A β 1–40. To study the binding site further, we carried out a digestion experiment with trypsin. We chose A β 12–28 as the trypsin substrate; the cleavage site was just next to the central hydrophobic region. It was clearly shown that K₈[P₂CoW₁₇O₆₁] could prevent the digestion of this fragment (see Figure S7B), which indicates that K₈[P₂CoW₁₇O₆₁] did bind this region of the A β peptide. Studies both *in vivo* and *in vitro* have suggested that low-molecular-weight (LMW) glycosaminoglycans (GAGs), such as LMW heparin or LMW chondroitin sulfate, might decrease the cytotoxic effect of A β and prevent the deposit of amyloid plaques

through binding to A β to interfere with its fibril formation.^[24] The binding site of heparin analogues to A β was speculated to be the region of His13–Lys16.^[25] Our results clearly show that POMs bind exclusively to a positively charged recognition motif, presumably HHQK, that is located in the 12–28 sequence of A β .

Previous studies have shown that A β -recognition agents modified with bulky groups, such as steroids, can be used as a strategy to hinder further A β association.^[26,27] We assumed that POMs could act as both recognition elements and agents to hinder A β fibrillization owing to their charged nature and bulky size. There are two possible mechanisms by which POMs could inhibit A β fibrillization. Our observations clearly showed that POMs could bind to the A β monomer. This binding would lower the concentration of the free monomer and shift the equilibrium away from fibrillization. Another possibility is that the interactions between the large POM surface and A β oligomers could result in unfavorable conditions for nucleation and fibril growth through the blocking of direct contact between monomers. This situation would lead to the depletion of sub- and near-critical oligomers and to partial blockage of the kinetic pathway until the surface is saturated and new nuclei can be grown.^[28]

One issue that needs to be addressed is the stability^[16] of the Keggin and Wells–Dawson anions in dilute aqueous solution. POM structures are known to depend on concentration, the pH value, and the buffer characteristics. We quantified the stability of these compounds experimentally by UV/Vis spectroscopy (see Figure S8). Under the most commonly used conditions for the experiments reported herein, all the POMs used remained intact.^[29–31]

The ability of POMs to inhibit A β assembly suggested that they might be useful in blocking A β -mediated cellular toxicity. To address this question, we used PC12 cells in 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assays to probe cellular metabolism. A β was incubated with POMs for 7 days, and then cells were exposed to the A β /POM mixtures. The results of the assays were normalized by using the results for cells not treated with A β fibrils as a positive control. In the presence of POMs, the survival of cells increased to about 90 % at most (Figure 4A). POMs themselves were nontoxic at the same concentrations (Figure 4B). Thus, POMs are not only effective *in vitro* inhibitors, as shown by the ThT fluorescence assay, but their inhibition effects can be demonstrated in PC12 cells as well.

In summary, through the use of a high-throughput screening method based on the fluorescence of an A β –ECFP fusion expression system and a ThT fluorescence assay, we have shown that four kinds of POMs serve as A β inhibitors. An electrostatic effect is the main factor in the interactions between POMs and A β . The size of POMs might play a key role in A β recognition and amyloid inhibition. Experiments based on enzyme digestion, fluorescence competitive binding, fluorescence quenching, and the use of a different A β fragment indicated that POMs bind to the positively charged His13–Lys16 cluster region of A β . To the best of our knowledge, it has not been reported previously that polyoxometalates can inhibit amyloid- β aggregation. We expect that

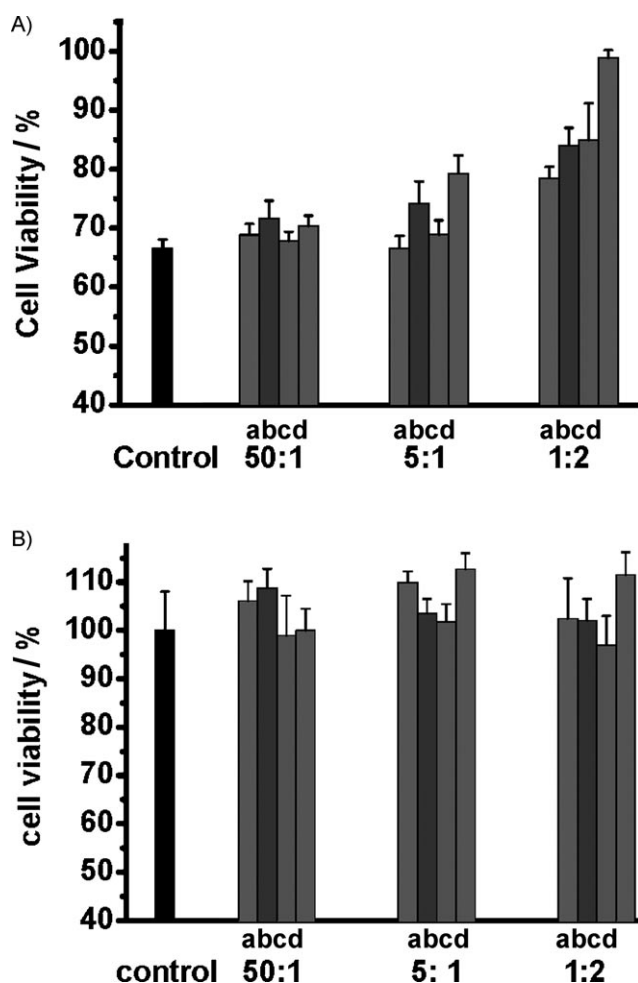


Figure 4. Effect of POMs ($K_7[PTi_2W_{10}O_{40}]$ (a); $K_8[\beta-SiW_{11}O_{39}]$ (b); $\alpha-Na_9H[SiW_9O_{34}]$ (c); $K_8[P_2CoW_{17}O_{61}]$ (d)) on the cell toxicity of A β . Samples were prepared according to the Experimental Section in the presence (A) or absence (B) of A β (5 μ M). The cytotoxic effect on PC12 cells was determined by using an MTT assay. A β /POM ratios are given.

this study will prompt the design and screening of inorganic compounds as therapeutic agents for AD.

Experimental Section

High-throughput screening of A β inhibitors: The A β -ECFP fusion system was constructed as described with some modification.^[17] Briefly, *Escherichia coli* strain BL21 (DE3) was transformed by the vector ALC (A β -linker-ECFP) or the control vector CFP (linker-ECFP) and cultured at 37°C in lysogeny broth (LB) containing ampicillin (50 μ g mL⁻¹). Different POMs were added to the culture medium 30 min prior to protein expression induced by isopropyl- β -D-thiogalactopyranoside (IPTG; 1 mM). After expression of the recombinant proteins for 3 h, all samples were diluted to an optical density of 0.1 at 600 nm. The fluorescence of each sample was measured at 512 nm (excitation 433 nm) with a JASCO FP6500 spectrofluorometer. Compounds were tested in triplicate at a final concentration of 100 μ M. The identification of hits was consistent across several repeated experiments.

Sample preparation: A β 1–40 and A β 12–28 were purchased individually from American Peptide (lot no. U10012) and Sigma (lot no. 32K12201). Peptides were prepared as previously de-

scribed.^[32–34] Briefly, the powdered A β peptide was first dissolved in 1,1,1,3,3,3-hexafluoro-2-propanol (HFIP) at a concentration of 1 mg mL⁻¹. The solution was shaken at 4°C for 2 h in a sealed vial for further dissolution and was then stored at –20°C as a stock solution. Before use, the solvent HFIP was removed by evaporation under a gentle stream of nitrogen, and the peptide was dissolved in 10 mM Tris buffer (pH 7.4). Fibril formation was carried out essentially as described earlier.^[32–34]

ThT fluorescence spectroscopy: The kinetics of A β aggregation were monitored by using the dye ThT, the fluorescence of which is dependent on the formation of amyloid fibrils. Fluorescence measurements were carried out with a JASCO FP6500 spectrofluorometer. The fluorescence signal (excitation at 444 nm) was recorded between 460 and 650 nm; 10 nm slits were used for both emission and excitation measurements. The peptide concentration was 1 μ M, and the ThT concentration was 10 μ M. At different times, aliquots of the A β solution were taken for fluorescence measurements.

Native gel electrophoresis: Samples (10 μ L) were analyzed by 20% native PAGE. Gels were run in a Tris/glycine system and developed by the silver-stain method.

Transmission electron microscopy: Samples (10 μ L) were spotted onto carbon-coated copper grids and left for 30 min. The grids were then blotted with filter paper to remove excess buffer, and the sample was stained with 1.5% (w/v) phosphotungstic acid (pH 7.4). Grids were blotted again and air-dried before analysis on a transmission electron microscope (JEOL JEM-1011) operating at a voltage of 100 kV.

Trypsin digestion: A β (20 μ M) was preincubated in 10 mM Tris buffer (pH 7.4) in the absence or presence of POM (in different A β /POM ratios) for 30 min at 37°C and then subjected to proteolysis by trypsin (0.1 mg mL⁻¹) under the same conditions for 10 min. At the end of the reaction, all samples were supplemented with SDS-PAGE reducing sample buffer, heated at 100°C for 5 min, and subjected to SDS-PAGE (20%). The gels were then silver-stained.

Fluorescence titration: The intrinsic tyrosine fluorescence was examined as previously described.^[20] The concentration of A β 1–40 was 3 μ M. The excitation wavelength was 278 nm, and the emission intensity at 305 nm was used for analysis. The 1:1 binding stoichiometric equation was used to calculate the binding constants.

Bis-ANS displacement: Solutions of freshly prepared A β 1–40 or A β 12–28 with bis-ANS were incubated in 10 mM Tris buffer (pH 7.4) for 30 min at room temperature, and then bis-ANS fluorescence was measured. The POM was titrated into the solution every 10 min. The excitation wavelength was 395 nm.

Cell-toxicity assays: PC12 cells (rat pheochromocytoma, American Type Culture Collection) were cultured in Iscove modified Dulbecco medium (IMDM, Gibco BRL) supplemented with 5% fetal bovine serum and 10% horse serum in a humidified 5% CO₂ environment at 37°C. Cells were plated at a density of 10000 cells per well on 96-well plates in fresh medium (90 mL). After 24 h, A β 1–40 peptides (5 μ M) that had been aged with or without POMs were added, and the cells were further incubated for 48 h at 37°C. Cytotoxicity was measured by using a modified MTT assay kit (Promega). Absorbance values of formazan were determined at 490 nm with an automatic plate reader.

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