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Can size alone explain some of the differences in toxicity between β -amyloid oligomers and fibrils?

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Abstract

β -amyloid ($A\beta$) peptide is believed to play a key role in the mechanism of Alzheimer's disease (AD). $A\beta$ tends to aggregate to form amyloid fibrils. A variety of evidence indicates that $A\beta$ aggregates are toxic *in-vitro* and *in-vivo*. An early ' $A\beta$ hypothesis' postulated that AD was the consequence of neuron death induced by insoluble deposits of large $A\beta$ fibrils. Newer findings indicate that small soluble $A\beta$ oligomers are the neurotoxic species, yet, their structure is still unknown. Many researchers have tried to probe the differences in molecular structure between $A\beta$ oligomers, protofibrils, and fibrils that give rise to their unique toxicities, but with limited success. In this report, we examine the hypothesis that differences in the toxicity of different aggregated $A\beta$ species are the result of differences in species concentration and diffusivity. Using a simple mathematical analysis based on the assumption of a diffusion limited reaction, we demonstrate that near 10 fold differences in toxicity between spherical oligomers and fibrils can be explained from size and concentration arguments. While this work does not suggest that $A\beta$ oligomers and fibrils have identical molecular structures, it highlights the possibility that simple physical phenomena may contribute to the biological processes induced by $A\beta$.

Keywords

Alzheimer's; oligomer; protofibril; diffusion; structure; biological activity

Aggregated β -amyloid ($A\beta$) is one of the major protein constituents of senile plaques found in Alzheimer's disease (AD) and is believed to play a significant role in the cascade of events that ultimately lead to AD (Selkoe 2001). $A\beta$ spontaneously aggregates *in-vitro* into fibrils that resemble the fibril morphology of $A\beta$ found *in-vivo* in plaques (Walsh and Selkoe 2007). Because $A\beta$ monomers appear nontoxic, while $A\beta$ aggregates are toxic to neurons both *in-vitro* and *in-vivo* (Klein 2006), it was widely accepted that the buildup of $A\beta$ fibrils extracellularly in the brain was the cause for the neurodegeneration associated with AD. In the last decade, however, accumulating evidence suggest that a soluble $A\beta$ aggregation intermediate, not the fibril, is the species that has the highest neurotoxicity (Klein 2006; Lee et al. 2007; Lih-Fen Lue 1999).

The revised $A\beta$ hypothesis led to extensive research towards identification and characterization of the neurotoxic intermediate. Several soluble intermediates have been characterized ranging in size and shape from short fibril-like aggregates called protofibrils (Kheterpal et al. 2006), to spherical structures that are 5–35 nm in diameter (Chimon et al. 2007; Hoshi et al. 2003; Walsh and Selkoe 2007). At the micro- to nanometer scale there are

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obvious structural differences between A β aggregation intermediates (oligomers and/or protofibrils) and fibrils. At the molecular level, it has been more challenging to characterize the structural differences between species that could give rise to their differences in biological activity.

Despite the limited molecular structural data characterizing A β intermediates, the view that the toxic A β intermediate is structurally different from A β fibrils prevails. The hypothesis that the toxic intermediate is structurally distinct from the fibril is based on three main pieces of evidence:

1. The high biological activity of the intermediate compared to the fibril (Klein 2006; Lee et al. 2007);
2. Micrographs that show morphologically distinct entities (Chimon et al. 2007; Lee et al. 2007), and;
3. The specificity and selectivity of some antibodies and small molecules for aggregation intermediates (Kayed et al. 2007; Mamikonyan et al. 2007).

In our laboratory we have examined the structure specific biological activity of A β , and tried to characterize the structural differences between different A β aggregated species (Lee et al. 2007; Wang et al. 2002; Zhang et al. 2009). We have shown that A β oligomers killed approximately 3 times more cells than fibrils, when equal concentrations of monomer were used to prepare either oligomer or fibril (Lee et al. 2007). Our findings indicating a highly active intermediate relatively to the fibril are consistent with data from other laboratories. Whether the biological activity being assayed involves lipid bilayer conductance (Sokolov et al. 2006), protein kinase activity and toxicity (Hoshi et al. 2003), or changes in intracellular Ca²⁺ (Demuro et al. 2005), oligomers or aggregation intermediates have repeatedly been found to be more active than fibrils.

While we have consistently found differences in A β biological activity as a function of A β structure, we have had much less success elucidating molecular level structural differences between A β oligomer or aggregation intermediate and fibril. We observed some differences in protein stability between oligomer and fibril (Lee et al. 2007), and modest differences in A β backbone amide hydrogen exchange (Zhang et al. 2009). Wetzel has also reported differences in A β backbone amide hydrogen exchange between protofibrils stabilized by a small molecule and fibrils (Kheterpal et al. 2006), but the largest differences in backbone solvent accessibility were found in residues 21–30, while we saw the biggest differences between our aggregation intermediate and fibril in the N-terminal segment (residues 1–16). In contrast to the hydrogen exchange data, recent NMR data indicated that A β spherical aggregates, had a molecular structure identical to the fibril at the hydrophobic core and the C-terminus, the two regions studied (Chimon et al. 2007).

Some A β antibodies recognize both A β fibrils and certain A β oligomers, suggesting that a fibril-like soluble oligomer exists (Kayed et al. 2007). On the contrary, conformation specific antibodies have been able to distinguish between several different A β aggregation intermediates and A β fibrils (Kayed et al. 2007; Mamikonyan et al. 2007), suggesting that there must be structural differences between different species, however the molecular level structural features that give rise to the differences in binding specificities between these antibodies is still unknown.

The apparent controversy between the accepted paradigm and the available data, led us to explore an alternative explanation, which is consistent with the high biological activity of the intermediate. The model presented here accounts for the differences in the aggregates'

macroscopic structures, but does not assume molecular structural differences between the A β intermediates and fibrils.

The model considers two parameters which are derived from the aggregate's size: concentration and diffusivity. In *in-vitro* experiments, for equal monomer concentrations, the concentration of A β aggregates decreases as the size of the aggregates increases. Thus, the concentration of aggregation intermediates, composed of dozens to hundreds monomers per aggregate, would be higher than the concentration of fibrils, which contain hundreds or thousands monomers per unit if pure species of intermediate and fibril were formed. In addition, the diffusivity of large A β aggregates is smaller than that of A β oligomers or protofibrils. Hence, the low mobility of the large A β aggregates may affect their reactivity with cells if the reaction becomes diffusion-limited.

The rate of reaction of a diffusing species on a non-diffusing spherical surface such as a cell, where the reaction of the diffusing species is first order, is given by (Shoup and Szabo 1982):

$$rate = \frac{4\pi DRkC_{A\beta}^{\infty}}{4\pi DR + k} \quad (1)$$

where D is the diffusivity of the diffusing species, R is the cell's radius, k is the intrinsic first order rate constant on a per cell basis and describes the rate at which A β reacts with the cell surface once it has encountered the cell surface, and $C_{A\beta}^{\infty}$ is the bulk concentration of the diffusing A β aggregates. The equation is derived by solving a steady state spherical diffusion equation, assuming that the flux of A β at the cell surface is equal to the rate of reaction of A β with the cell. Implicit in the steady state assumption is that the rate of reaction of A β with the cell surface, once the A β had encountered the surface is of comparable order to the rate of A β diffusion through the solution. When the rate of reaction is fast compared to the rate of diffusion ($4\pi DR \ll k$), equation (1) is reduced to:

$$rate = 4\pi DR C_{A\beta}^{\infty} \quad (2)$$

In order to evaluate the relative magnitudes of reaction and diffusion terms (k and $4\pi DR$, respectively) in the expression shown in equation 1, and the validity of the diffusion limited reaction assumption, we considered the possible reactions of A β with the cell surface and their approximate magnitudes. Current hypotheses of mechanisms of A β cytotoxicity include A β induced oxidative stress or free radical generation (Butterfield 2002), changes in bilayer conductance (Sokolov et al., 2006), membrane pore formation or disruption (Demuro et al., 2005), or receptor mediated signal transduction (Hoshi et al, 2003). Associated intrinsic rate constants might be on the order of 1 s^{-1} or less for free radical generation or membrane disruption, but much smaller for reactions associated with signal transduction. We have observed changes cell membrane permeability in as little as 15 minutes (Patel and Good, 2007), suggesting to us that the intrinsic reaction rate of A β with the cell is fast. Given that A β aggregates have large hydrodynamic radii and therefore relatively small diffusivities, we believe that the diffusion limited reaction assumption is reasonable.

As illustrated by equation (2), under the diffusion-limited reaction assumption, the reaction rate of A β on the cell surface that leads to biological activity, depends linearly on the diffusion coefficient and the bulk concentration of the A β aggregate. The diffusion coefficients can be obtained using Einstein's equation:

$$D = \frac{k_B T}{f} \quad (3)$$

where k_B is Boltzmann's constant, T is the temperature and f is the friction coefficient. Stoke's law was used to calculate f for the spherical A β oligomers ($f = 6\pi r\eta$), where η is the solution viscosity and r is the radius of the sphere. The friction coefficients of fibrils and protofibrils were calculated using the translational friction coefficient of a cylinder, estimated as an average of sideways and lengthwise movements (equation 4) (Bloomfield et al. 1967):

$$f = \frac{6\pi\eta(2a)}{2\ln(2a/b) - \gamma} \quad (4)$$

where a is the half height and b is the radius of the cylinder, and γ equals 0.62 for infinitely long fibrils.

We modeled A β fibrils and intermediates using representative dimensions we have observed (Lee et al. 2007, Qi et al. 2008, Zhang et al. 2009) and as reported by others. A β fibrils were modeled as cylinders 500 $\pm$ 100nm in length and 14 $\pm$ 6nm in diameter (Antzutkin et al. 2000; Stine et al. 1996). Three types of intermediates were modeled; (1) a spherical aggregate with a radius of 15 $\pm$ 3nm (Huang et al. 2000), (2) a rod-like protofibril, as a cylinder with the same diameter as the fibril but only 200 $\pm$ 50nm long (protofibril I) (Nichols et al. 2002), and (3) a rod-like protofilament, as a cylinder 200 $\pm$ 50nm long with a diameter of 3 $\pm$ 1nm (protofibril II) (Stine et al. 1996). We specifically chose to use A β aggregate dimensions that were comparable to what we have observed in the laboratory via our methods of aggregation such that we could compare model estimates with our own biological activity data. In order to calculate the bulk concentration of the aggregates ($C_{A\beta}^\infty$), we assumed a homogeneous environment that consists of only one type of aggregate. We estimated the number of monomers per aggregate based on the aggregate volume and by assuming that all monomers within an aggregate had the similar secondary structure configuration and dimensions as in an A β 40 fibril. Monomer dimensions were measured from the available A β 40 fibril NMR structure (Petkova et al. 2006).

Table 1 summarizes the values obtained using this model for the number of monomers per aggregate, the bulk concentration, the diffusivity and the rate of reaction at the cell surface. The estimated rates of reaction are depicted also in Figure 1 for comparison with A β cell binding data. The binding data, collected from aggregated species prepared via the same methods as oligomer and fibrils reported in Table 1, and in previously reported viability assays (Lee et al., 2007), clearly demonstrate that fibrils bind less to cells than aggregation intermediates (species formed at after approximately 10 and 24 hour aggregation under quiescent conditions). Binding is reported in molecules bound per cell on a monomer basis. If data were corrected to show aggregates species bound per cell, then the difference in binding between oligomer and fibril would be even greater, given that there are more monomers in a fibril than in aggregation intermediates. Data reported exceed limits of detection of the method by 100 fold or more.

The results, shown in Table 1 and Figure 1, predict the rate of biological activity of both the protofibrils and the spherical aggregates to be much greater than the rate of activity of the fibrils, consistent both with cell binding results (Figure 1) and experimental toxicity (Lee et al. 2007; Qi et al., 2008; Patel and Good, 2007). The predicted rates of biological activity of

the different intermediate structures are comparable to each other given the uncertainties in the estimates. Notably, the predicted diffusivities of intermediates are comparable to the diffusivity of the toxic species that was previously estimated experimentally to be $2.6 \pm 0.3 \times 10^{-7} \text{ cm}^2/\text{sec}$ (Wang et al. 2002).

The concentration of spherical oligomers estimated by this method is only twice that of the concentration of fibrils, in part because we estimated concentrations based on a fairly large and tightly packed spherical oligomer, and a relatively short fibril. Use of a longer fibril, and/or a smaller sphere (or hollow sphere) would have resulted in even larger predicted differences in rates of biological activity between A β oligomer and fibril.

The results demonstrate that A β aggregate's size and concentration may contribute to differences in observed biological activity of oligomer, protofibrils and fibril species. The analysis presented is oversimplified and its limitations include geometric approximations, as well as the first order reaction kinetics assumed at the cell surface. Additionally, we considered homogeneity of A β aggregated species, while in reality a mixture of species coexist. While the dimensions of actual A β aggregates may differ from those we considered, the trends in the rates of reaction would be similar (oligomer or other intermediate would be more reactive than fibrils) and support the same conclusion. Despite the simplicity of this model, this analysis highlights some of the factors that may contribute to differences in biological activity observed between different A β aggregated species.

Our proposed model in no way precludes there being differences between A β aggregate structures at the molecular scale or differences in stability between aggregated species. In fact, the selectivity of certain inhibitors and antibodies towards the toxic intermediate support the hypothesis that there are molecular level structural differences between different aggregated species that have been difficult to elucidate (Kayed et al. 2007; Mamikonyan et al. 2007). However, our model does suggest that certain experimental factors that have been difficult to control in the past, such as the concentration of particular A β aggregates in solution, and the size distribution of aggregates within a mixture may contribute significantly to measurements of A β toxicity or other measures of biological activity, thus obscuring the effects of molecular level differences in the different A β aggregated species.

Material and Methods

N2a cell were grown in a humidified incubator at 37°C containing 5% CO₂, and using minimal essential media. Cells were plated in a 96-well plate at 50,000 cells/well for 24 hours prior to A β addition to allow cell adherence to plate. A β 1-40 (Anaspec, San Jose, CA) containing 1% N-terminus labeled TAMRA-A β (Anaspec) was dissolved in ddH₂O at 10mg/ml in ddH₂O with 0.1% TFA, then diluted in PBS to 100 μ M, and incubated at 37°C and for different time periods prior to addition to cells. Cells were incubated with the different aggregated A β species for 2 hours. Fluorescently labeled peptide incorporated into A β aggregates bound to the cell surface was detected using a BD FACSArray flow cytometer. Median fluorescence intensity was correlated to amount of A β bound to cells (on a monomer basis) using calibration beads.

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Figure 1.

A β binding to cell surface (closed circles) and estimated rate of biological activity (bars) as a function of aggregation time prior to addition to cells and structure, respectively. Binding was measured using a mixture of 1:100 TAMRA labeled A β 40 to unlabeled A β 40, at 100 μM total concentration (on an A β monomer basis), in PBS, an aggregated at 37°C for different time periods as indicated on the x axis. Fluorescently labeled peptide incorporated into A β aggregates bound to the cell surface was detected using flow cytometry, and was reported as total A β bound to the cell on a per monomer basis. Rate of biological activity was estimated for spherical oligomer (S), protofilament (PII), protofibrils (PI), and fibril (F). Bars are placed on graph at approximate times during aggregation when spherical oligomer and fibril have typically been observed (Lee et al. 2007). Protofilament and protofibrils should appear in aggregating solutions after oligomers but before fibrils, therefore their time placement on the graph should be as a qualitative estimate of their time of formation.

Estimated concentrations, diffusion coefficients and rates of biological activity of different A β aggregates. Cell radius (R) was assumed to be 10 μ m. Concentrations were estimated assuming each aggregate was formed from a 100 μ M monomer solution. Uncertainties in concentration and diffusion coefficient were estimated from the uncertainty in aggregate dimension.

Table 1

A β aggregate species	Dimensions (r or a,b) (nm)	Number of monomers per aggregate	$C_{A\beta}^{\infty}$ (nM)	$D \times 10^8$ (cm ² /s)	Rate $\times 10^{20}$ (moles/cell·s)
Fibril	250 $\pm$ 50, 7 $\pm$ 3	2083	48 $\pm$ 0.5	5.2 $\pm$ 1.3	3.1 $\pm$ 0.9
Spherical oligomer	15 $\pm$ 3	1107	90 $\pm$ 41	22 $\pm$ 4	25 $\pm$ 12
Protofibril I	100 $\pm$ 25, 7 $\pm$ 3	833	120 $\pm$ 15	10 $\pm$ 3	15 $\pm$ 5
Protofibril II	100 $\pm$ 25, 3 $\pm$ 1	417	240 $\pm$ 60	13 $\pm$ 3	39 $\pm$ 14