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Stabilizing Highly Pathogenic Nanoparticles Associated with Alzheimer's Disease

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Abstract

When the Aß peptides aggregate into soluble oligomeric clusters, they form central nervous system neurotoxins known as amyloid-ß-derived diffusible ligands (ADDLs). The assembly of ADDLs is currently thought to be a pathogenic process in Alzheimer's disease (AD). Understanding the molecular structure and properties of ADDLs could prove useful in the design of treatments. Because ADDLs easily denature, it is difficult to isolate them for study using classical methods such as gel electrophoresis or SDS-PAGE. This research developed a technique for isolating intact ADDLs oligomers. Using photo-induced cross-linking of unmodified proteins (PICUP) enabled the formation of covalent bonds between Aß polypeptide chains to stabilize the ADDLs. The strength of the cross-linked ADDLs was tested through SDS-PAGE. The surface of the proteins was mapped with atomic force microscopy (AFM) to confirm that the PICUP process did not alter the shape of the oligomers. This work also demonstrated that the PICUP method stabilizes ADDLs without altering their innate properties.

Introduction

Alzheimer's disease (AD) is a debilitating neurodegenerative disease that afflicts more than 4 million Americans, and burdens the economy with an annual cost exceeding $100 billion.[1] The absence of both a cure and an accurate means for diagnosing AD stresses the urgent need to better comprehend the disease's pathogenic mechanisms. Various studies examining AD brain samples assign amyloid-ß (Aß) peptides with a leading role in AD's characteristic neuronal damage.[2] When the Aß peptides aggregate into soluble oligomeric clusters, they form central nervous system neurotoxins known as amyloid-ß-derived diffusible ligands (ADDLs).[3] ADDLs bind to specific synapses in the brain, generating the rapid and aberrant signal transduction necessary for proper memory function. The errant accumulation of ADDLs has been linked to the memory dysfunction characteristic of AD.[4]

One strategy for managing AD involves interfering with the function of ADDLs through competitive inhibition of its synaptic binding. This interference might be accomplished by constructing a benign version of the toxin capable of binding to the same synaptic receptors to which ADDLs bind. If these innocuous ADDLs were to have greater affinity for the ADDL receptor sites than do the toxic ADDLs, it might be possible to prevent further degeneration of the brain.

To create innocuous ADDLs, the characteristics of ADDLs — specifically molecular weight and structure — must be thoroughly understood. As an initial step to achieving that goal, a method is presented for the isolation and characterization of ADDLs without denaturing them, a procedure that heretofore has not existed.

Background

Lesné et al. characterized Aß peptides in mice expressing Aß precursor proteins (APP), which are known variants of proteins associated with AD. The goal of their research was to evaluate the basis for memory decline in AD prior to the development of neurodegeneration. They attributed irregularities in their mice to the accumulation of soluble 56 kD Aß oligomers. They also showed that the 56 kD Aß species from the impaired mice damaged memory function when administered to healthy young rats, a finding that stimulated further examination of the 56 kD Aßs.

When Aß oligomers were (1) exposed to hexafluoroisopropanol (HFIP), a solvent with strong hydrogen-bonding properties; and (2) subjected to SDS-polyacrylamide gel electrophoresis (SDS-PAGE), a process that can be shown to denature protein assemblies; they disintegrated into their composite Aß trimer, dimer, and monomer proteins.[5]

The obstacle in determining the native size of the peptides became the buffers because they denatured the proteins. The experiments reported in the present paper demonstrate that the original size of Aß can be determined using SDS-PAGE by cross-linking the ADDLs to strengthen their intermolecular bonds.

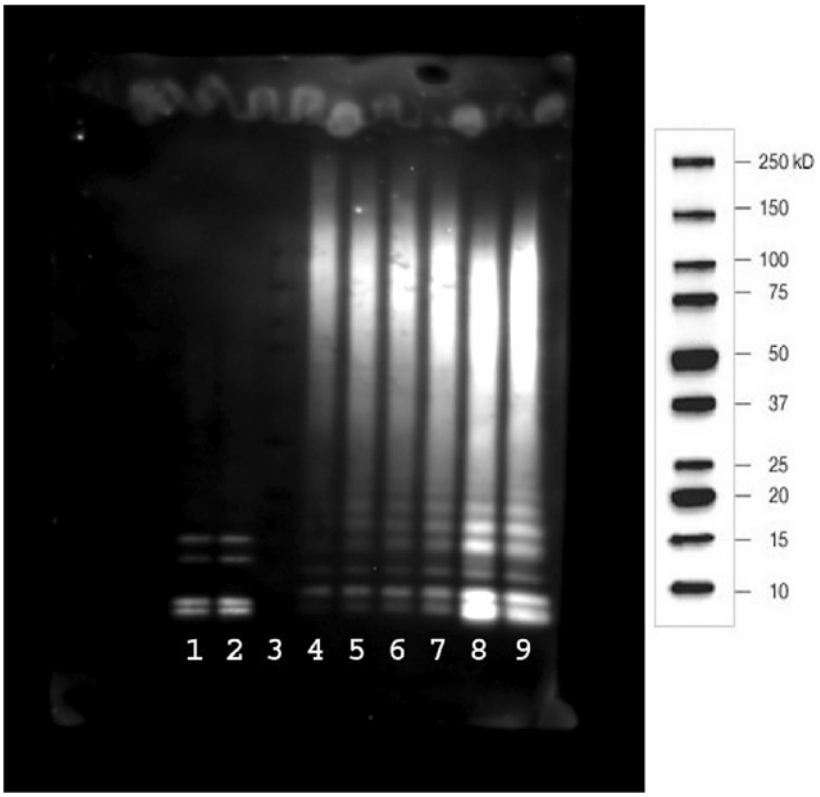

Figure 1. The Western blot (above) viewed with chemiluminescence indicates which samples contain cross-linked ADDLs, as well as the degree to which cross-linking was successful. The areas that appear white within each lane indicate where protein was present when the SDS-PAGE was run. In lanes 1 and 2, which contain the control sample, no cross-linking occurred, and therefore no protein is visibly present within the 37–100-kD band range. Because protein is visible only at the 10-kD band, it is reasonable to deduce that the ADDLs broke down mainly into dimer and monomer protein species. The heavy presence of proteins above the 37-kD band range in lanes 4–9 indicates that a significant portion of these samples contain ADDLs that did not break down into monomers and dimmers. The ability of these proteins to withstand the SDS-PAGE denaturing capabilities suggests that the cross-linking was successful.

Approach

The key to stabilizing ADDLs against denaturing lies in chemical cross-linking techniques that deter any modifications of the proteins. A useful method for experimentally determining ADDL size that concurrently mirrors in vivo conditions is photo-induced cross-linking of unmodified proteins (PICUP). PICUP facilitates cross-linking by enabling the formation of covalent bonds between polypeptide chains. In PICUP, the cross-linking reaction is induced by rapid visible-light photolysis of a tris-bipyridyl Ru(II) complex (RuBpy) in the presence of an electron acceptor.

PICUP thus establishes a population of oligomers whose original conditions have been petrified by covalent cross-linking. The PICUP technique was adapted to determine the oligomeric state of synthetic ADDLs under physiologic conditions. PICUP enables the cross-linking of proteins within time intervals shorter than 60 sec. Although previous PICUP protocols have been used to successfully cross-link other types of oligomers, including Aß, a specific procedure was needed to induce cross-linking among ADDLs.[6]

There were three critical determinations to honing the PICUP technique to successfully cross-link ADDLs: the ideal quenching reagent for the reaction, the optimal irradiation time (T1) for observing cross-linking, and the most favorable ADDL concentration (C1).

Dithiothreitol (DTT) and 2-mercapto-ethanol (ß-ME), two strong reducing agents, were tested separately as potential quenching reagents. Once added to a solution, a quenching reagent should stop a reaction. Eight samples were made to test quenching abilities. Six of the samples contained 20-µL mixtures of ADDLs, PBS buffer, APS, and RuBpy. The individual samples were dispensed into 0.2-mL polymerase chain reaction (PCR) tubes. Each sample was then irradiated for 30 sec in a black box in which the only light source was a 200-watt white light bulb placed 15 cm from the sample. The samples were quenched immediately after exposure; three samples were quenched with 1 µL of DTT, while the other three were quenched with 1 µL ß-ME. Two control samples contained the same ADDL, PBS, APS,and RuBpy solution as the experimental tubes, but the designated quenching reagent was added to the PCR

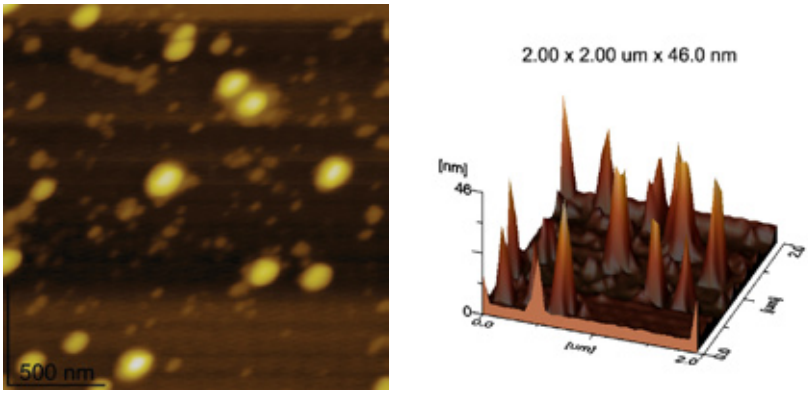

Figure 2. The image on the left contains noncross-linked ADDLs imaged on the AFM. The figure on the right is a computer-generated 3-D mapping of the proteins imaged on the left. In this sample the tallest protein height obtained was 45.73 nm.

tube prior to other substances in the controls. This step ensured that no reactions took place within the control samples because the quenching reagent was already present in the tube. Unlike the experimental samples, the controls were not irradiated, which prevented any potential cross-linking reaction within the tube.

SDS-PAGE was used to determine which quenching reagent was most effective. Samples were analyzed by electrophoresis followed by Western blotting and immunostaining. The immunostaining was performed using chemiluminescence so the samples could be transferred to a photographic imager. At this point the samples were viewed at a Kodak Image Station to determine which of the two quenching reagents was more effective. No cross-linking or reaction was visible in either control. The experimental samples quenched with DTT had large bands between 50 kD and 70 kD, indicating that the cross-linking of these samples was successful. On the other hand, the samples quenched with ß-ME showed minimal presence at the 50 kD band and a greater presence at the 20 kD and 15 kD bands. This indicated degradation of the native oligomer into shorter fragments.

Determining the Optimal Irradiation Time
Ten samples were prepared in this experiement. Nine samples contained 20-µL solutions of ADDL, PBS, APS, and RuBpy. Three of these samples were individually irradiated for 10 sec and subsequently quenched with 1 µL DTT. The next three samples were each irradiated for 20 sec and subsequently quenched with 1 µL DTT. The final 3 samples were all irradiated for 30 sec before 1 µL DTT was added. The 10th sample was a control sample where the DTT was added to the PCR tube before the other 20 µL of solutions. This sample was irradiated for 30 sec. SDS-PAGE, Western blot transfer and immunostaining protocols were performed before the samples were viewed on the Kodak imager. No cross-linking occurred in the control sample, and those irradiated for 20 sec yielded the highest amount of cross-linked ADDLs.

Similar steps were employed to perform PICUP to obtain the final variable. Here, samples with ADDL concentrations of 2 µM, 4 µM, and 6 µM were prepared, and each was then mixed with PBS, APS, and RuBpy prior to irradiation and quenching with DTT. The three samples were then subjected to an SDS-PAGE, transferred via Western blot technique and immunostained. It was evident that ADDLs had been cross-linked in each of the samples from the presence of molecular weight bands between 37 and 100 kDa. In the 6-µM concentration sample of ADDLs, there was a greater presence of cross-linked proteins than in the 2-µM and 4-µM samples (Figure 1).

In Figure 1, when lanes 4 and 5 (which contain 2-µM ADDLs) are compared with lanes 6 and 7 (which contain 4-µM ADDLs), it appears that both produce nearly the same amount of ADDLs within the 75–100 kD bands, where the cross-linked ADDLs are visibly present. In lanes 8 and 9 (which hold the 6 µM ADDLs sample), the accumulation of cross-linked ADDLs within the 37–100 kD bands indicate that in order to generate the most effective cross-linking among the ADDLs samples, 6 µM concentration of ADDLs must be used together with a 20 sec irradiation period and DTT as a quenching reagent.

PICUP experiments using the newly-developed protocol demonstrated that cross-linking occurred in the samples. It was still necessary to determine if the cross-linking within each sample had left ADDLs entirely intact, or if the white bands interpreted as cross-linked ADDLs were partially denatured by SDS-PAGE.

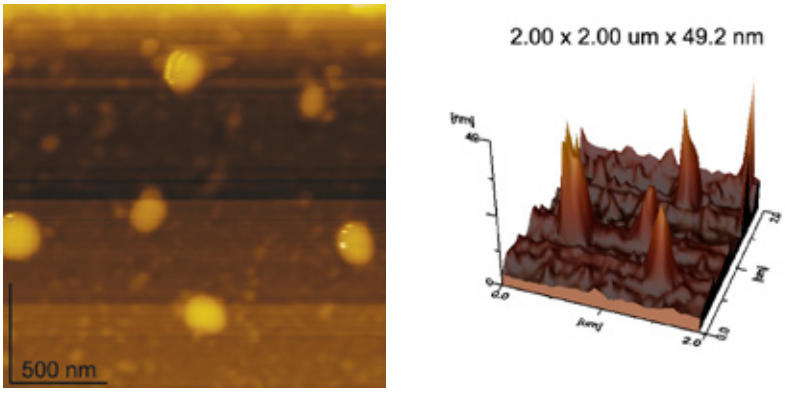

Figure 3. The image on the left contains cross-linked ADDLs imaged on the AFM. The figure on the right is a computer generated 3-D mapping of the proteins imaged on the left. In this sample the tallest protein height obtained was 48.69 nm.

To ascertain the precise characteristics of cross-linked specimen, an atomic force microscope (AFM) was used to examine samples of ADDLs before and after cross-linking. AFM can be used to image structures with nanometer scale and provides a three-dimensional surface profile of the sample in question. ADDLs not subjected to any form of experimental modification and cross-linked ADDLs post-PICUP should be the same size. Partial denaturing would be detected as differences between the AFM-generated surface profiles of the cross-linked ADDLs and those of unmodified ADDLs.

Three types of samples were prepared for viewing by AFM, which requires samples to be placed on a hard surface capable of withstanding the microscope's prodding probe. The samples were placed on the top face of mica discs with a diameter of 1 cm. The mica was first rinsed three times with double-distilled water. The water was then aspirated from the mica's surface and replaced with the sample solution.

The first of the three samples acted as a control: one disc of mica was rinsed with the double-distilled water that was subsequently aspirated off and not replaced. Instead, the mica was left bare to permit visualization of the surface of plain mica, allowing differentiation of dust and barren mica from the proteins of interest. The next two samples were similarly prepared. For each sample one mica disc was rinsed with double-distilled water, the water was aspirated off, and the sample substance was left on the disc. One disc contained a sample of non-cross-linked ADDLs, while the second disc was coated with 10 µL of cross-linked ADDLs. The cross-linked sample was an aliquot of solution that had already gone through PICUP, SDS-PAGE, Western blot transfer, and immunostaining to confirm that the proteins had indeed cross-linked.

The AFM was operated in acoustic mode, in which its cantilever's oscillation amplitudes are controlled by a feedback loop. The optimal oscillation frequency was determined using WinSPM system processing software, and the force exerted on the sample was automatically assigned by the computer. When the cantilever tip passed over bumps on the sample's surface, it had less room to oscillate, which decreased the amplitude of oscillation. The amplitude increased and there was more room to oscillate when the cantilever probe passed over a depression. The oscillation amplitude of the tip was sensed by the detector connected to NanoScope III controller electronics. A digital feedback loop adjusted the cantilever tip–sample separation to maintain steady amplitude and force on the sample. As the probe tip tapped the surface of the sample, the computer generated a 3-D map.

Once the samples were imaged with AFM, the heights of proteins found in both samples were measured, and the average heights for the samples were calculated. This process was repeated four times, and each replication was performed with fresh protein samples.

Results and Discussion

The average height of the ADDLs in the non-cross-linked samples was 47.125 nm, and the average height of the proteins in the cross-linked samples was 50.175 nm. The average height values for the protein samples were calculated by adding the protein heights collected from a sample and dividing the total by the number of proteins measured. This resulted in four average-height values for both types of examined ADDLs. For the four non-cross-linked samples, the average heights were 46.7 nm, 49.5 nm, 43.4 nm, and 48.9 nm. The average heights for the four cross-linked samples were 51.3 nm, 48.7 nm, 49.4 nm, and 48.3 nm. The

average values of the heights were then calculated, yielding a final average-height value for the type of protein imaged. The absolute error (E_{abs}) for the measurements obtained was 6.47; this was calculated using the formula for E_{abs} where E_{abs} = [(measured value – expected value)/ expected value]*100. ADDL heights measured using the AFM are presented in Figures 2 and 3.

The samples of cross-linked ADDLs had nearly the same heights as the non-cross-linked ADDLs. The cross-linked proteins had slightly increased elevation because of reduced mobility. It is unlikely that cross-linked proteins could glide off dust particles or solution lying beneath it, behavior typical of non-cross-linked proteins. Since the samples visualized with the AFM are on an exposed stage, and not in a vacuum, the presence of residual buffer solution and microscopic airborne debris particles is expected and explains minor inconsistencies between the two types of proteins.

The insignificance of the difference between cross-linked and normal ADDLs indicates that PICUP protocol to cross-link ADDLs successfully strengthens the proteins without altering their physical characteristics. Because the cross-linked ADDLs are also capable of interacting with SDS in a gel electrophoresis without being affected by the vigorous denaturing power of SDS, further studies can determine properties of ADDLs that use SDS. This ability is useful primarily because SDS is a substance required by numerous protocols and for which there is rarely an available substitute. Before the successful cross-linking of ADDLs, these tasks could not be successfully performed in a consistent fashion.

Conclusion

The pressing need to find a mechanism for treating Alzheimer's disease motivates the study of ADDLs and their properties. The determination that ADDLs — and not the plaques and tangles once considered to be the cause of the illness — are the neurotoxins responsible for the neurodegenerative process[7] is a significant advance in understanding the pathophysiology of AD. Defining the structure and composition of these neurotoxins is a necessary initial step in the construction of a pharmacologic treatment for the disease. Using the novel mechanism presented in this paper for the isolation of ADDLs without denaturing the proteins, it will now be possible to fully characterize their structure and construct pharmacologically active congeners.

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